UNIVERSITAT DE
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Biological invasions: a temporal, spatial and plastic point of view: the case of *Discoglossus pictus* in Europe

Invasions biològiques: un punt de vista temporal, espaial i plàstic: el cas de *Discoglossus pictus* a Europa

Eudald Pujol Buxó

PART A: Intraspecific variation along the invasive range of *Discoglossus pictus*

Chapter 1: Geographical variations in adult body size and reproductive life history traits in an invasive anuran, *Discoglossus pictus*.

Chapter 2: A genomics study of population structure within the invasion range of the Mediterranean Painted Frog (*Discoglossus pictus*).

PART B: Intraguild competition in tadpoles of *Discoglossus pictus*

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Chapter 4: Differential trophic traits between invasive and native anuran tadpoles.

Chapter 5: Fast evolutionary responses in a native–invasive competitive system after several generations of coexistence.

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Biological Invasions:
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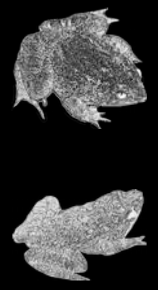
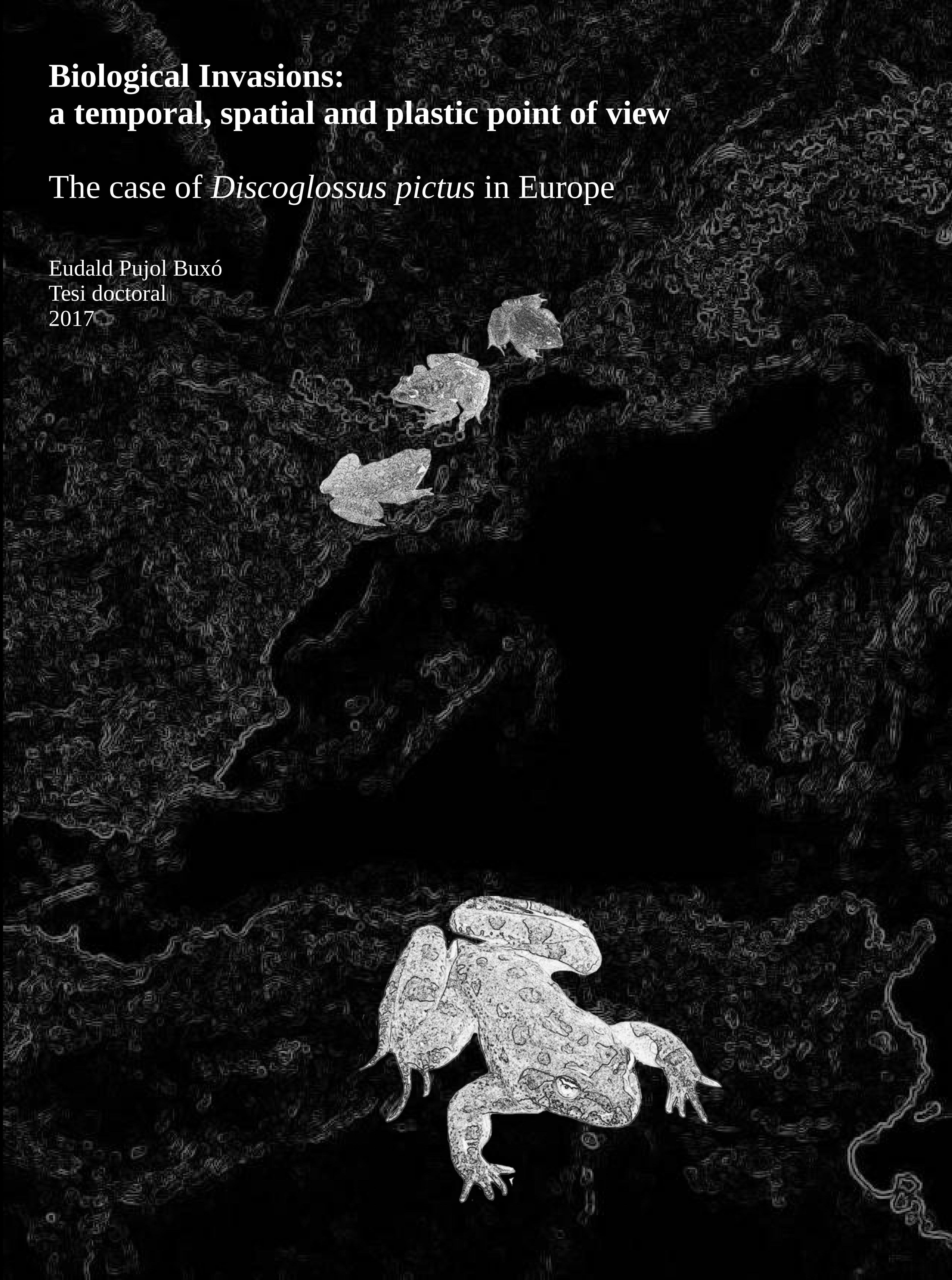
Tesi doctoral, 2017





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Departament de Biologia Evolutiva, Ecologia i Ciències Ambientals
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Memòria presentada per l'Eudald Pujol Buxó per optar
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El doctorand
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Agraïments

«Canta, oh, deessa, la ira d’Aquil·leu, fill de Peleu, còlera fatal que ocasionà als aqueus innombrables dolors, i estimbà a l'Hades les ànimes valeroses de multitud d'herois, fent-ne pastura de cans i aus»

Una tesi doctoral és tota una aventura, i com a tal en té tots els ingredients: una introducció, un nus, un desenllaç, uns personatges bons i dolents, moments d'angoixa i moments d'alegria... o d'alleujament, perquè a vegades ja no et queden energies per l'alegria (article accepted? Pffff ja era hora collonssss que llarg que s'ha fet!!!). I com tota aventura, al final, quan acaba bé (o acaba, vaja, que això ja és bo tant per la Ilíada com en aquest cas), tot s'amoroseix, s'endolceix, es suavitza, el nostre cap fa allò tant típic de començar a esborrar de la memòria les part dolentes de la història. Per més llarga que sigui la llista de successos i personatges, els que passen a la posteritat són els que et donen bons records... junt amb els grans dolents de la pel·lícula. I és que com va dir el gran filòsof Gandalf: «tota gran història mereix ser guarnida».

De fet, un dels processos que ajuda a Gandalfitzar la tesi és escriure aquests agraïments. Comences a pensar en els personatges bons i tot va agafant un toc gairebé nostàlgic (i encara no he acabat la tesi ben bé que es digui...), allò de «Òstia vam patir però al final la merda aquella va sortir bé. Quins moments! Hahaha» així amb un to de la típica colla d'homes de mitjana edat explicant històries de la mili. Els humans som així. Així doncs, acceptem-ho, i fem una llista dels personatges bons de la pel·lícula, i que serveixi per arrencar un petit somriure a tothom! Espero no deixar-me ningú. I als grans dolents? No penso fer com l'Homer: regalem-los-hi l'oblit, en aquest cas, expressament.

M'aixeco al matí. Tard pel meu gust, com sempre «puta vida, ets una puta marmota, Eudald». És possible que sigui culpa del senyor Dan Simmons o en George RR Martin, entre altres escriptors que fan que m'adormi als matins i que m'agradaria agrair-ne la seva obra. Però a aquelles hores no agraeixo res a ningú. Em dutxo, esmorzo, surto del pis. Arribo al departament, en Gustavo probablement ja fa 1508970832 hores que hi és, si hi ha alguna cosa important per comentar m'hi acosto a la porta, sinó, ens veurem cap allà les tres o les quatre de la tarda que és la hora no-oficial però efectiva de reunions o trobades breus per comentar la tesi, o un article interessant, o rajar dels problemes de la universitat, planificar experiments nous, la vida política del país, que si ara ja no plou com abans, jo què sé. Aquí haig d'admetre que m'ha tocat un bon director i tutor. Me'n recordo no fa massa quan en el moment que li van donar per dir unes paraules a la presentació de la tesi d'en Marc Franch, i ell va fer-ne tot un elogi, i amb raó (després et torno a agrair l'existència, Marc), amb una conclusió clara: «és una molt bona persona». Doncs jo aquí haig de fer el mateix: «Gustavo, ets una molt bona persona», de veritat, sense cap dubte. I a més a més, t'interessa el que fas, el que es fa, el que passa al teu voltant, i ets un bon biòleg amb el qual comentar-hi les discussions més interessants, sobre qualsevol tema. Pot semblar una ximpleria, però per un petit padawan, un mestre jedi amb interès sincer pels resultats dels experiments que tanta suor porten, acaba siguent molt important. Com aquell típic amic amb el qual comentes les aficions frikis, durant una tesi un doctorand necessita algú que quan li dius: *«Òstia, els putos capgrossos de Discoglossus dels mesocosmos estan sobrevisquent molt més que els de calamita a la presència de les larves de Sympetrum, crec que és perquè creixen més ràpid i els cicles de desenvolupament no estan ben bé acoblats entre depredador i presa!»* en comptes de fer una cara de porteu-lo al manicomi o de no sé de què collons em parles, et comenci una conversa que acaba en especulació d'hora i mitja llarga sobre els capgrossos, les libèl·lules, la saga Star Wars, el funcionament de la investigació actual en biologia i pràcticament sobre la teoria de l'evolució en sí. En fi, que hem fet un bon equip, merci per tot. Per altra banda, el paper de filòsofa-mestra jedi a la meva breu estada a Portugal li va correspondre a l'Antigoni. Tot i que l'hora no oficial de reunions acabava siguent abans de dinar, el cas era el mateix... també som un equip perillós! A pensar idees, experiments, línies científiques, hahaha també podríem encadenar hores filosofant. També t'haig d'agrair el teu exemple de biòloga apassionada, jefa d'estada molt guai (com a jefa i com persona), i la teva empenta de «fes-ho» que m'ha servit per fer-me perdre la por d'anar més i més enllà amb les meves capacitats estadístiques. Vols sentir una punxadeta d'orgull científic? L'apartat «Geometric morphometrics» de materials i mètodes de la tesi, i de molts capítols de la tesi no haguessin estat ni de lluny el que són sense tu, i l'apartat «Permutation tests», bé, potser no hi hagués sigut, simplement. La ciència també és transmetre maneres de pensar entre científics, a nivell personal, i tant Gustavo com Antigoni ho heu fet molt bé. M'heu estimulat les neurones, cadascú amb la seva manera de pensar, o ens les hem estimulat mútuament, i us ho agraeixo molt.

El dia segueix el seu curs, i el cuquet de la gana comença a fer forat... les neurones comencen a fer figa. El típic moment que et treus els auriculars i dónes conversa al despatx. I aquí, uf, la de gent que hi ha per agraïr la companyia en moments de baixada de sucre... un toc d'humor àcid amb l'Eloy i la Débora per riure una mica, o uns comentaris sobre morfometria? Yeah, ho trobaré a faltar, una abraçada morfòlegs. Un cafetet amb cara de son, o uns palitos compartits, o uns comentaris trivials sobre herpetofauna? Marc i Olatz, seguiu així de guais, també us trobaré a faltar. A vosaltres també us haig d'agrair que hi fóssiu i que sigueu com sou, per seguir-vos l'estela de l'herpetòleg de camp, de les persones que els hi encanta la sortida a fora del despatx... Marc encara me'n recordo de les primeres sortides que vaig fer anant a marcar tortugues al Garraf, aquestes coses semblen xorrades però motiven molt quan ets un pringui que tot just està acabant la carrera! Vas inspirar-me a fer el mateix amb els nous «ajudants d'aprenents» (TFGs, TFMs...) del departament: a motivar-los amb el camp... espero haver-ho aconseguit, més endavant us agraeixo a vosaltres (TFGs, TFMs...) també la vostra companyia. Molta més gent ha passat pel despatx, a ajudar-nos mútuament, a comunicar-nos complicacions... Urtzi, vas ser un molt bon company de batalla durant el màster i primers passos de la tesi. Núria, vas tindre la paciència en ser la primera persona a ensenyar-me l'anàlisi estadístic dels residus, i el funcionament del puto Statistica. Després he acabat fent ANCOVAs o models mixtes amb l'R, i ara ja en cada cas m'invento alguna cosa diferent, però al començament em va ajudar molt que estiguessis per allà per aguantar el pringadet que començava la tesi o el treball de màster i no n'entenia ni un borrall d'estadística. De tant en tant també apareix l'Albert Montori, que entra dins del pack d'herpetòlegs apassionats que et motiven a sortir al camp tot el sovint que sigui possible. El típic que t'enganya per anar a la Vall d'Aran a buscar bitxos, per exemple. Merci pel bon humor i les agradables converses. I aquí també haig d'agrair l'existència de la companyia d'en Pol i en Felipe, bons col·legues de cansament mental al despatx (quantes vegades ens hem queixat entre nosaltres de cansament neural a les set de la tarda? No puc meessss tius un cafeeeee), i com tots els altres, a part de companys de feina també sou bons companys de borratxera als congressos hehe. Ara toca la herpetocelebració de la tesi, important! Prou de cansament al despatx, BBQ? O què? Que no quedi només en paraules!

A tots, espero que ens seguim veient sovint.

Finalment arriba l'hora de dinar, i obro el mail per confirmar que la «Dinar 13:45 crew» estigui a lloc. Aquí entrem en un top5 de moments de la tesi: quin és un dels millors records de la tesi? Quan no feia tesi i dinava amb els amics. Ha!. En aquest punt tots sabeu qui sou. Ho sabeu tant que passo d'anomenar-vos un(a) a un(a), imagineu-vos... a més molts tornaríeu a sortir més endavant... no diré noms, no cal. You know. Metanorfosis. Operació Montcortès. David. I això del procés així doncs què. Berta. Mays. Vaig veure un estudi molt guai que deia que. Muntanyades. Alba. Això dels referees maleducats s'hauria de solucionar. Marc. Anem a fer el cafè allà al bar? Nora. A qui li toca anar a buscar els cafès? Demi. Val, al final, us he anomenat dissimuladament entre els keywords... tots trobarem a faltar aquests dinars. Ja quedarem per fer un skype-dinar13:45 de post-doc no? Tanta conya però weno que això s'ha de fer eh!

De tant en tant, a les hores de dinar hi ha assemblees, de coses i tal. De precaris sobretot. Algun dia aconseguirem millorar les coses penya, de moment aprofito per agrair no només als assemblearis d'ara (tant als «beecaris» com als precaris) l'esforç i el companyerisme, sinó també als assemblearis de temps anteriors el fet que jo hagi pogut cotitzar per treballar durant la tesi, per exemple. Algun dia d'aquí molts anys llegiré alguns agraïments d'una tesi que agraeixi la feina feta per la meva generació de precaris en lluita... o no. Hahaha. Però l'intent hi haurà estat, hi haurà estat.

Ah, i si és dimarts, després de dinar s'ha d'anar a buscar la cistella de verdura ecològica: doncs bé, què menys que agrair a la gent que ha fet possible aquest projecte? A la gent que ha fet possible que mengés mig sa durant la tesi? I de proximitat? I sense intermediaris? I les converses sobre masses pomes o si això és bròquil verd o blanc o bròcoli i romanescu joquèsé! Com es cuina la remolatxa? Una abraçada a tota la genteta de la coope de la facultat des d'aquí!

A la tarda, sovint la jornada es fa llarga perquè com que m'he aixecat tard doncs s'ha de compensar (com collons vols acabar la tesi sinó, Eudald?), i a les tantes a vegades apareix del no-res gent... Àlex Sánchez! Aquí t'haig d'agrair molt la companyia i les riures en aquelles hores tant rares de la vida, no hem rigut de xorrades ni res, i també hem arreglat el món unes quantes vegades que jo recordi. Fins i tot tenim un article de mussaranyes a mitges entre conya i conya. Per altra banda, quan arribes és allò de: osti, hi ha l'Àlex, avui realment estic allargant la feina... haig de millorar els meus horaris o acabaré sortint a pelis de vampirs... Però és difícil agafar horaris normals a vegades, ja que de tant en tant, també a la tarda i vespre, toca anar a camp. A buscar coses, en plan capgrossos i granotes que ponen ous i a fer coses de biòleg. I qui em ve el cap? Tots els TFM i TFG que heu passat per aquí. Molts m'heu agraït que hagi intentat currar-m'ho perquè gaudíssiu i n'aprenguéssiu molt de la vostra aventura particular del treball de grau o màster, i espero haver-ho aconseguit, però la veritat és que el simple fet d'intentar-ho ha estat un plaer. Tots i totes heu estat molt bona gent, una motivació extra i una sèrie de persones amb les quals ens hem ajudat mútuament de puta mare i ens ho hem passat molt bé al camp. Ander, Egoitz, Bàrbara, Gerezti, Berta, Gabri, Cristian, les dues Martes... deu n'hi do la penya que heu passat per aquí, i amb alguns, quines aventures eh? Que si no plou, que si a les tantes per allà l'Albera, que si avui enganxem la nit del diluvi universal i entra aigua per tots els racons del cotxe i ens morim d'hipotèrmia, que si les granotes no ens crien quan volem, puta sequera, que si giro i no miro i embarranco el land-rover en una cuneta i ens l'ha de treure una puta excavadora d'un pobre home que es deia Pere i corria treballant el camp per allà, cap problema seguim amb el dia de camp... tot plegat hem rigut, i també al laboratori, no fotem, amb els capgrossos que si es mouen que si no es mouen que si la libèl·lula se'ls menja o no. La ciència, a vegades, és molt divertida, i més amb gent divertida com vosaltres. Una abraçada molt forta! I parlant de laboratori, els dies que tocava laboratori de genètica, hi ha una sèrie de gent que també li dec agraïment per la paciècia i la companyia: Pol (ja repeteixes!), Xènia, i Àlex, sembla que al final ens n'hem ensortit amb això de la genètica... merci a tots tres per la paciència si les coses no sortien prou bé a la primera, o amb les meves novatades i preguntes constants... una abraçada molt forta! I molta altra bona gent amb qui he col·laborat i discutit sobre projectes científics, alguns d'ells encara a mitges, i que ara no puc anomenar-vos a tots i totes perquè sinó no acabaria, gràcies per tot també, seguim!

Si hi ha sort, quan s'acaba la jornada laboral, i em sembla que hem anat perdent sort a mesura que avançava la tesi, toca anar a sopar a fora a fer unes birres. Si estigués a Portugal el director d'orquestra seria l'inefable Xavier Santos, que em va ensenyar els millors bars per fer gintònics, restaurants bàsics per la supervivència humana a VdC i proximitats, i em va marcar el ritme de vida sopar-birres-dormir-treballar-sopar-gintònic de l'estada, bàsic per la productivitat científica. Patacrack, et torno el complit que em vas fer i ho faig sincerament: tu també vas ser un molt bon company de pis! Algun dia tornarem a coincidir a Portugal i ens fotrem un chateaubriand o un bacalhao a doca parlant de política com als vells temps.

I si estic a barna? Doncs aquí hauria d'agrair a molta gent les estones passades fora de la feina i no podré anomenar una llista de tothom... alguns perquè hem acabat visquent lluny per exemple, la vida moderna, quines coses, separa els amics. N'hi ha d'alguns que hem aconseguit trobar-nos més sovint amb ganes d'entrepans «al terra» o de kebab «al musta» i ten thousand beer per exemple, que encara no han sortit i que els hi haig d'agrair especialment els vespres de desconnexió, com per exemple l'Albert, que no ha fet mai tesi i ens ha aguantat sovint els rotllos... i a «companys de tesi» del dinar 13:45 crew que també sou grans colegues, que no hem fet la tesi «junts» però com si l'haguéssim fet i si em dóna la gana aquí diré que som companys de tesi perquè hem passat per les mateixes muntanyes russes i punt, perquè són els meus agraïments i dic el que em dóna la gana. I a d'altres dels meus mamonassos preferits que han de sortir aquí, com l'Edu – i els altres mosqueperros i tartanyans que no us anomeno again and again expressament perquè us repetiríeu com l'all en aquest text! – que ara s'està traient el doctorat en fàider, i altres grans frikis com la resta de Nyctipenya que algun dia fins i tot farem alguna cosa seriosa, de moment ens ho hem passat bé planificant coses hahaha!

Finalment, hi ha la gent més bàsica de la més bàsica, que no puc adscriure a moments determinats del dia a dia predoctoril, perquè hi son sempre presents. Allò sense el qual és com impossible emprendre cap projecte amb cara i ulls, els que fan que la vida pugui ser més vida. Primer, la família. Pare, mare i germana, us estimo molt. Per la vida en general necessitaria un llibre per descriure-us el que us dec, però per la tesi en sí deu n'hi do també, déu n'hi do... M'heu aguantat caps de setmana catatònics després de setmanes dures, m'heu animat quan estava rebentat, m'heu aixecat amb una grua quan estava espatarrat en mil trossos al terra per mil motius, m'heu donat converses genials amb el sopar si les meves neurones responien. Moltes gràcies, de veritat. I també per altres coses més «materials», com el robatori constant i consentit del Land Rover (hi hagués hagut tesi sense Defender? NO), com pels tàpers ocasionals per sobreviure a les setmanes dures, i fins i tot entrepans per anar al camp... tinc una mare tant adorable i que m'estima tant que no em deixarà fer-me els entrepans per anar a buscar granotes ni quan tingui seixanta anys perquè els vol fer ella, que els fa millor (això últim no ho negaré). I si fins i tot he pogut anar acompanyat al camp amb algú de la família a vegades! L'equip pare-fill va ser bo en algunes ocasions, i Clara tu també hi has vingut a buscar granotes! Això és family power! Seguim així! A la resta de familiars, que també us vull dir que us estimo molt, i que m'alegra que ara ja s'haurà acabat allò de: així doncs de què treballes exactament? I això de fer un doctorat és estudiar doncs?

I finalment, tu, pitxiflun (sí, tu! potxi! o potxirrainins! putxiflutxins! Ei, no dissimulis! La que està pensant que cabró en daldins que m'ha fotut els noms ridículs per escrit a la tesi i va dir que no ho faria, sí, tu! Carinyo, estimada! Ariadna!). Deu n'hi do la paciència que ens tenim eh? El que m'has aguantat. Aquí estem... més feliços que mai, amb la meva companya d'aventures... Espero no haver-te espantat per si vols fer una tesi, qui sap, tu potser ho portaràs de puta mare sempre. O potser fins i tot potser trobes una feina normal i t'ho estalvies... sigui com sigui el que ens vingui, allà estaré, te'n dec molta de paciència, per totes les carretades de problemes i frustracions que he portat a casa i que m'has aconseguit posar a lloc amb la teva màgia femenina que tant feliç m'ha fet aquests anys. Merci per tot, i em quedo curt dient-ho. Per cert, tens alguna cosa per fer els propers quaranta o cinquanta anys?

Gràcies a tothom!

Eudald

SUMMARY
RESUM

SUMMARY

The rates at which allochthonous species are introduced and become invasive worldwide are unprecedented, and the economic and ecological losses linked to these processes are overwhelming. Accordingly, the number of scientific studies on invasion biology have greatly increased during the past years. However, even though biological invasions have now been largely studied from an applied ecology and conservation point of view, they have been more overlooked by fundamental ecology and evolutionary biology. Despite great opportunities for the study of phenotypic or genetic evolution, biological invasions are still seemingly underused as natural laboratories to test essential biological theory. This obviously means a loss of opportunities to test basic biological principles, but on the other hand it can also leave conservation biology and management practices unarmed to understand the causes behind the unpredictability of the outcomes and the dynamics of biological invasions, or the patterns and processes that invasive species create and undergo.

In this sense, the nine studies presented in this thesis try to globally give an evolutionary – apart from ecologic – point of view on the ongoing invasion of the Mediterranean Painted Frog (*Discoglossus pictus*) in Europe. This frog is native to Northern Africa and was introduced from Algeria in Banyuls de la Marenda (Southern France) approximately a century ago. The introduced individuals overcame all possible barriers to establish self-sustainable populations and eventually became invasive, expanding its range and being nowadays present on a wide coastal strip of approximately 250km from Montpellier (SE France) to Sant Celoni (Barcelona, NE Spain). The invasive species seems to have accelerated its pace of expansion in the last decades, and is now continuously colonizing new areas at an approximate rate of two or three km per year.

The nine studies presented are ordered in three parts, which function as thematic blocks. The aim of **"PART A: Intraspecific variation along the invasive range of *Discoglossus pictus*"** is the examination – at a phenotypic and genomic level – of possible differences across populations within the invasive range of the species. The objective is to explore evolutionary diversification, either due to intrinsic – e.g. range expansion dynamics – or extrinsic factors – environmental limitations –, that the invasive species may be experiencing within its invasive range. In this case, we observe that the Mediterranean Painted Frog is not a uniform unit in its invasive range in Europe, neither from a phenotypic nor a genomic point of view: mean size of adults and life-history traits related to mean annual precipitation, while substantial genetic differences among populations are most probably created by the expansion history itself.

The aim of **"PART B: Intraguild competition in tadpoles of *Discoglossus pictus*"** is to study and discuss the role of *Discoglossus pictus* tadpoles as potential competitors for the larvae of native anurans. In this block there are five studies, that range from community-wide assessments of physiological traits, to a deep focus in the ecology of a native-invasive competitive relationship – *Epidalea calamita* versus *Discoglossus pictus* –, with several evaluations of the possible influence of previous co-evolution in their interactions. In this section, we see that the Mediterranean Painted Frog represents, physiologically, a unique addition to the local tadpole guild, characterized by a very rapid and consumption-oriented growth, and that these tadpoles are able also to increase the quality and quantity of its food intake when these possibilities are provided. This poses these tadpoles as potentially very disruptive of the natural dynamics of native anurans. Concerning the competitive relationship between the Natterjack Toad (*Epidalea calamita)* and the Mediterranean Painted Frog, we can observe that it changes according to previous evolutionary history of populations. In this sense, populations of the native toad that have had a greater number of generations of contact with the invasive frog are able to inflict a greater competitive distress on the invasive tadpoles, pointing at an optimistic long-term scenario. Both competitors also differ mildly in their breeding preferences, and direct competitor avoidance seems also plausible according to the results here presented. Nevertheless, both species seem forced to end up competing very often at a pond level. Within ponds, the trophic position of both species can respond either to evolutionary or to ecological patterns according to the data gathered hitherto. Information from the most genetically differentiated population with isotopic samplings – La Jonquera –, will probably disentangle which is the dominant pattern.

Finally, in **"PART C: Tadpoles of *Discoglossus pictus* as prey for aquatic native predators"** I present two studies which examine the degree in which tadpoles modify its phenotype in front of a chosen array of – native and invasive – predators, including behaviour and several aspects of morphology. Linked to these changes, it is also studied how these tadpoles are affected by them in the present – survival – and in subsequent phases of its life-history. Interestingly, the invasive frog presents a pattern of inducible defences which would be expected for a native anuran, responding to all native predators effectively, lacking its responses in front of introduced or invasive predators. Given the apparent lack of naiveté in front of native predators, additional predation costs for the invasive frog in comparison to native species should be discarded.

Globally, the complex evolutionary history of the local anuran assemblages poses the Mediterranean Painted Frog as a very particular case of invasive species, in which the echo of previous recurrent shared evolutionary history among similar competitor / predator species is probably present. Hence, the Mediterranean Painted Frog is a good model for ecological and evolutionary studies reaching beyond invasion biology, and signals that an evolutionary focus is probably essential for realistic long-term predictions of the impacts and ecological role of any invasive species.

RESUM GENERAL

Estem una època sense precedents, a nivell mundial, en relació al ritme al qual s'introdueixen noves espècies al·lòctones arreu. Algunes d'aquestes es converteixen en invasores, i les pèrdues econòmiques i ecològiques relacionades amb aquests processos poden acabar essent aclaparadores. D'acord amb aquesta tendència, el nombre d'estudis científics centrats en invasions biològiques s'ha incrementat molt en els últims anys. No obstant això, tot i que han estat àmpliament estudiades des del punt de vista de l'ecologia aplicada i de la conservació de la biodiversitat, les invasions biològiques s'han passat més per alt des del punt de vista de l'ecologia i biologia evolutiva més fonamental. Tot i les grans oportunitats creades per a l'estudi de l'evolució fenotípica o genètica, les invasions biològiques estan siguent aparentment infrautilitzades com a laboratoris naturals per posar a prova certs aspectes teòrics essencials per la biologia. Això implica no només una pèrdua d'oportunitats en aquest sentit, sinó que també possiblement pot acabar deixant tant la biologia de la conservació com la gestió aplicada desarmades per entendre les causes darrere de la imprevisibilitat de les conseqüències, dinàmiques, patrons i processos que les espècies invasores creen i al quals també se sotmeten.

En aquest sentit, els nou estudis presentats en aquesta tesi intenten donar una punt de vista globalment evolutiu – a part d'ecològic – sobre la present invasió de la granota pintada (*Discoglossus pictus*) a Europa. Aquesta granota és originària del Nord d'Àfrica i es va introduir des d'Algèria a Banyuls de la Marenda (Catalunya Nord) fa aproximadament un segle. Els individus introduïts van superar tots els obstacles possibles per establir poblacions auto-sostenibles i arribar a l'etapa de propagació. Per tant, aquestes poblacions de granota pintada han de ser referides com a invasores actualment. Així doncs, després d'expandir-se la granota pintada és avui dia present en una àmplia franja contínua costanera d'aproximadament 250 quilòmetres, des de Montpeller com a extrem nord, fins a Sant Celoni com a extrem sud, tot i que hi ha també poblacions aïllades al voltant de Barcelona. L'espècie invasora sembla haver accelerat el seu ritme d'expansió en les últimes dècades, i ara està colonitzant contínuament noves àrees a una velocitat aproximada de dos o tres quilòmetres per any.

Els estudis que es presenten estan ordenats en tres parts, que funcionen com blocs temàtics. Dins dels blocs, els treballs s'ordenen per formar una línia científica coherent. L'objectiu de la **"PART A: Variabilitat intraespecífica al llarg de la distribució invasora de *Discoglossus pictus*"** és el d'examinar – a un nivell fenotípic i genòmic – possibles diferències entre poblacions, sobretot a dins de la distribució invasora de l'espècie. L'objectiu doncs, és explorar la diversificació evolutiva que pot estar patint aquesta espècie, ja sigui per causes intrínseques – per exemple, la mateixa dinàmica d'expansió – o per factors extrínsecs – limitacions ambientals. En aquest cas, s'observa que la granota pintada no forma un conjunt uniforme de poblacions en la seva distribució invasora a Europa, ni des d'un de vista fenotípic ni genòmic: la mida mitjana i els trets de la història vital dels adults es relacionen amb la precipitació mitjana anual de cada zona, mentre que hi ha un cert grau de diferenciació genètica entre les poblacions que ha estat molt probablement creat pel procés d'expansió en sí.

L'objectiu de la **"PART B: Competència intra-gremi en capgrossos de *Discoglossus pictus*"** és estudiar i analitzar el paper dels capgrossos de *Discoglossus pictus* com a potencials competidors per les larves d'anurs autòctons. En aquest bloc hi ha cinc estudis que van des d'avaluacions dels trets fisiològics de tota la comunitat de capgrossos, a un enfocament profund en els patrons de reproducció i d'ecologia tròfica en una relació competitiva autòcton-invasor – *Epidalea calamita* versus *Discoglossus pictus* –, així com avaluacions de la influència de la co-evolució prèvia de cada població en les seves interaccions. En aquesta secció, veiem que la granota pintada representa, fisiològicament, una addició única al gremi local de capgrossos, que es caracteritza per un creixement molt ràpid i orientat al consum, i que els seus capgrossos són capaços d'augmentar la qualitat i quantitat de la seva ingesta d'aliments quan n'hi ha possibilitats. Això planteja aquests capgrossos com a potencialment molt perjudicials per la dinàmica natural dels amfibis autòctons. Pel que fa a la relació de competència entre el gripau corredor (*Epidalea calamita*) i la granota pintada mediterrània, podem observar que canvia d'acord amb la història evolutiva de les poblacions. En aquest sentit, els capgrossos de les poblacions del gripau autòcton que han tingut un major nombre de generacions de contacte amb la granota invasora són capaços d'infligir un major estrès competitiu en els capgrossos invasors, apuntant un escenari optimista a llarg termini en aquest cas. Els dos competidors també difereixen lleugerament en les seves preferències de llocs de cria, mentre que l'evitació directe entre ells també sembla plausible, segons els resultats aquí presentats. No obstant això, les dues espècies semblen estar obligades a acabar competint molt sovint a nivell de bassa. Dins de cada bassa, la posició tròfica d'ambdues espècies de capgròs pot respondre tant a patrons evolutius com a patrons ecològics amb les dades recollides fins ara. Les dades de la població més diferenciada genèticament – La Jonquera – probablement seran bàsiques per desemmascarar el patró dominant.

Finalment, a la **"Part C: Capgrossos de *Discoglossus pictus* com a presa per depredadors aquàtics autòctons"** s'hi presenten dos estudis que examinen el grau en què els capgrossos modifiquen el seu fenotip davant d'una matriu de depredadors – tant autòctons com introduïts –, incloent-hi el seu comportament i morfologia, entre d'altres aspectes. Lligat a aquests canvis, també se n'estudien els seus efectes tant en el mateix estadi de desenvolupament – p. ex. la supervivència –, com en posteriors fases del seu cicle vital. Curiosament, la granota invasora presenta un patró de defenses induïbles que s'emmotlla al que s'esperaria per a un anur autòcton, responent a tots els depredadors nadius amb eficàcia, però mancant les respostes davant de depredadors introduïts o invasors. Això ens fa descartar costos addicionals deguts a la falta de coneixement previ dels depredadors autòctons per part de la granota invasora en comparació amb les espècies natives.

A nivell global, la complexa història evolutiva dels conjunts d'anurs del mediterrani occidental posa la granota pintada com un cas molt particular d'espècie invasora, en la que hi ha efectes actuals d'una història evolutiva compartida prèvia amb espècies similars. Un enfocament evolutiu és probablement essencial per tal de generar prediccions realistes a llarg termini dels impactes i del paper ecològic de les espècies invasores.

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INTRODUCTION

GENERAL INTRODUCTION

1. INVASIVE ALIEN SPECIES:

Basic concepts and framework.

Perhaps one of the most visible consequences of economic globalization on natural systems is what could be named as the “biological globalization”. Concerning terrestrial and freshwater animals, aspects like sport fishing and hunting, pet and experimental trade, even including management of local plagues with allochthonous species, add to the increased transport and communication worldwide to increase exponentially the number of releases of alien animals in the environment (Hulme *et al.* 2008). Only a part of the “released” species survive the new environment, a smaller part gets to reproduce in the wild and even a tinier fraction establish self-sustainable populations. Among them, in turn, only some of them have the ability to advance even forward and become “invasive alien species”, creating in some cases serious environmental and economic losses (Vilà *et al.* 2010).

But, what is exactly an invasive species? At which point do we need to consider that a species has become invasive? Are all invasive species necessarily harmful or hazardous for the native biota? Perhaps the first step to undertake a serious discussion on this issue is to clearly define a series of concepts. Although several frameworks to define “invasive” – and several other related concepts – exist in the scientific literature (e.g. Williamson 1996, Williamson & Fitter 1996, Alpert *et al.* 2000, Richardson *et al.* 2000, 2011) perhaps the most complete, comprehensive and useful up to date – in my opinion –, is the recently proposed by Blackburn *et al.* (2011, 2014). Among other important details, this framework clearly differentiates the population dynamics of a species or population (Blackburn *et al.* 2011) from their several possible impacts (Blackburn *et al.* 2014). Thus, the framework does not assume a coincidence among both processes, and allows to classify, for instance, a naturalized – but not yet invasive – species as moderately dangerous due to its competitive or predatory impact on natives, and a clearly invasive species as a minor concern for native biota.

According to this framework (Fig. 1), a “*fully invasive species*” has a “*self-sustaining population in the wild beyond limits of its native range*” with “*individuals dispersing, surviving and reproducing at multiple sites across a greater or lesser spectrum of habitats and extent of* *occurrence*". This differentiates invasive species from simply "naturalized" or "established" species which have not yet – if they indeed can – started to expand its range, even though self-sustaining populations in the wild. Thus, following this criteria, independently from the assessment of the possible impacts of the species of interest here in this thesis – the Mediterranean Painted Frog, *Discoglossus pictus* –, we should classify it as "invasive" simply by is geographic and population dynamics (see section 4 of this introduction for more detailed information).

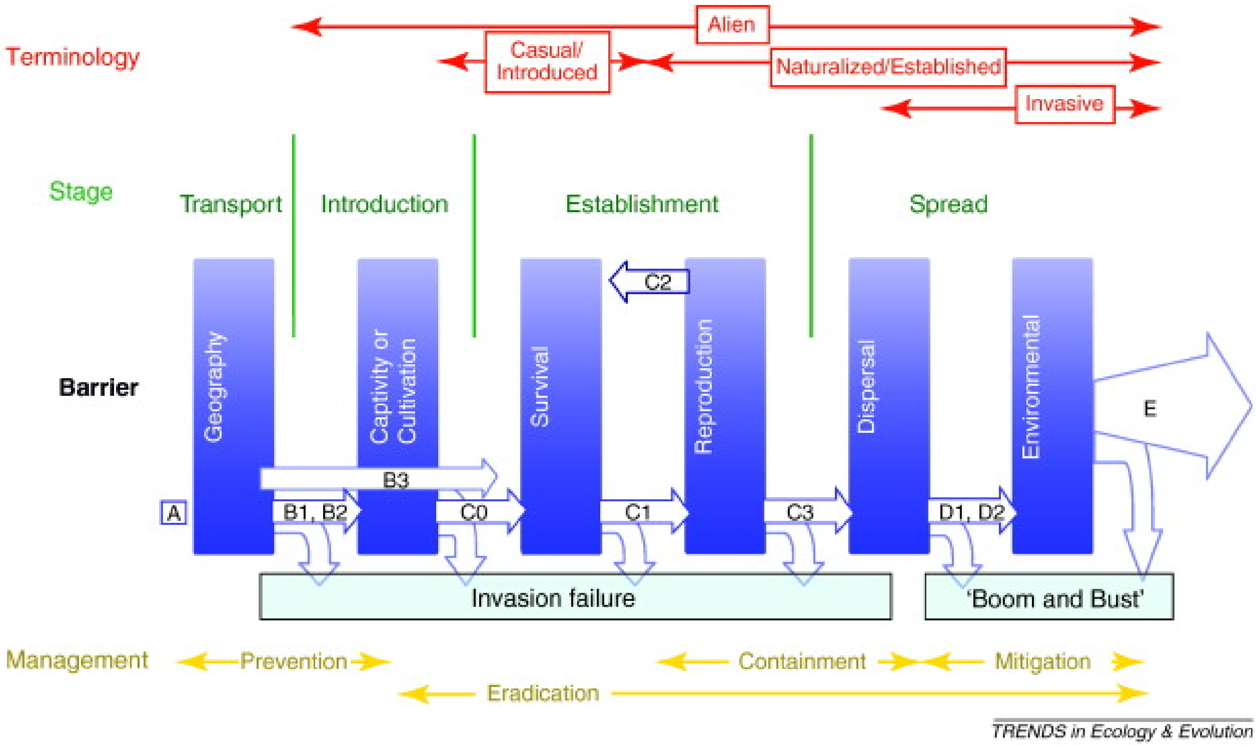


Figure 1. Simplified scheme of the unified framework for biological invasions, from Blackburn (2011).

As a second part of the classification, and mainly for management purposes, as an alien species it should be also categorized according to the magnitude of its environmental impacts (Blackburn *et al.* 2014). Therefore, since in this second step species are not categorized simply based on geographic dynamics and distributions, for this second categorization we will need a series of scientific studies to serve as an assessment of their possible impacts. In this case, for *D. pictus*, this means that the reader will have to wait until the last part of the Discussion of this thesis (in "5. Future directions and management perspectives") to know the proposed categorisation of the species in terms of the magnitude of its impacts.

2. INVASION BIOLOGY and EVOLUTIONARY ECOLOGY:

The temporal and spatial point of view, or the necessary communication between fundamental theory and management practice.

Invasion biology has become an essential field of study for conservation biology and ecology nowadays (Richardson *et al.* 2011, Richardson & Ricciardi 2013, HilleRisLambers *et al.* 2013). The rates at which allochthonous species are introduced worldwide – and at which some of them become invasive – are unprecedented (Vilà *et al.* 2010, Richardson & Ricciardi 2013), and the economic and ecological losses linked to these processes are overwhelming (Perrings *et al.* 2000, Pimetel *et al.* 2005, Vilà *et al.* 2010, Richardson & Ricciardi 2013, Simberloff *et al.* 2013). Accordingly, the number of scientific studies dealing with – among other topics – the monitoring, ecological role and management of invasive species has increased constantly in the last two decades (Fig. 1). The literature on biological invasions is now enormous (Lowry *et al.* 2013), and managers, policy makers, and the general public have become increasingly aware of the economic and ecological issues raised.

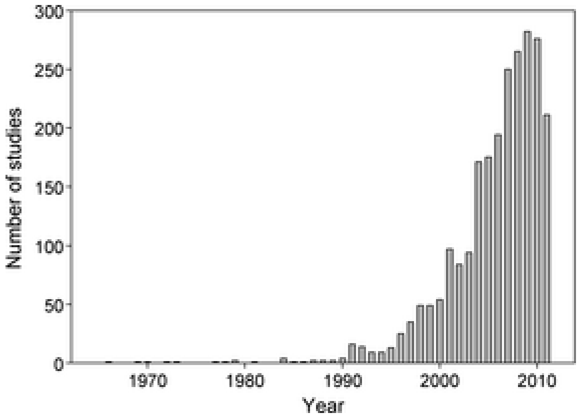


Fig. 1 The number of studies published per year related to biological invasions indexed on the Web of Science (2011 only includes records through September). Extracted from Lowry *et al.* 2013.

However, although largely studied from an applied ecology and conservation point of view, biological invasions have been more overlooked by fundamental ecology and evolutionary biology (HilleRisLambers 2013, Lowry *et al.* 2013, Moran & Alexander 2014). That is, despite great opportunities in terms of “accidental experiments” for the study of phenotypic or genetic evolution (Sax *et al.* 2007, HilleRisLambers 2013, Moran & Alexander 2014), biological invasions are still seemingly underused as natural laboratories to test essential biological theory (Fig. 2).

Concerning fundamental principles of evolutionary biology, it is important to note that Cox (2004) made a first compilation of reported cases of fast evolution linked to biological invasions in his book *Alien Species and Evolution* only thirteen years ago. Importantly, the book states in its preface that *"Despite the numerous, well-publicized cases of evolutionary shifts […] the public at large remains largely unaware of the accelerated pace of evolution resulting from the massive introduction of species to new geographical regions. In particular, systems of governmental regulation of alien species tend to regard them as stable genetic entities. In education* [George W. Cox may speak for the USA, but this largely applies to other countries]*, the concept of evolution is treated as a controversial theory of the history of life on earth, rather than an on-going process of great relevance to human welfare".* Luckily, from then, an increasing number of scientific studies related to biological invasions incorporate evolution – or what could be classified as evolutionary ecology – in their principles. However, as for 2013, only 1-2% of the studies published in evolutionary journals related to invasion biology somehow (Fig. 3B). This means not only a loss of opportunities to test basic principles for evolutionary biology and evolutionary ecology, but has also left conservation biology unarmed to understand the causes behind the unpredictability of the outcomes and dynamics of biological invasions, or the patterns and processes that invasive species create and undergo. Worryingly, the lack of a (co-)evolutionary point of view in the scientific literature of biological invasions ends up translating also to the the management policies – for example, as dogmatic approaches (Martínez-Abraín & Oro 2013) – and to the public opinion and media, which usually exaggerate, prejudge, mix or simplify several aspects of the issue.

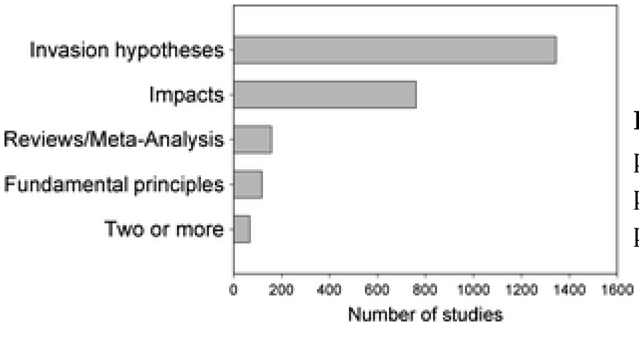


Fig. 2 The focus of the studies on biological invasions published until September 2011. "Fundamental principles" lies at the fourth – *i.e.* last – numerical position. Extracted from Lowry *et al.* 2013.

Thus, despite the progressive incorporation of evolution into invasion biology, a broad awareness of the potential importance of the field is far from present. Probably due to a lack of abundance (Fig, 3B) and influence (Fig. 3C) of evolutionary ecology or evolutionary biology on the field of biological invasions, there is still a general lack of temporal or co-evolutionary point of view in the applied management of allochthonous species. Recent studies and ongoing experimentation linking rapid evolution to management – notable cases like studies on the Cane Toad *Rhinella marina* in Australia (Kelly & Phillips 2015, Phillips *et al.* 2016), which will be further commented later – shed hope in this sense, but an alarming number of studies still consider together ancient, mildy recent and onsetting invasions, assuming their impacts to be independent from previous evolutionary history (also discussed in Martínez-Abraín & Oro 2013).

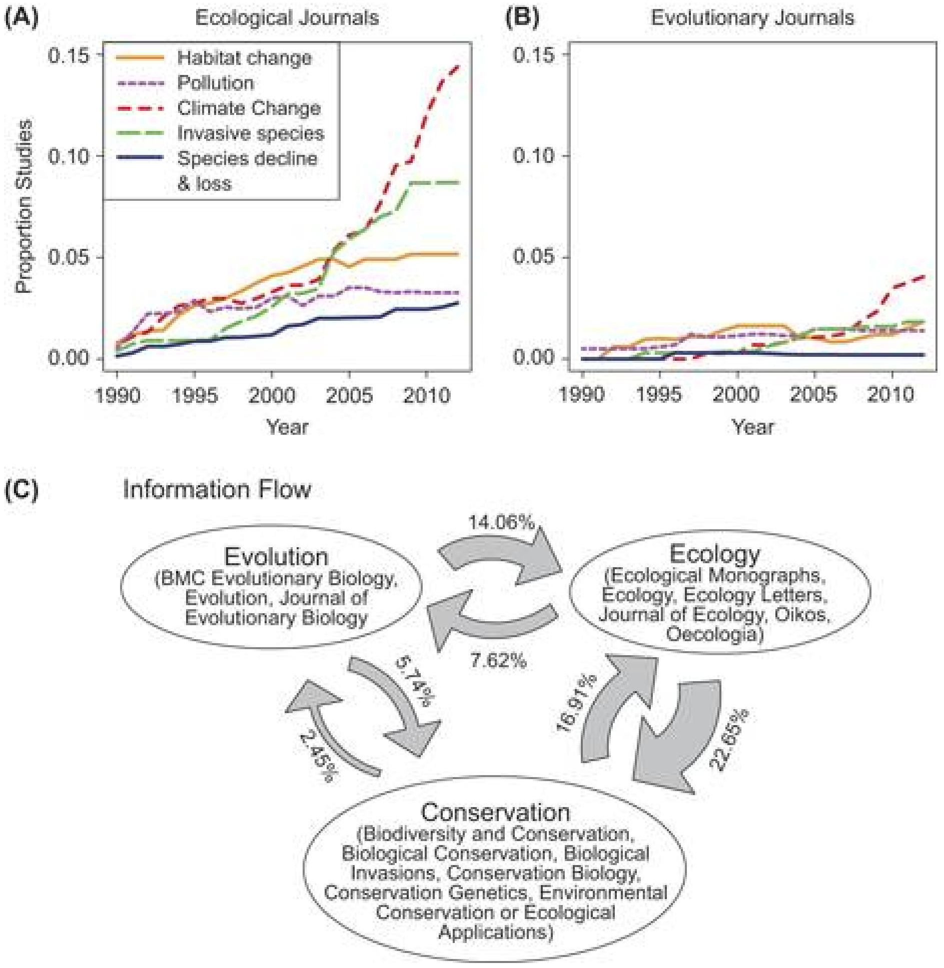


Figure 3. Proportion of studies in ecological (A) and evolutionary (B) journals including specific keywords in the title, abstract or keyword list that relate to the five global change factors. (C) The flow of information (according to number of citations) between ‘basic’ and ‘applied’ journals. The size of each arrow and percentage indicates the proportion of articles in one subdiscipline (that the arrow is pointing to) that cite at least one article published in journals in the other subdiscipline (that the arrow is pointing from). Extracted from HilleRisLambers *et al.* 2013.

In this sense, the nine studies presented in this thesis try to globally give an evolutionary – apart from ecologic – point of view on the ongoing invasion of the Mediterranean Painted Frog. Hopefully, this will serve to reflect the real complexity behind the ecological role and possible impacts this species may have on the native biota. Summing up, at least in the opinion of the PhD candidate here writing this words, the (co-)evolutionary point of view is a mandatory step that should be added in our to-do list for a correct assessment, discussion and management of any future invasion. More importantly, instead of “useful simplifications” – and even white lies –, I believe the general public deserves to know the real story, to understand the complexity behind the categorization and the management of some introduced species.

3. INVASIVE ANURANS:

A few species, some serious impacts.

Among animals, and among vertebrates, anurans are not usually listed as one of the most problematic “potentially invasive” taxonomic group (Vilà *et al.* 2010). Thus, with some important taxonomic and biogeographical exceptions (Bomford *et al.* 2009, Tingley *et al.* 2010, Van Boxclaer *et al.* 2010), anuran species do not easily become introduced, established, and eventually invasive. However, of the few species that have managed to overcome all the possible barriers to invasion, some have become, indeed, largely problematic in ecological and even economic terms (Kraus *et al.* 2009).

In Europe, apart from the species studied in this thesis, there are other cases of established alien species of anurans, with some invasive populations that need to be shortly commented. Hence, in Europe we already have established populations of two globally invasive anurans: the African Clawed Frog (*Xenopus laevis*) and the American Bullfrog (*Lithobates catesbeianus*). The African Clawed Frog is native from sub-Saharan Africa and was commonly exported in the second half of the 20th century to be used in laboratory tests or, lately, as a pet (Tinsley & McCoid 1996). Nowadays it holds established and invasive populations in the wild in several places around the world, including Chile, Sicily and Portugal (Lobos & Jaksic 2005, Faraone *et al.* 2008, Rebelo *et al.* 2010). The main concerns for this species are its roles as possible predator and competitor of native anuran species (Lobos & Jaksic 2005), and as probable carrier of the amphibian pathogen fungus *Batrachochytrium dendrobatidis,* the agent of chytridiomycosis (Weldon 2002). On its side, the American Bullfrog (*Lithobates catesbeianus*) is native to eastern North America, being introduced during the last century in over 40 countries in four different continents (Ficetola *et al.* 2007a). At least 25 independent introductions have already occurred in Europe, and nowadays Belgium, France, Germany, Greece, and Italy support established or invasive populations of the species (Ficetola *et al.* 2007b). Large tadpoles of this species usually outcompete natives and force them to alter their preferred dietary preferences or microhabitats, while adults are generalist predators that can prey on other juvenile and adult amphibians (Blaustein & Kiesecker 2002, Kats & Ferrer 2003). Moreover, the species can be a carrier and vector of *Batrachochytrium dendrobatidis* (Miaud *et al.* 2016) as well. Bullfrogs are now usually considered to be among the worst invasive species worldwide (Lowe *et al.* 2000, Vilà *et al.* 2010). Accordingly, eradication and containment plans of this species are a priority for amphibian conservation in several countries (Ficetola *et al.* 2007b).

Outside Europe, there are two other cases which deserve to be commented here, namely the Common Coquí (*Eleutherodactylus coqui*), and the Cane Toad (*Rhinella marina*). The Common Coquí is a mainly terrestrial frog native to Puerto Rico, that became established in the late 1980s on the Hawaiian Islands (Kraus *et al.* 1999). It was introduced via the horticulture trade and expanded very rapidly: nowadays from 250 to 300 populations are known on all four main islands. This frog can attain amazingly high densities in some Hawaiian areas – more than 50 000 frogs/ha (Woolbright *et al.* 2006) – consuming approximately 350 000 invertebrate prey items per night and hectare (Beard 2007). Thus, since invertebrates comprise the large majority of the endemic fauna of the islands (Beard & Pitt 2005), the main ecological impact this species may pose is as a predator. In addition, recent studies highlight that in the densest populations the frog can even alter nutrient cycling dynamics (Sin *et al.* 2008). Concerning economic losses, due to its loud mating call (80–90 dB at 0.5m) it has also affected Hawaii tourism industry (Kraus & Campbell 2002).

Finally, the Cane Toad (*Rhinella (Bufo) marina*) is a paradigmatic case of invasive species, and is perhaps one of the most diversely and intensively studied anuran invader. These large toads, native to the neotropics, were introduced to NE Australia (from French Guiana, via Puerto Rico and Hawaii) in 1935 (Slade & Moritz 1998, Lever 2001). From then, they expanded at an increasing rate across Australia (Phillips *et al.*, 2006; Urban *et al.*, 2008), at the same time increasing its range of suitable habitats (Urban *et al.* 2007). The toad is a competitor to other tadpoles (Williamson 1999, Cabrera-Guzmán *et al.* 2013a,b), and venomous as adult to most native potential predators (Phillips & Shine 2004). Most interestingly, the evolutionary point of view has been very important in the study of this biological invasion. Thus, several studies demonstrate changing interactions, impacts, speed of invasion and morphology of the invasive toad (Phillips & Shine 2004, Phillips & Shine 2005, Phillips *et al.* 2006, Ducatez *et al.* 2016) along its geography. In addition, the use of evolutionary differences among long-established and expanding populations – e.g. using targeted gene flow from non-dispersive populations to dispersive populations (Kelly & Phillips 2015, Phillips *et al.* 2016) – is already projected as a possible key to halt its invasion. As another positive addition of holding an evolutionary point of view, based on phylogenetic similarities in "invasiveness" among Bufonids (Van Boxclaer *et al.* 2010), similar species have been readily targeted as potentially catastrophic for native biota in other locations, like Madagascar (Kolby *et al.* 2014).

4. THE MEDITERRANEAN PAINTED FROG (*Discoglossus pictus*) IN EUROPE

4.1. Phylogenetic and palaeontological context

If this thesis is indeed going to discuss, among other aspects, the effects of previous co-evolution in the ecological relationships, it might be useful to highlight several potentially important aspects of the recent evolutionary history of the study species, from phylogeny and past distribution of the Discoglossinae subfamily to the present and recent distribution of the genus *Discoglossus*.

The Discoglossinae subfamily is grouped within the Alytidae family, which forms an ancient group not very speciose nowadays. The Discoglossinae subfamily nowadays only comprises the genus *Discoglossus* and the re-discovered "living fossil" *Latonia nigriventer*, the last living species of the genus *Latonia* (Biton *et al.* 2013). All species in the subfamily are morphologically similar – and named accordingly: Painted Frogs –, but several genetic and phenotypic characters allow to distinguish both genera, placing nowadays the genus *Discoglossus* as native only to the Western Mediterranean Basin (Fig. 4, Pabijan *et al.* 2012, Biton *et al.* 2013). Within *Discoglossus*, all currently recognized populations were long considered to be a single species because of their morphological similarity. However, phenotypic – including morphology, osteology and advertisement calls – allozymatic, and genetic data helped to delimit and corroborate the present species tree (Vences & Glaw 1996, Zangari *et al.* 2006, Pabijan *et al.* 2012). The present consensus (Fig. 4) recognizes among five and six species – depending on the nature of the Iberian complex – all with parapatric or allopatric distributions excepting Corsica, the only area where two *Discoglossus* species coexist.

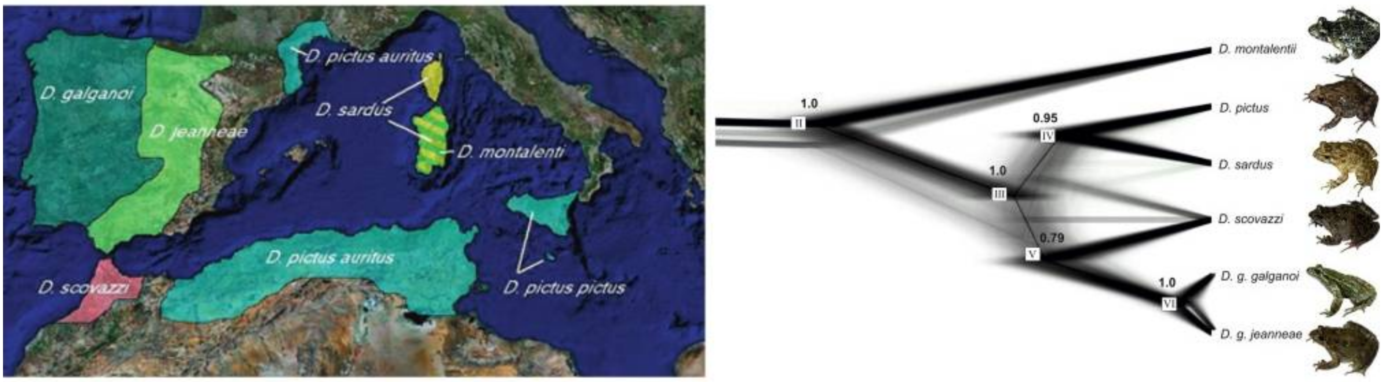


Figure 4. Left: Current distribution of the genus *Discoglossus*, extracted from Montori *et al.* (2007). Note that *D. montalentii* should be placed in Corsica (errata). **Right:** Phylogenetic relationships among the genus *Discoglossus*, represented as a "cloudogram", extracted from Pabijan *et al.* (2012). These are posterior density of 2700 species trees, colouring more densely the configurations where many trees agree. The tree with highest posterior probability – with values indicating posterior probabilities – is also represented within the set. Note that, while the left panel considers two species of Iberian *Discoglossus*, the most recent studies (right) consider them as subspecies of *Discoglossus galganoi*.

However, it might be obvious to the reader that, most probably, the distribution of the genus *Discoglossus* has not always been the presently observed (Biton *et al.* 2013, Torres-Roig *et al.* 2017). Actually, there are hundreds of locations where Discoglossinae fossils (*Discoglossus* and *Latonia*) have been found far from their present distributions (Fig. 5). Concerning *Discoglossus*, curious findings include Miocene records from Hungary, Oligocene records from Belgium and the central Pyrenees, Pliocene records from the Medes Islands – within the area of study of this thesis –, and Pleistocene records from Crete, Mallorca, Southern France, and the Mediterranean coast of the Iberian Penninsula (Biton *et al.* 2013).

Thus, although Pleistocene fossils strictly within the current invasion area of *Discoglossus pictus* are lacking, the area is surrounded by its presence (Southern France, Mallorca, Centre and the eastern coast of the Iberian Peninsula), arguably dating the absence of *Discoglossus* in the study area not earlier from 2.5 my - 12000 years ago. The western Mediterranean area has had a very complex recent geology (Hsü *et al.* 1973, Rosenbaum *et al.* 2002), which has greatly affected the local herpetofaunal assemblages and biogeography, recurrently isolating and allowing secondary contacts among different areas (Rage & Roček 2003). This, in combination with several important climate changes (Rage & Roček 2003) has shaped the current distribution not only of the painted frogs, but also of their competitors and predators. Concerning for example, the main native competitors of *Discoglossus pictus* – which are *Epidalea (Bufo) calamita* and *Pelodytes punctatus*, as will be discussed – it is important to note that Discoglossidae, Pelodytidae, and Bufonidae have disappeared and reappeared – and therefore competed – several times in Europe during the tertiary

(Rage & Roček 2003). In fact, considering the Cenozoic biogeography of the area as a whole, the lack of a species of *Discoglossus* in the study area might represent more an exception than a common biogeographical situation.

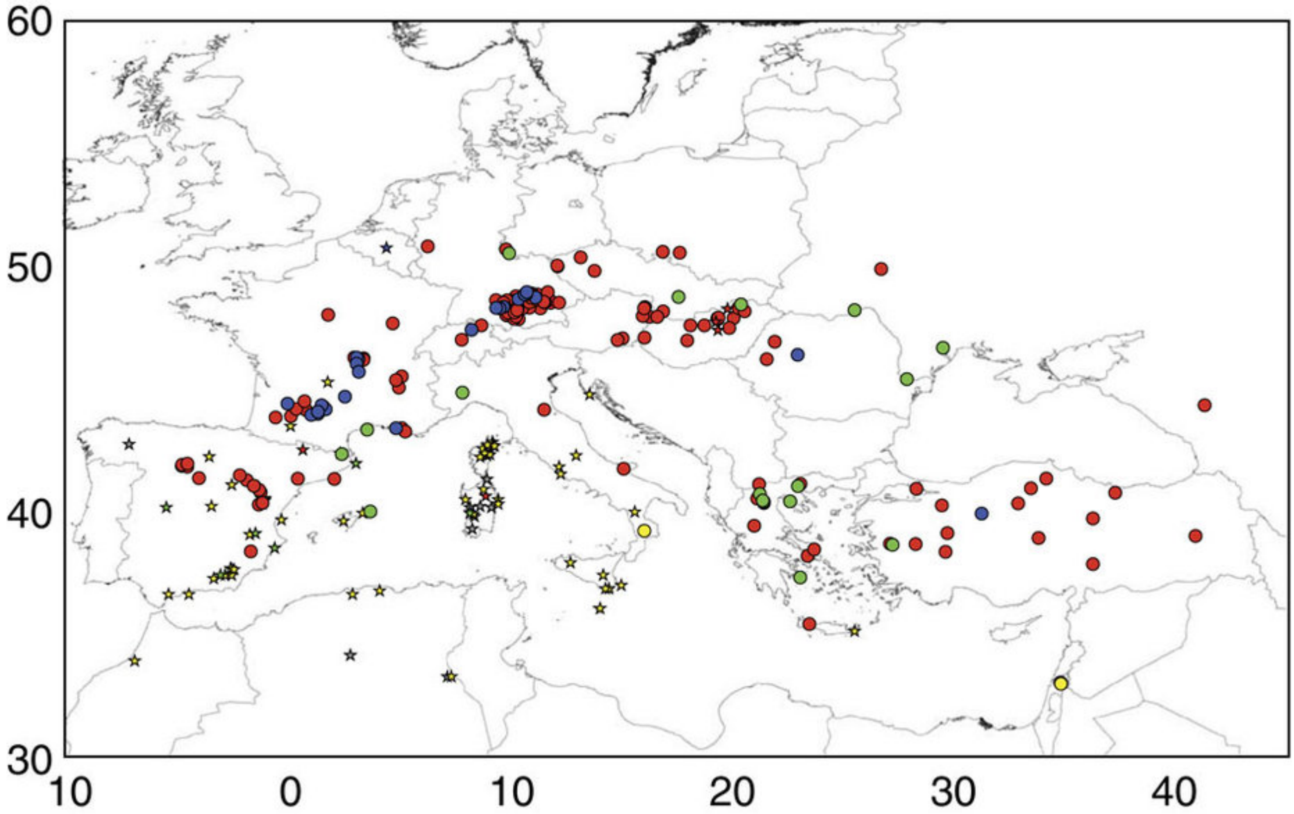


Figure 5. Distribution of Discoglossinae fossils in Europe. Shapes denote genera (star=*Discoglossus*, circle=*Latonia*), and colours denote geological periods (red=Miocene, blue=Oligocene, green=Pliocene, yellow=Pleistocene and grey=Holocene). Extracted from Biton *et al.* (2013).

4.2. *Discoglossus pictus*: distribution, invasion history, and general biology.

Nowadays, within the *Discoglossus* genus, the Mediterranean painted frog (*Discoglossus pictus*, Fig. 6) is considered to comprise the populations native to the NE tip of Morocco, Northern Algeria, Tunisia, Sicily, Malta and Gozo (Zangari *et al.* 2006). The native distribution of the species is therefore limited either by the Mediterranean Sea or an excessive aridity – Sahara desert, or the Moulouya valley in Morocco. In its native area, according to Ben Hassine & Nouira (2009, 2012) and Escoriza & Boix (2014), the species is an habitat generalist that can reproduce all year long and in most available water bodies.

Figure 6. Left: Male adult of *Discoglossus pictus*. **Right:** group of adults gathered for reproduction in a temporary pond. Photographs: Eudald Pujol Buxó.

Most probably due to its use for experiments on reproductive biology (Knoepffler 1962, ACEMAV 2003), the species has been introduced in mainland France at least four times during the late 19th and early 20th centuries (Fretey *et al.* 2003): in Saint-Hilaire (Val-de-Marne, near Paris), in Amboise (Indre-et-Loire), in the Jardin des Plantes (central Paris), and in Banyuls de la Marenda (Languedoc-Roussillon). Among them, the species seems to have disappeared from the first two locations, but it is still possibly present in the Jardin des Plantes in Paris as a naturalized and enclosed population (Fretey *et al.* 2003), and became also established in Banyuls de la Marenda (Wintrebert 1908). According to genetic studies, populations from Banyuls are most related to the native ones from northern Algeria (Zangari *et al.* 2006). These populations expanded its range and are nowadays present on a wide coastal strip of approximately 250km from Montpellier (SE France) to Sant Celoni (Barcelona, NE Spain). The expansion seems to have accelerated its pace in the last decades (Geniez & Cheylan 2012, Montori *et al.* 2007) as the species is now continuously colonizing new areas at an approximate rate of two or three km per year (Montori *et al.* 2007). Thus, according to the aforementioned unified framework for biological invasions (Blackburn *et al.* 2011), these populations of *D. pictus* have overcome all the possible barriers to reach the spread stage, and should clearly be referred to as invasive.

The species is largely terrestrial as an adult, becoming more aquatic during reproductive season when it is usually found near the water bodies. Adults feed mainly on terrestrial – but including also aquatic – invertebrates and arthropods (Montori *et al.* 2007, Ben Hassine & Nouira 2009). According to Knoepffler (1962), adults will try to capture nearly any prey not excessively large that moves near enough the head of the animal, including conspecific juveniles. Concerning the larval phase, which is the most studied here in this thesis, the species has the ability to reach metamorphosis in a very short time (as short as 21 days, García París *et al.* 2004), with great plastic abilities in terms of the duration of the larval period, even overwintering as a tadpole (*pers. obs.*). Accordingly, and similarly as in its native range, the invasive populations show great versatility in its choice of reproduction points (Montori *et al.* 2007, Richter-Boix *et al.* 2013, Escoriza & Boix 2014, Escoriza *et al.* 2014). Interestingly, some comparative studies suggest that the breadth of pond choice in the invasion area could be indeed narrower than in the native area, choosing in Europe significantly smaller and shallower ponds than the randomly available (Escoriza & Boix 2014). Similar comparative studies nonetheless (Escoriza *et al.* 2014), although observing the same tendency, do not find them as significant. In any case, all published literature to date highlights the ability or the preference of invasive populations of *Discoglossus pictus* to breed in ephemeral and temporary ponds, which have become the most visibly and commonly occupied (Montori 2007, Richter-Boix *et al.* 2013, Escoriza & Boix 2014, Escoriza *et al.* 2014).

Finally it is important to remark several previous similar – or important – studies to the ones presented here in this thesis. Hence, contrarily to its role as prey to native and invasive species – which had not been explored earlier – before and during this thesis several other studies have explored the possible impacts of *Discoglossus pictus* on the native anuran assemblages, that is, its potential competitors (Montori *et al.* 2007, Escoriza & Boix 2012, Richter-Boix *et al.* 2013, Escoriza & Boix 2014, Escoriza *et al.* 2014, San Sebastian *et al.* 2015). In this sense, all published community-wide studies to date highlight that, in terms of ecological overlap in pond occupancy – therefore, in their larval form –, the most possibly affected species are the Common Parsley Frog *Pelodytes punctatus* and the Natterjack Toad *Epidalea (Bufo) calamita* (Montori *et al.* 2007, Richter-Boix *et al.* 2013, Fig. 7).

Among these two species, the Natterjack Toad seems the most probably affected by the invasion of the frog due to its exaggerated ecological similarities with the invasive species (Escoriza & Boix 2012, Richter-Boix *et al.* 2013, Escoriza & Boix 2014), a fact that will be further studied and discussed in this thesis. Concerning microhabitat within ponds, larvae are assumed from its morphological features to be largely bottom-dwelling (Escoriza & Boix 2012), coinciding again with both *P. punctatus* and *E. calamita*, among others. In fact, very recent studies using stable isotopes – which encouraged the project of Chapter 7 of this thesis – show possible effects of the invasive tadpoles on the dietary habits of the larvae of the native toad in natural ponds (San

Sebastian *et al.* 2015). As a further addition, concerning adult habits, the same studies (Montori *et al.* 2007) also indicate that the species with a major dietary overlap with *D. pictus* is, again, the Natterjack Toad. However, this might simply be correlated with the overlap in pond use – and therefore terrestrial habitat – during the reproductive season.

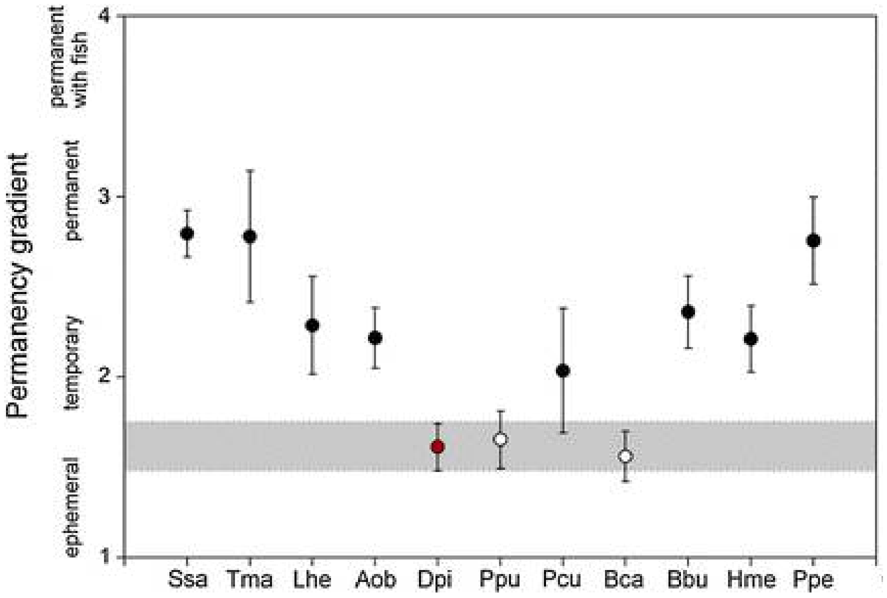


Figure 7. Pond preferences for the amphibian species present in the study area (mean ± SE). *Dashed lines* limit the overlap zone with *Discoglossus pictus*, species with the highest overlap with the invasive frog are shown as *white circles*. Abbreviations: Ssa: *Salamandra salamandra*; Tma: *Triturus marmoratus*; Lhe: *Lissotriton helveticus*; Aob: *Alytes obstetricans*; Dpi: *Discoglossus pictus*; Ppu: *Pelodytes punctatus*; Pcu: *Pelobates cultripes*; Bca: *Bufo calamita*; Bbu: *Bufo bufo*; Hme: *Hyla meridionalis*; Ppe: *Pelophylax perezi*. Extracted and adapted from Richter-Boix et al. (2013).

All these previous works mainly served as the empirical basis behind the decision to focus on the deep study of the competitive relationship between *Discoglossus pictus* and *Epidalea calamita*, leading to the series of works presented in **PART B** of this thesis.

AIMS AND OBJECTIVES

AIMS AND OBJECTIVES

If we were to pinpoint this PhD thesis in the hypothetical space where all biological areas of study lie, we should find it, perhaps, on the multivariate mean point among zoology, ecology, and invasion and evolutionary biology. Indeed, this is unavoidably a zoological thesis – nearly all organisms that appear in this thesis are animals –, which focuses on the effects of the interplay between ecology and evolution on the ongoing invasion of *Discoglossus pictus*. Thus, the reader may find from discussions regarding the effects of evolution on the actual ecology of the frog, to arguments on the effects of its ecology on evolutionary processes within its invasive range.

As the reader may have observed in the table of contents, the nine studies are ordered in three parts, which function as thematic blocks. Within blocks, studies are ordered to form an understandable scientific pipeline. Curiously – or predictably, in fact – this coincides almost perfectly with a chronological record inside each part, reflecting also an implacable tendency of the PhD candidate to leave behind purely zoological or ecological studies to explore more evolutionary questions, integrating them in a current view of zoology. Finally, in the Annex, a series of small publications also related to the topic of interest – and therefore important for the global discussion – are presented. These are also authored or co-authored by the PhD candidate but considered, due to low personal implication or more meagre scientific importance, not to be enough relevant to become chapters. Anyhow, these articles have applied interest in the management of the species studied here and are useful for the final discussion of the thesis.

The aim of **PART A: Intraspecific variation along the invasive range of *Discoglossus pictus*** is to examine – both at a phenotypic and genomic level – potential differences among several populations of the invasive species, including native locations but mainly exploring differences within the invasive range. The objective here is clear: to diagnose and understand possible patterns of evolutionary diversification that the invasive species may be experiencing, either due to intrinsic – e.g. range expansion dynamics – or extrinsic factors – e.g. environmental limitations. To this end, in Chapter 1 we examine life-history and mean adult body size in several populations of the invasive frog, including both native and invasive locations, and try to elucidate if the observed differences could correlate with any environmental variable. Thus, in this chapter we mainly explore if the Mediterranean Painted Frog displays phenotypic modifications in response to environmental differences, even across populations sharing a very recent common evolutionary history. On the other hand, in Chapter 2 we explore only the invasive range of the frog, in this case from a genomic point of view. We use genotyping-by-sequencing (GBS) to study the variation in genetic diversity and the possible population structure within the invasion range. This section, although with a clear focus on microevolutionary patterns of genetic variation, gives interesting insights that will be important to discuss from an ecological point of view in subsequent studies in **PART B**, as for example patterns of habitat selection or competitive differences found across populations.

PART B: Intraguild competition in tadpoles of *Discoglossus pictus* is the lengthier block of this thesis, encompassing up to five chapters. Here we explore and discuss the role of the tadpoles of *Discoglossus pictus* as potential competitors for the larvae of native species of anurans. While the first study (Chapter 3) of this block is community-wide, the rest of four studies in it (Chapters 4 to 8) focus in the competitive relationship between the invasive frog and the Natterjack Toad *Epidalea (Bufo) calamita*, its most common competitor (Fig. 7 & Fig. 8 right).

The objective of the first chapter here (Chapter 3) is to compare a series of growth-related physiological traits between the invasive frog and the rest of the tadpole community – and thus assess its competitive and disruptive abilities. Although it is not presented as an invasion biology study in its form but rather a community study, the voluntary avoidance of mainly focusing on the invasion had to be in fact an editorial decision. In other words, the study could have been presented in its present form – *i.e.* a community-wide comparison of physiological traits in relation to the main selective pressure – or rather proposed to compare the physiology of the invasive species with the rest of the guild present in the invaded area. In any case, the most important ideas from the point of view of invasion biology could still be exposed in the final text of the article.

On the other hand, the rest of Chapters are less flexible in their objectives, deeply focusing in a series of different aspects of a native-invasive competitive interaction. These aspects comprise a comparison of feeding abilities among the invasive frog and the Natterjack Toad (Chapter 4), a study of the possible evolutionary changes in this competitive relationship according to patterns of previous coexistence (Chapter 5), similarities and fine segregation in the use of ephemeral and temporary pond choice between both competitors (Chapter 6), and an isotopic study of trophic relationships along the invasion range (Chapter 7), in this last two cases also evaluating potential variations across populations. Since the time of coexistence between both species varies across populations, differences are mainly explored as a cause of different degrees of co-history between competitors. Hence, generally speaking, the objective of this part of the thesis is to delve into the competitive dynamics of the invasive species, and the possible causes behind the persistence of the native Natterjack Toad, examining how it co-occurs, is affected by, and copes with a previously absent competitor in the tadpole guild. As previously hinted, this block progressively moves from an ecological point of view to search – and test – for answers in evolutionary theory.

Figure 8. **Top left:** *Anax imperator*, and **Bottom left**: *Sympetrum striolatum*, both photographed in the area of study. Nymphs of these native species are used as predators in part C (photographs: Eudald Pujol Buxó). **Right**: Densely populated ephemeral pond in Girona, typically comprising only *Discoglossus pictus* (brownish) and *Epidalea calamita* (black) tadpoles (photograph: Gabriel Mochales Riaño).

Finally, in **PART C: Tadpoles of *Discoglossus pictus* as prey for aquatic native predators** two studies are presented. In this case, the second work (Chapter 9) can be considered a scientifically unavoidable consequence of the first (Chapter 8). The first study (Chapter 8) is – even with a clear hypothesis – mainly an exploration of the potential effects that previous coexistence may exert on the expression of inducible defences in tadpoles confronting predators. Hence, this part of the thesis mainly focuses on the use of phenotypic plasticity by tadpoles acting as prey, and its dependence on previous coevolution between predator and prey. To that end, more concretely, we examine the degree in which tadpoles modify their phenotypes in front of a chosen array of native and invasive predators, mainly examining their behaviour and their morphology, also including juveniles in order to detect the possible consequences of displaying these inducible defences. The second study (Chapter 9) clearly aims at generalizing an unexpected but important conclusion of the first: that *Discoglossus pictus* may be able to effectively reduce predation risk from native predators (e.g. Fig. 8 left) by means of phenotypic plasticity. In this case, interestingly, we do not only examine the presence of inducible defences but also their effectiveness in reducing predation risk.

(SOME)

MATERIALS AND METHODS

(SOME) MATERIALS AND METHODS

THE MOST USED METHODOLOGIES

Since a wide range of experimental and analytical tools are used in this thesis, I considered that an unconstrained summary of some of them would be helpful for a reader possibly unused to certain methodologies. However, for obvious reasons of space and effort, I cannot summarize all major methodologies presented here, and therefore I chose to comment on the most repeatedly used: (1) laboratory and mesocosms block experiments, (2) geometric morphometrics, (3) permutation and randomization tests.

1. Laboratory and mesocosm block experiments

This is, perhaps, both the simplest and the keystone methodology of this thesis. It is used in chapters 4, 5, 8 and 9, representing in most cases the main body of data in them. Theory behind these experiments lies on the main and plain, but sometimes difficult to achieve, premises of biological experimentation: isolation of variables and repeatability. This is perhaps, easier to achieve in a laboratory experiment, where we can easily control most variables potentially affecting the survival, behaviour, growth, development and morphology of tadpoles. Thus, we control the feeding possibilities of tadpoles – usually fed *ad libitum*, meaning that we consider that food has always to be readily available for tadpoles under experimentation – and the features of their surrounding environment – water levels, general temperature, photoperiod, lack of rain or wind perturbations. This is done by using great amounts of patience for a daily control of the experiment, and using always the same interior laboratory in case the experiment is repeated twice to compare the results.

The variations we create among different water tanks – the real experimentation – will obviously depend on the hypotheses of interest. For example, in predation experiments we add in some water tanks a series predators placed in cages that allow water – but not the predators – to go through. This allows tadpoles to be in chemical communication – a main signalling path for them – with predators and detect them, assuming they “know their scent”. Since eaten conspecifics are also used to detect predation risk, we feed the predators conspecific tadpoles. In fact, if they detect or not the potentially dangerous situation is one of the main points of Chapters 8 and 9. On the other hand, in competition studies the methods are simpler, and consist in variations in the location or types of food available (Chapter 4), or in controlled drops in water levels with combined variations in the composition or abundance of the tadpole guild in each tank (Chapter 5). The treatments will always consist in several water tanks in each case in order to be able to correctly assess the repeatability of the results.

Figure 9. Several images of the preparation of a mesocosm experiment. **Top Left:** Provisional array of mesocosms. **Top Right:** Mesocosm with a sapling of *Cyperus eragrostis* being filled with water. Saplings of *Ranunculus aquatilis* and accompanying fauna were added later. **Bottom left**: Mesocosm prepared for experimentation after a few days. In this case – this mesocosm was prepared for another experiment not presented in this thesis – a cage with holes is used to keep chemical but not physical communication between predators and tadpoles. **Bottom Right**: Mesocosm covered with a net, as is kept during experimentation. (photographs: Eudald Pujol Buxó).

Mesocosms used in chapter 9 are, in fact, an extension of the same idea, aiming at an increased realism (see Fig. 9 for similar examples). This poses a series of trade-offs and dangers in terms of variable isolation, but at the same time allows the biologist to be able to infer more reliably the possible outcomes in natural environments. Thus, the artificial creation of a more complex environment is assumed to allow tadpoles to survive free ranging predators – or increase their possibilities at doing so –, but this means that the “natural” environment indeed needs to be correctly – realistically – created. This means some hard work, like taking – preferably from the same ponds as the tadpoles – and planting some saplings – *e.g.* Ranunculaceae, Cyperaceae –, adding some leaf litter, and introducing the usual accompanying animal species like water fleas, fairy shrimps, ostracods, copepods and freshwater snails. As mesocosm experimentation is done outside the lab environment, you need to cover the mesocosms with nets to avoid the unwanted entry of other predators or accompanying species. All this helps to create several similar versions of a standardized small temporary pond where, then, you can add different types and densities of predators and competitors. Here, although water levels can also be kept under control, the environment is under the natural fluctuations of light and darkness present along the reproductive period of the species, meaning you no longer have to induce an artificially constant photoperiod and water temperature.

2. Geometric morphometrics

Traditional morphometrics (Reyment 1996), consisting mainly in applying univariate and multivariate tests to sets of linear measures, dominated the 20th century and are still in use. In fact, this is the methodology used in Chapter 8, which is, chronologically, the first study of this thesis. However, over time it became apparent to biologists – and to the PhD candidate writing these words – that this methodology had several shortcomings which excessively dwindled the ratio between work effort and possibilities for biological insights. Realistic, accurate, and illustrative graphical depictions of shape changes are difficult to be generated using linear morphometrics (Rohlf & Marcus 1993, Adams *et al.* 2004) and, in my case, great variation in tadpole size forced the continuous use of covariates, complicating even more the plotting of results. Finally, the biological answers extracted from linear morphometrics are rather limited. Probably, similar caveats urged biologists in the end of the 20th century to invest some effort in creating a more powerful technique to analyse shape changes. And, I say, they were right to do so: a comparison among plots and analyses present in Chapter 8 and Chapters 5 and 9 might be sufficient to acknowledge that.

The “revolution” was named “geometric morphometrics” (Corti 1993, Rohlf & Marcus 1993) and embodied both a shift in data acquisition and in the underlying statistical theory. Thus, we move from linear measures to a system of cartesian coordinates of anatomical landmarks (Bookstein 1991, Bookstein 1996). These coordinates are fixed points placed on anatomical traits defining the shape of the organism – in my case, tadpoles. Analytical procedures from mathematical theory (Kendall 1981, 1984, 1985) allow to correctly eliminate size and orientation differences, superimposing the obtained landmark configurations and yielding shape variables (Rohlf 1999). This process, based on least sums of squares methods, is called the Procrustes superimposition – named after the greek myth of Procrustes. After superimposition, the new shape coordinates – the "procrustes coordinates" – describe the location of each specimen in a curved space (similar to Kendall 1981, 1984, 1985). Orthogonal projections onto a tangent lineal space yield a final set of coordinates (Thin Plate Spline or Kendall's tangent space coordinates, Dryden and Mardia 1993, 1998; Rohlf 1999) on which multivariate analyses of shape variation can be conducted. At this point we can use the available army of multivariate statistics and techniques to represent, test and explore our data.

Figure 10. Tadpoles photographed for morphological analyses. Observe that the main potential problem is the transparency of the tail, forcing the researcher to take dark photographs or manipulate the light of the images posteriorly. This possible problem is much more less present with the darker tadpoles of *E. calamita*, for example. (photographs: Marta Garcia Maireles and Marta Olivé Muñiz)

The basic initial set usually comprises a principal components analysis (PCA) to explore the data, with thin-plate spline transformation grids (Bookstein 1989, 1991) to provide visual description of axes of the PCA and shape differences between tadpoles (see for example Fig. 3 in Chapter 5). Then, usually, a non-parametric randomization multivariate analysis of variance (NP-R-MANOVA, see next section for permutation and randomization tests) is used to test for differences among groups. Going further, it is possible to explore possible patterns of modularity, integration, and multivariate variance of shape within groups, among others. Graphically, transformation grids can be generated for actual specimens, imaginary mean shapes for populations or treatments, or even predicted specimens along regression lines, even being able to exaggerate differences in order to ease visualization to the biologist. The combination of powerful multivariate techniques with a robust and flexible graphical output makes geometric morphometrics a promising tool for studying morphology.

3. Permutation and randomization tests

If sir Ronald Aylmer Fisher came back to life today, he would probably be disappointed at the use most biologists make of his main biostatistical breakthrough, the analysis of variance (ANOVA). To start with, the F statistic tables were created under a number of assumptions like normality, continuity, and homocedasticity. But more importantly, they were generalized distributions created in order to avoid creating defining a new statistic distribution for each dataset and hypothesis test. This was done specifically – or most probably – because at that time, given the lack of computers, it was totally unthinkable to do it every time an hypothesis had to be tested. But computers were eventually invented, and nowadays, thanks to the amazing advances in computation, we can use, among others, a generalization of the Fisher exact test – the true breakthrough behind the common ANOVA –, and we can also create a statistical distribution for each dataset and hypotheses in a minute in our laptop.

As a general definition, permutation tests are a type of resampling technique specifically aimed to test an hypothesis for statistical inference. Resampling techniques are – were? – computer-intensive methods that take many samples – all shuffled, or random subsets, with resampling or not, depending on the technique – from the original dataset and analyse the data based on them (Crowley 1992). As good examples, the perhaps “best known” resampling techniques are bootstraping and jackknife, which allow to determine confidence intervals or effects of stochastic processes. As previously implied, these methods mainly differ in the specificities of the sampling, like the possibility that some values are sampled more than once or not (Manly 1991, Crowley 1992). Concerning tests, the resampling aims at creating a distribution of a number of possible outcomes, usually by randomizing the dataset, that is, the dependent variables (Edgington 1987, Manly 1991). Assuming there are several groups of data – whose assignations are not shuffled along with the dependent variables – this allows to calculate a distribution of the possible values of a statistic of interest, like Fisher's F, for example. Then, if we “place” the real value of the statistic – that is, the value calculated with the original distribution of the data – on this distribution of possible random outcomes we can assess the likelihood that our data structure – mean differences among groups, for example – could be found by chance. This likelihood is nothing else than our p-value.

Thus, in fact, when conducting these types of test, we return to the real root and definition of the p-value, without an “intermediary” like a generalized F distribution. We directly calculate likelihoods, not approximate likelihood according to a generalized distribution that should – or even might not if I did not account for several assumptions – resemble mine. Generalizations of this idea, like shuffling residuals instead of the dependent variables, or resampling techniques for complex multivariate tests, etc. also apply and are in fact widely used in geometric morphometrics and genetics nowadays, for example. Concerning names, it is interesting to say that if all possible permutations can be calculated – this simply depends on the trade-off between the number of subsets wanted and the computation time – the test will be called an exact permutation test (like the Fisher's exact test, Sokal & Rohlf 1995). Exact tests are used in this thesis for example to assess differences in survival among treatments (Chapter 9), which involve few data and are therefore feasible in a laptop. On the other hand, if the number of possibilities is larger, a subset of possible samples – typically 1000 or 10000 – has to be used. This will be called randomization test (Manly 1991), and is repeatedly used throughout the thesis in all types of situations, from manually created R scripts if the wanted test is not in any statistical package yet (e.g. Supplementary Statistics in Chapter 5) to the use of already optimized functions from several R packages. As the only drawback in this case, the p-values will not be the same every time you run a randomization test. This is because the random sample is, obviously, random, and therefore the inferred p-value fluctuates accordingly. The instability of the p-values is easily solved by repeating the test many times to observe the range of possible values for the p-value or directly increasing the number of samples at the cost of some extra computation time. On the other hand, as previously hinted, among the good reasons to choose permutation or randomization tests there is the lack of underlying assumptions – normality or homoscedasticity – due to the creation of a frequency distribution that does not need to resemble any other that has been previously described. This is specially useful when samples are very small – sometimes even posing problems in terms of continuity – or when the underlying distribution is utterly unknown. Randomization tests do not use ranks (Edgington 1987; Manly 1991) but the actual data, meaning that have higher power than other non-parametric possibilities. For all this, I have sincerely fallen in love with the flexibility and utility of specific and manually created randomization tests, applying them more and more often to solve any statistical problem. As extreme examples, in Chapter 4 there is a randomization test comparing a real experimental outcome with a monte-carlo created control – both with a combination between binomial and Poisson distributions –, and in Chapter 5 there are nearly no examples of “traditional” – in opposition to randomization or permutation – tests.

SUPERVISOR'S STATEMENT
INFORME DEL DIRECTOR

Informe del Director de la Tesi

El director, Dr. Gustavo A. Llorente del Departament de Biologia Evolutiva, Ecologia i Ciències Ambientals de la Facultat de Biologia de la Universitat de Barcelona fa constar que :

El doctorand Eudald Pujol Buxó, presenta dins de la seva tesi doctoral titulada: **"Biological Invasions: a temporal, spatial and plastic point of view: The case of Discoglossus pictus in Europe)",** 14 treballs originals dels quals 10 estan publicats, dos sotmesos i dos en preparació. Els treballs son d'una gran qualitat científica i estan publicats en revistes de reconegut prestigi (incloses en el Science Citation Index) dins de l'àmbit de la Biologia Evolutiva, Zoologia i Ecologia. També s'inclouen treballs publicats sense índex d'impacte però que tenen importància científica ja que aporten dades complementàries d'alt interès.

A continuació es detallen els articles referits i els seus factors d'impacte que formen part del nucli de la Tesi Doctoral:

1.- Pujol-Buxó, E., San Sebastián, O., Garriga, N. & Llorente, G. A. 2013. How does the invasive/native nature of species influence tadpoles' plastic responses to predators? *Oikos*, 122: 19–29 pp. Doi: 10.1111/j.1600-0706.2012.20617.x
Factor d'impacte : 3,44

2.- San Sebastián, O., Pujol-Buxó, E., Garriga, N., Richter-Boix, A & Llorente, G. A . 2015. Differential trophic traits between invasive and native anuran tadpoles. *Aquatic Invasions* 10 (4): 475–484.
Factor d'impacte: 1,61

3.- Oromi, N.; Pujol-Buxó, E.; San Sebastián, O.; Llorente, G.A.; Aït Hammou, M.; Sanuy, D., 2016. Geographical variations in adult body size and reproductive life history traits in an invasive anuran, *Discoglossus pictus*. *Zoology*. 119 (3) 216-223.
Factor d'impacte: 1,69

4.- Pujol-Buxó, E;.; García Guerrero, C.; Llorente, G.A 2017. Alien versus predators: effective induced defenses of an invasive frog in response to native predators *Journal of Zoology* .301 (3): 227-234..
Factor d'impacte: 1.819

5.- Pujol-Buxó, E.; Garriga, N.; Richter-Boix Á.; Llorente, G.A,; 2016. Growth strategies of tadpoles along the pond permanency gradient *Evolutionary Ecology* 30(6): 1117-1132
Factor d'impacte): 1.875

6.- Pujol-Buxó, E.; Kaliontzopoulou, A.; Unanue-Goikoetxea, G.; Ambrós, B.; Llorente, G.A. Fast evolutionary responses in a native-invasive competitive system after several generations of coexistence (Sotmès) *Journal of Animal Ecology*.

Factor d'impacte: 4.827

7.- Pujol-Buxó, E.; Mochales-Riaño, G.; Llorente, G.A. (Sotmès) Mild ecological segregation in the breeding preferences of an invasive anuran (*Discoglossus pictus*) and its native competitor (*Epidalea calamita*). Amphibia-Reptilia.
Factor d'impacte: 1,396

8.- Pujol-Buxó, E.; Mochales-Riaño, G.; Llorente, G.A (in prep.) Preliminary exploration of changes in native–invasive trophic competition according to coevolutionary history.

9.- Pujol-Buxó, E; Garcia-Cisneros, A.; Llorente, G.A. (in prep.) A genomics study of population structure within the invasion range of the Mediterranean Painted Frog (Discoglossus pictus)

A continuació es detallen els articles referits que formen part de l'annex de la Tesi

1.-Llorente, G.A.; Montori, A.; Pujol-Buxó, E., 2015. El sapillo pintojo mediterráneo *Discoglossus pictus*) en la península ibérica *Boletín de la Asociación Herpetológica Española* 26(2): 12-17
Factor d'impacte: --

2.- Ayllón, E.; Santos, X.; Arribas, O.;Bertolero, A.; Bosch, J.;Cabido, C.;Carranza, S.;Carretero, M.A.; Díaz-Paniagua, .;Egea-Serrano, A.; Garin-Barrio, I.; Giménez, A.: Gosá, A.; Graciá, E.; Guicking, D.; Llorente, G.A.; 13, Martínez-Solano I.;Mateo, J.A.; Montori, A.; Palomar, G.; Perera, A.; Pinya, S.; Pretus, J.L.; Pujol-Buxó, E.; Rato, C.; Recuero, E.; Sanz-Azkue, I.; Silva-Rocha, I.; Vasconcelos, R.; Velo-Antón, G.; Vörös, J.;. Pleguezuelos,; J.M. 2015. Propuesta de revisión de los listados y catálogos nacionales y autonómicos de especies amenazadas o protegidas y del Catálogo Nacional de Especies Invasoras *Boletín de la Asociación Herpetológica Española* 26(2): 108-112
Factor d'impacte --

3.- Pleguezuelos,; J.M.; Ayllón, E.; Arribas, O.;Bertolero, A.; Bosch, J.;Cabido, C.;Carranza, S.;Carretero, M.A.; Díaz-Paniagua, .;Egea-Serrano, A.; Garin-Barrio, I.; Giménez, A.: Gosá, A.; Graciá, E.; Guicking, D.; Llorente, G.A.; 13, Martínez-Solano I.;Mateo, J.A.; Montori, A.; Palomar, G.; Perera, A.; Pinya, S.; Pretus, J.L.; Pujol-Buxó, E.; Rato, C.; Recuero, E.; Sanz-Azkue, I.; Silva-Rocha, I.; Vasconcelos, R.; Velo-Antón, G.; Vörös, J.;. Santos, X. 2015. Conclusiones y propuesta de gestión de las poblaciones y especies de anfibios y reptiles alóctonos en España. *Boletín de la Asociación Herpetológica Española* 26(2): 113-115.
Factor d'impacte: --

4.- Santos, X.; Ayllón, E.; Arribas, O.;Bertolero, A.; Bosch, J.;Cabido, C.;Carranza, S.;Carretero, M.A.; Díaz-Paniagua, .;Egea-Serrano, A.; Garin-Barrio, I.; Giménez, A.: Gosá, A.; Graciá, E.; Guicking, D.; Llorente, G.A.; 13, Martínez-Solano I.;Mateo, J.A.; Montori, A.; Palomar, G.; Perera, A.; Pinya, S.; Pretus, J.L.; Pujol-Buxó, E.; Rato, C.; Recuero, E.; Sanz-Azkue, I.; Silva-Rocha, I.; Vasconcelos, R.; Velo-Antón, G.; Vörös, J.;. Pleguezuelos,; J.M., 2015 Síntesis de las introducciones de anfibios y reptiles en España *Boletín de la Asociación Herpetológica Española* 26(2): 98-108..
Factor d'impacte: --

5.- Pujol-Buxó, E.; Mochales-Riaño, G.; Llorente, G.A. 2016. Predation of Bufo calamita eggs by Discoglossus pictus tadpoles. . *Boletín de la Asociación Herpetológica Española* 27(2): 27-29
Factor d'impacte: --

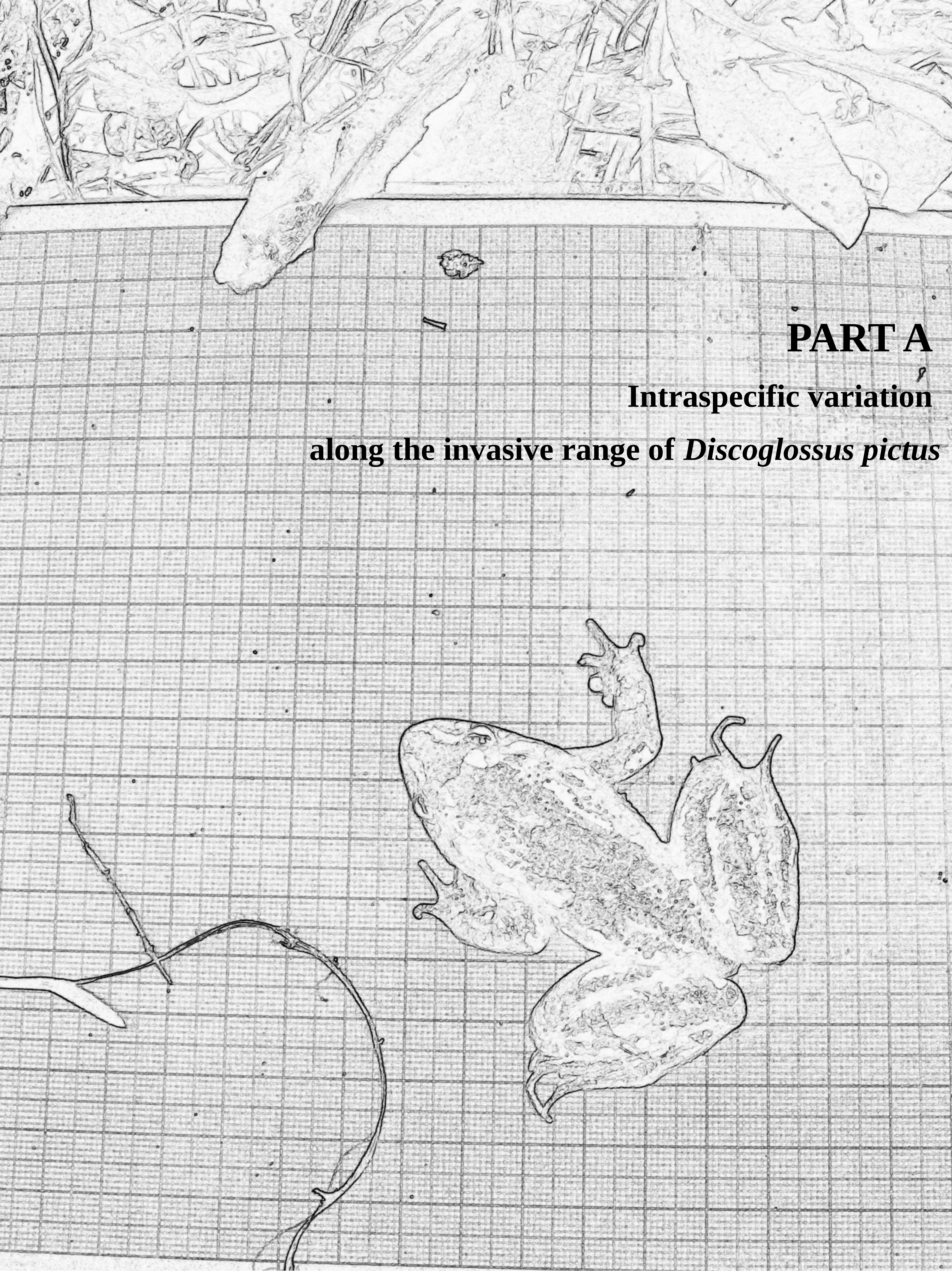
La contribució científica del doctorand ha estat molt important, com demostra que sigui el primer autor en 8 d'ells . Ha participat activament en el disseny, mostreig i anàlisi de les mostres així com en la discussió i redacció científica de tots els treballs presentats.

El director informa que cap dels coautors participants en els articles que componen aquesta tesi han utilitzat implícitament o explícita caps d'aquests treballs per a l'elaboració de la seva pròpia tesi doctoral a excepció dels articles: Pujol-Buxó, E., San Sebastián, O., Garriga, N. & Llorente, G. A. 2013. How does the invasive/native nature of species influence tadpoles' plastic responses to predators? *Oikos*, 122: 19–29 pp. Doi: 10.1111/j.1600-0706.2012.20617.x i San Sebastián, O., Pujol-Buxó, E., Garriga, N., Richter-Boix, A & Llorente, G. A . 2015. Differential trophic traits between invasive and native anuran tadpoles. *Aquatic Invasions* 10 (4): 475–484 dels quals la coautora Olatz San Sebastián Mendoza els ha utilitzat en el marc de la seva tesi doctoral presentada el 2 de novembre de 2016.

Barcelona a 7 de juny de 2017.

Dr. Gustavo A. Llorente

Departament de Biologia Evolutiva, Ecologia
i Ciències Ambientals
Facultat de Biologia
Universitat de Barcelona.



PART A

Intraspecific variation along the invasive range of *Discoglossus pictus*

PART A

Intraspecific variation along the invasive range of *Discoglossus pictus*

Chapter 1

Geographical variations in adult body size and reproductive life history traits in an invasive anuran, *Discoglossus pictus*.

[published in *Zoology*]

Variability in life history traits positively affects the establishment and expansive potential of invasive species. In the present study, we analysed the variation of body size in seven populations − two native, and five invasive − of the Mediterranean Painted Frog (*Discoglossus pictus*, Anura: Discoglossidae), which is native to North Africa and introduced in southern France and the north-east of the Iberian Peninsula. Additionally, other life history traits (age and size at maturity, longevity, median age and potential reproductive lifespan) were analysed in a subset of individuals comprising a native and an invasive population with clearly different mean adult sizes.

We observed geographic variations in adult body size, which correlated mainly to mean annual precipitation. Thus, populations seem to have greater adult body size as mean annual precipitation in the area is higher. This not only created differences across population but generally results in larger specimens found in the invasive European populations. Adult body size and growth rates also varied between sexes in all studied populations, being males always significantly larger than females. Finally, age distribution varied between the native (1–5 years) and the invasive populations (2–4 years) analysed and also between sexes. Globally, our results suggest that higher precipitations can promote several changes in the life-history of the species, mainly, faster growth rates and larger adult body size. This could even facilitate the successful establishment of invasive populations, a possibility which has yet to be explored.

PART A

Variació intraespecífica al llarg de la distribució invasora de la granota pintada (*Discoglossus pictus*)

Capítol 1

Variacions geogràfiques en la mida dels adults i el cicle de vida de la granota pintada (*Discoglossus pictus*).

[publicat a la revista *Zoology*]

La variabilitat en els diversos trets que marquen el cicle de vida d'una espècie invasora afecta positivament a la capacitat d'establiment i per tant al potencial expansiu d'aquesta. En aquesta línia, el present estudi analitza la variació de la mida mitjana corporal dels adults de granota pintada (*Discoglossus pictus*, Anura: Discoglossidae) en set poblacions: dues de distribució autòctona, a Algèria, i cinc d'invasora, al nord-est de la Península Ibèrica. Altres trets addicionals del cicle de vida – l'edat i mida de maduresa, la longevitat, l'edat mitjana i el temps potencial de vida reproductiva – es van analitzar més profundament en una població autòctona i una d'invasora. Quant a resultats, s'observen variacions geogràfiques en la mida corporal mitjana dels adults, amb un patró, pel que sembla, relacionat principalment amb la precipitació a cada zona: les poblacions tenen una mida més gran de l'adult a mesura que augmenta precipitació mitjana anual. Això marca no només un patró general sinó també que les poblacions invasores acostumin a ser de més mida. Per altra banda, les taxes de creixement per tant d'assoliment de la mida d'adult reproductor també va variar entre sexes i entre les poblacions estudiades, siguent els mascles significativament més grans que les femelles. La distribució per edats dels adults reproductors va variar entre la població autòctona estudiada (1-5 anys, més variable) i la població invasora (2-4 anys, més restringida) i també entre sexes. Els nostres resultats suggereixen que l'augment de la precipitació promou majors taxes de creixement i mida corporal dels adults en la granota pintada. Està per veure si això – o alternativament, la mateixa flexibilitat del cicle vital de l'espècie – pot facilitar l'establiment de noves poblacions invasores.

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Geographical variations in adult body size and reproductive life history traits in an invasive anuran, *Discoglossus pictus*

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ABSTRACT

Variability in life history traits positively affects the establishment and expansive potential of invasive species. In the present study, we analysed the variation of body size in seven populations – two native and five invasive – of the painted frog (*Discoglossus pictus*, Anura: Discoglossidae), native to North Africa and introduced in southern France and the north-east of the Iberian Peninsula. Other life history traits (age at maturity, size at maturity, longevity, median age and potential reproductive lifespan) were analysed in a native and an invasive population. We observed geographic variations in adult body size, related mainly to mean annual precipitation. Thus, populations had greater body size as mean annual precipitation increased, resulting in bigger specimens in the invasive populations. Adult body size and growth rates also varied between sexes in all studied populations, with males significantly larger than females. Age distribution varied between native (1–5 years) and invasive populations (2–4 years) and also between sexes. Our results suggest that higher precipitation promotes faster growth rates and larger adult body size that could facilitate the successful establishment of invasive populations.

1. Introduction

Introduced species commonly fail to establish in a new environment (Williamson, 1999). However, successful ones may find biotic and abiotic conditions that, in conjunction with their particular biological attributes, drive the temporal process of occupation and dispersion in the new environment (Sakai et al., 2001). The debate on which factors determine successful introduction is complex, pointing to a mixture of event-level factors and particular species features. Although propagule pressure and other introduction event-level factors seem crucial determinants of a successful introduction of vertebrates (Cassey et al., 2004; Lockwood et al., 2005), there are several studies on fishes reporting species-specific features to be of importance to invasion success. Invasive fishes usually show wide latitudinal ranges and variations of life history traits along biotic or abiotic gradients (Bohn et al., 2004; Alcaraz and Garcia-Berthou, 2007; Benejam et al., 2009) that allow a successful establishment in a new environment. In contrast, the failure of some introduced species to successfully invade has been attributed to their poor ability to survive and adapt to geographical gradients (Becker et al., 2005).

In amphibians, a study by Rago et al. (2012) reports that pathway of introduction, introduction locality, and favourable climatic conditions are the most important features determining the establishment success of an introduced amphibian population, with little support for a role of species characteristics or phylogeny. However, there is also evidence suggesting that some phenotypic features – such as clutch size and reproductive strategies – can contribute to the success of range expansion on both ecologically and evolutionary time scales (Van Bocxlaer et al., 2010). Thus, it is arguable that adaptable amphibian species can more easily overcome habitat or climatic differences and become invasive. For example, the invasive American bullfrog (*Lithobates catesbeianus*) shows morphological variation along an invaded area in response to different

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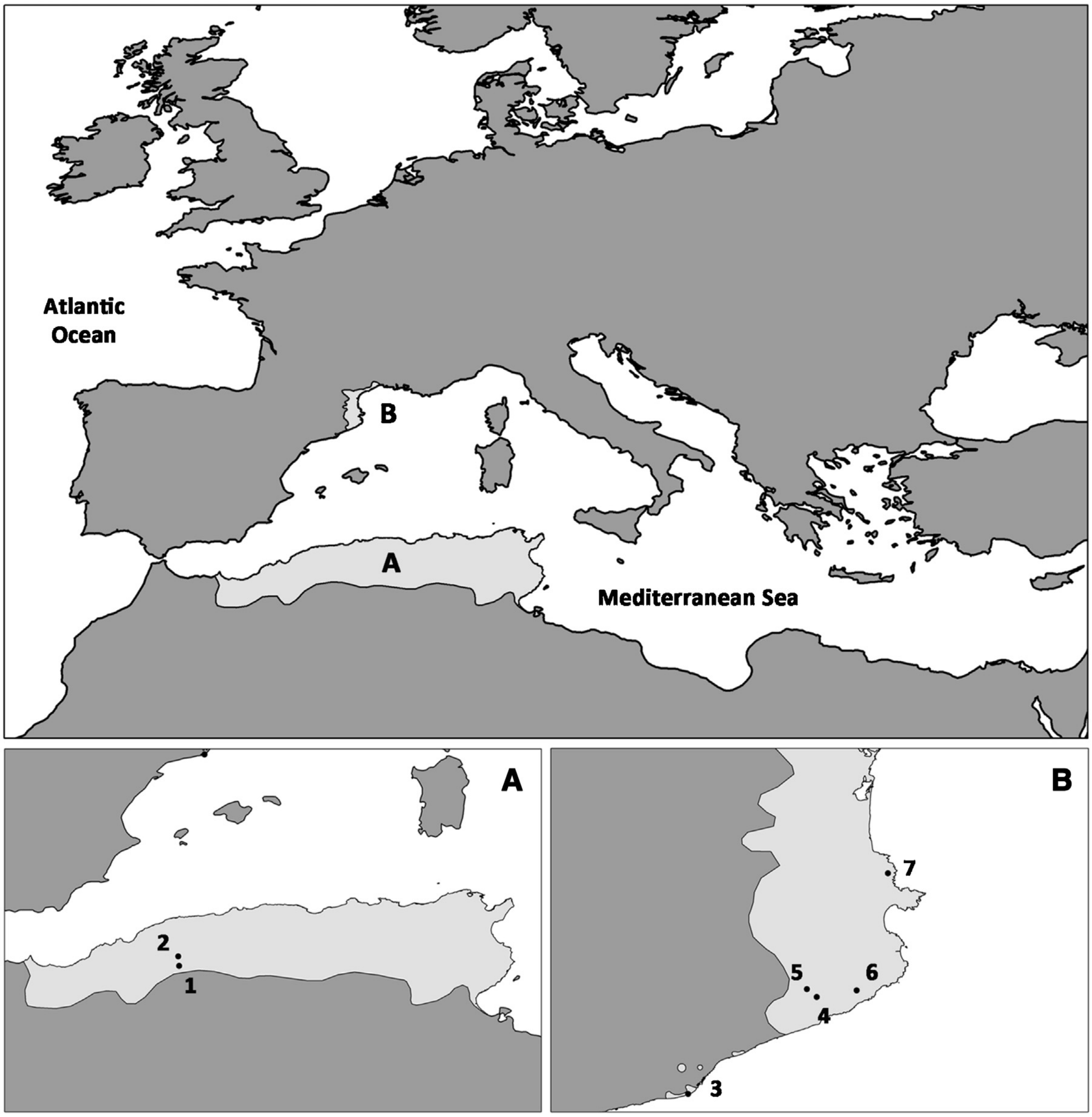

Fig. 1. Distribution of *D. pictus* and the locations of the study populations. (A) Native area: La Fontaine Juba (1) and Jementrie (2). (B) Invasive area: Can Camins (3), Vidreres (4), Riudarenes (5), Mas Pla (6) and Portbou (7).

environmental gradients (Xuan et al., 2010). Another example is the invasive cane toad (*Rhinella marina*) in Australia that has evolved greater tolerance to extreme abiotic conditions, tripling the invasive range predictions based on their native distribution (Urban et al., 2007). And in this sense, tolerance to diverse environmental conditions – through phenotypic plasticity or rapid adaptive shifts – seems to be an important factor in the establishment of a new population in a new site.

The study of life history traits across the current range of invasive amphibian species can be essential to estimate the potential success of establishment and further expansion (Mooney and Hobbs, 2000; Bohn et al., 2004). Moreover, geographical variations of the invasive species must be taken into consideration to evaluate potential impacts on native communities (McGarrity and Johnson, 2009). Life history traits in amphibians – such as longevity and age at sexual maturity – are estimated using individual age determination by skeletochronology (e.g. Castanet, 2002). This procedure is based on the observation of the growth marks (lines of arrested growth = LAGs) recorded in the periosteal part of the bones (phalanges are usually obtained by toe-clipping). LAGs are formed when growth is interrupted by hibernation or aestivation (Olgun et al., 2005). The correspondence of LAGs to seasonal cycles has been verified in many studies, especially for anurans inhabiting temperate environments and low-altitude regions in tropical climates (review in Sinsch, 2015).

In the present study we analyse the size variation in seven populations (two native and five invasive) of the anuran *Discoglossus pictus*. The Mediterranean painted frog, *D. pictus*, is a native species to North Africa and Sicily (Zangari et al., 2006). Some Algerian specimens were accidentally introduced in south-eastern France (Banyuls-sur-Mer) approximately a century ago (Wintrebert, 1908; Lanza et al., 1986). Currently, this species is present in a wide coastal strip from Montpellier in southern France to Vilassar de Mar (Barcelona) in northern Spain, and its distribution is still increasing (Montori et al., 2007; Geniez and Cheylan, 2012; SIARE, 2014). Recently, new introductions outside its range of expansion have been described along the Spanish and French Mediterranean coast (Fradet and Geniez, 2004; Franch et al., 2007). Other life history traits (age at maturity, size at maturity, longevity, median age and potential reproductive lifespan) available for some of the populations are also compared in order to discuss the potential establishment success of *D. pictus* in the invasive zones.

2. Materials and methods

2.1. Study areas and sampling

A total of 186 reproductive adults (see Martínez-Solano, 2014) of *D. pictus* were studied at seven localities (Fig. 1). Following sunset, as many individuals as possible were collected at the local breeding sites during the reproduction periods in 2008 and 2009 (Table 1). Individuals were caught by hand and released at the point of capture after measurement of the snout–vent length (SVL, to the nearest mm) using callipers (precision 0.05 mm). Sex was determined on the basis of secondary sexual characters (i.e., presence of nuptial calluses in males). The 3rd toe of the right hind limb was collected in the Riudarenes (invasive) and Jementrie (native) individuals for skeletochronological analysis. Local climate data were obtained from the meteorological station (period: 2000–2011; Servei Meterologic de Catalunya, Generalitat de Catalunya) closest to the breeding sites (Table 1). The Vidreres, Mas Pla and Riudarenes populations are exposed to the same climatic features because these three breeding sites are very close, so the corresponding meteorological station is the same.

The native populations (Jementrie and La Fontaine Juba) are situated in Algeria. These two populations live in stepped areas covered mainly by croplands, minor forests and scrublands. The invasive populations are located in the north of the Iberian Peninsula. Portbou (6 km from the first introduction record of *D. pictus*), Riudarenes, Vidreres and Mas Pla are covered by Mediterranean scrubland and forest. The population of Can Camins originated from anthropogenic translocation into the coastal swamp habitats of El Prat de Llobregat (Franch et al., 2007).

2.2. Skeletochronological analysis

The age structure was estimated in a subsample of 76 individuals from the Riudarenes (invasive, $n = 43$) and Jementrie (native, $n = 33$) populations. Skeletochronological analysis followed the standard protocols described by Smirina (1972) and Tejedo et al. (1997), modified for the species. The toe samples were decalcified in 3% nitric acid for at least 1 h. Cross-sections (16 μm) were cut using a freezing microtome (HM 50 N; Microm International GmbH, Walldorf, Germany) and stained with Ehrlich's hematoxyline. Cross-sections were examined for the presence of growth marks (strongly stained lines in the periosteal bone) using a light microscope at magnifications of 200x and 400x. For each individual, we selected diaphysis sections in which the size of the medullar cavity was at its minimum and that of the periosteal bone at its maximum (e.g., Fig. 2). These sections were used to assess the number of annual growth marks (LAGs) for each individual. We considered prolonged periosteal bone growth before deposition of the first visible LAG as an indicator of the first-year activity period. As it can be observed in the individuals with a metamorphosis line present, bone growth was maximal during the first year of life.

The age of each individual was estimated (number of LAGs) and was used to define the following five life history traits in both populations: (i) age at maturity (since all of the sampled individuals were collected during their reproductive period, the youngest sampled individuals in a given population were considered first breeders), (ii) size at maturity (the minimum SVL of all first breeders with the minimum number of LAGs), (iii) longevity (the maximum number of LAGs counted in reproductive individuals), (iv) median age (median of the age distribution), and (v) potential reproductive lifespan (the difference between longevity and age at maturity).

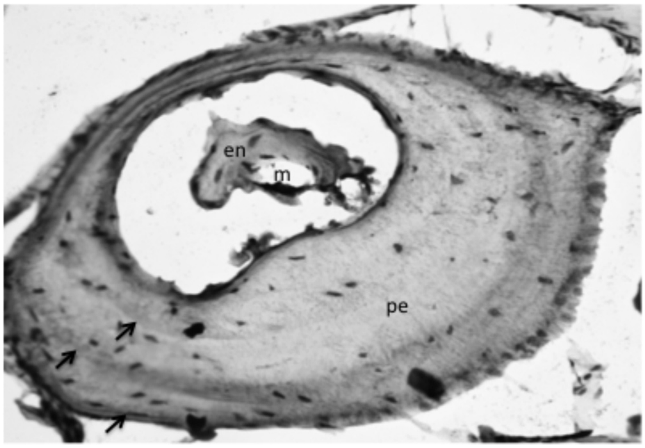

Fig. 2. Cross-section of the phalange of a *D. pictus* male from the Jementrie population. The presence of three lines of arrested growth (LAGs) is marked in the periosteal bone. Abbreviations: en, endosteal bone; m, medullar cavity; pe, periosteal bone.

Table 1
Locations, climate features and number of male (M) and female (F) *Discoglossus pictus* captured.

Locality	Coordinates	Altitude (m a.s.l.)	Precipitation (mm)	Temperature (min, max) (°C)	Date of collection	M/F	Origin
Can Camins	41°17’18.18“N 2°6’6.70“E	1	615.0	(12.1, 20.9)	May 2009	7/1	invasive
Vidreres	41°47’16.88“N 2°45’52.39“E	74	737.8	(6.9, 21.8)	May 2009	19/1	invasive
Portbou	42°25’29.56“N 3°7’52.98“E	80	595,7	(12.1, 19.5)	May 2009	3/5	invasive
Mas Pla	41°49’18.50“N 2°58’12.00“E	54	737.8	(6.9, 21,7)	May 2009	15/6	invasive
Riudarenes	41°49’40.62“N 2°42’48,92“E	93	737.8	(6.9, 21,7)	May, 2008	28/27	invasive
La Fontaine Juba	35°6’36.68“N 1°21’45.21“E	1164	505.3	(7.7, 20.3)	August 2009	8/8	native
Jementrie	35°23’5.67“N 1°20’11.44“E	1029	404.95	(8.1, 21.0)	August 2009	39/19	native

Table 2
Models to test the individual snout–vent length (svl) variation of *D. pictus*, according to AIC, BIC and LogLikelihood (logLik) tests. We considered sex, latitude, elevation (elev), precipitation (prec), mean maximum temperature (maxT), mean minimum temperature (minT), invasive or native origin (zone) and population (pop, random effect) as potential factors/covariables. * = best model. Likelihood ratios (L.Ratio) and associated *p*-values are tested against the best model.

model	df	AIC	BIC	logLik	L.Ratio	*p*-value
svl ~ sex + prec + (pop) *	5	1293.66	1309.79	−641.83		
svl ~ sex + prec + maxT + (pop)	6	1294.77	1314.12	−641.38	0.896	0.3437
svl ~ sex + prec + elev + (pop)	6	1295.52	1314.87	−641.76	0.092	0.7614
svl ~ sex + prec + minT + (pop)	6	1295.57	1314.93	−641.79	0.148	0.7004
svl ~ sex + prec + zone + (pop)	6	1295.61	1314.96	−641.80	0.056	0.8131
svl ~ sex + prec	4	1304.76	1317.66	−648.38	13.094	<0.0001
svl ~ sex + (pop)	4	1306.65	1319.56	−649.33	14.989	<0.0001
svl ~ prec + (pop)	4	1307.45	1320.35	−649.72	15.784	<0.0001

2.3. Statistical analysis

2.3.1. Size variation

In order to estimate the possible factors influencing individual SVL, we performed a model selection following the protocols proposed by Zuur et al. (2009) using the Akaike information criterion (AIC), the Bayesian information criterion (BIC) and likelihood ratio tests (LRTs). Linear mixed models (LMMs) were fitted using “lme” in the “nlme” package in R (Pinheiro et al., 2014), with individual SVL as response variable ($n = 186$). As potential fixed effects we included sex and zone (invasive range vs. native range) as factors, plus elevation, mean annual precipitation, mean maximum temperature (maxtemp) and mean minimum temperature (mintemp) of the area as covariates, not allowing for interactions. We did not add interaction effects to ease interpretation of the results and avoid overfitting, given the small sample sizes in some sex and population combinations. Population was included as a potential random effect (intercept) to account for possible local effects and lack of independence of SVL data of individuals belonging to the same population. First, a full model including all potential fixed and random effects was fitted using restricted maximum likelihood (REML). We tested this model against the same model without the random effect using the function “exactLRT” from the package RLRsim (Scheipl et al., 2008). Once the need of a random intercept had been determined (likelihood ratio = 6.98, $p = 0.0083$), we explored all possible combinations of fixed effects fitting the models using maximum likelihood (ML) and using the automated model generation algorithm implemented in the function “dredge” of the package MuMIn (Bartoń, 2013). Once the best model was found and validated through residual analysis, alternative models were tested against this best model using LRTs (Table 2). Although all tendencies were visually linear, additive and polynomial modelling was also explored in all covariates, using “gamm” in “mgvc” (Wood, 2011), but results never improved linear modelling (results not shown). Size variation analyses were done using R (R Development Core Team, 2013).

2.3.2. Size and age relationship

All variables were first tested for normality. As age and SVL distributions were significantly skewed, data were normalised by log(10)-transformation. The influences of sex and population, and log-normalised age (covariate) on log-normalised SVL were assessed using an analysis of covariance (ANCOVA). These analyses were performed using the procedures from Statgraphics Centurion, version XV (StatPoint Technologies, Inc., Warrenton, VA, USA).

Age data were unbalanced and had problems of continuity (very few age classes) and non-normality. Therefore, to compare the shape of age distributions among each group of data (population and sex) we tested pairwise differences in the mean and standard deviation using self-written routines for running non-parametric randomization tests (see Tables S1–S5 in the supplementary online Appendix). In addition, the relationship between size and age was analysed fitting Von Bertalanffy curves separately for each population and sex, using a medium-metamorphic size (according to Pujol-Buxó et al., 2013) as a starting size (age 0). Thus, four Von Bertalanffy curves were fitted using nonlinear least-squares in the function “vbfr” in the package “fishmethods” (Nelson, 2014). After fitting the model we obtained the parameters L_{inf} (mean length at infinity), K (mean growth rate), t_0 (hypothetical age at size 0) for the best parameter combination in each case, and the suitability of these curves to explain our data was evaluated through residual analysis. Significance level was always set at alpha = 0.05. Statistical analyses of age distribution and size variation growth curves were done using R (R Development Core Team, 2013).

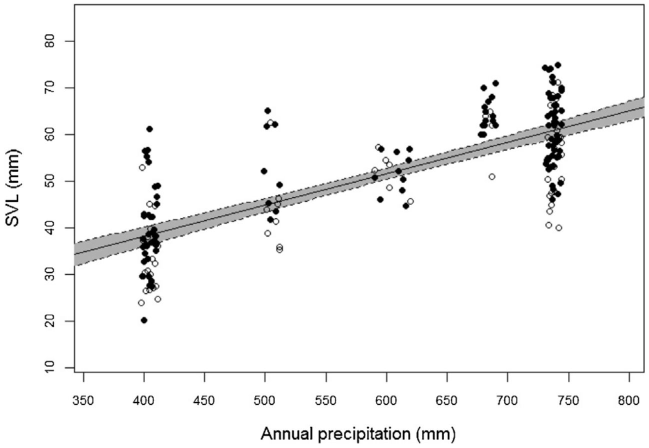

Fig. 3. Linear regression (with confidence intervals) of *D. pictus* snout–vent length (SVL) on annual precipitation. Black dots: males, empty dots: females. Dots are slightly dispersed horizontally to allow for better visualisation.

3. Results

3.1. Size variation

The best model included the fixed effects “sex” and “precipitation”, plus “population” as a random effect (Table 2). Residuals of the best model did not display patterns of heteroscedasticity or non-linearity, neither between populations nor along continuous variables.

The model shows that sex has an influence on the SVL of individuals. Concretely, adult males show a larger mean size (least squares (LS) mean ± SE = 53.59 ± 0.74) than females (LS mean ± SE = 47.34 ± 0.99). The mean annual precipitation is the only covariate that has a notable influence on SVL, with a mean increase of 6.73 mm per 100 mm rain/year (Fig. 3). Although there seems to be a relationship between size and mean maximum and minimum temperatures, none of these variables significantly improved the fit of the models explaining SVL, while excluding sex or precipitation clearly decreased the quality of the model (Table 2).

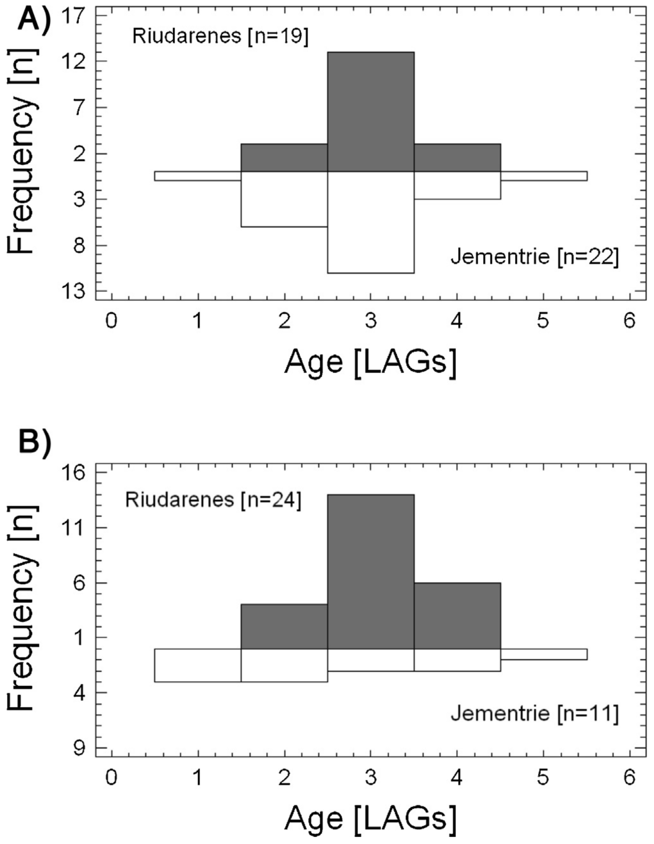

Fig. 4. (A) Male and (B) female age distribution of reproductive individuals in the *D. pictus* populations at Jementrie and Riudarenes.

Table 4
Von Bertalanffy curves for males and females of Jementrie (native) and Riudarenes (invasive) populations of *D. pictus*. L_{inf} = mean length at infinity, *K* = mean growth rate, t_0 = hypothetical age at size 0.

	Jementrie		Riudarenes	
	Males	Females	Males	Females
K	1.028	0.679	0.975	1.091
L_{inf}	40.90	37.46	67.39	56.90
t_0	−0.28	−0.55	−0.13	−0.18

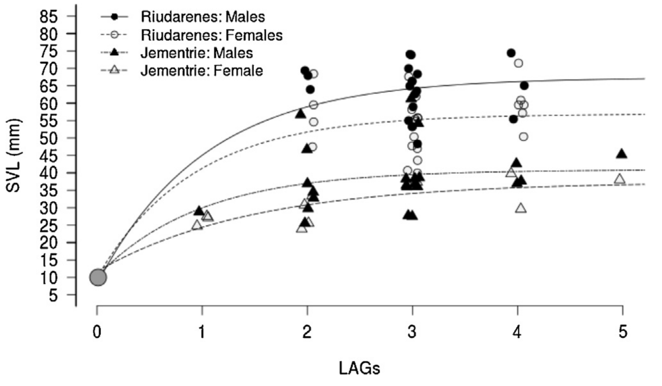

Fig. 5. Von Bertalanffy growth curves for males and females of the two *D. pictus* populations studied. Riudarenes: invasive population. Jementrie: native population. Dots are slightly dispersed horizontally to allow for better visualisation. Size at age 0 (large grey dot) was assumed to be the same for all populations.

3.2. Age structure and age–size relationship

Life history traits varied between the Jementrie (native) and Riudarenes (invasive) populations. The earliest age at which sexual maturity was attained in males and females ranged from 1 LAG (Jementrie) to 2 LAGs (Riudarenes) and the maximum longevity recorded was 5 LAGs in the Jementrie population (see Fig. 4 for more details). Mean age was not statistically different in any group combination (Tables S1, S2 and S3). However, there were differences in the standard deviation of age (Tables S1, S3 and S4), with the ages of females from Jementrie having significantly higher standard deviations than those of females (Δsd = 0.479, p = 0.017) and males (Δsd = 0.79, p = 0.007) from Riudarenes. Although males from Jementrie also had higher standard deviations than both sexes from Riudarenes, these differences were not significant according to our data (Tables S1, S3 and S4).

Log-normalised SVL distribution of individuals differed significantly between males and females and varied among the populations. Age (log-normalised LAGs) was related to SVL (Table 3 and Fig. 4). Size at maturity was 28.8 ± 1.50 mm (males) and 26.50 ± 0.87 mm (females) in the Jementrie population and was significantly smaller than the size of the first breeders of the Riudarenes population (males: 67.08 ± 4.07 mm, females: 57.53 ± 3.52 mm; ANOVA, population: F = 117.76, p < 0.0001, sex: F = 2.76, p = 0.14, interaction sex × population: F = 0.27, p = 0.62).

3.3. Growth curves

Growth curves for males and females from Riudarenes were correctly fitted (Shapiro–Wilk's test for normality: W = 0.94, p = 0.291 for males; W = 0.97, p = 0.769 for females). The presence of outliers (small females and big males) in the Jementrie population prevented these curves from achieving normality (Shapiro–Wilk's test for normality of residuals: W = 0.86, p = 0.0036 for males; W = 0.82, p = 0.015 for females). L_{inf} was always greater in males than in females, and notably greater in individuals from Riudarenes than from Jementrie (see Table 4 and Fig. 5). Growth rates were higher in Riudarenes individuals. However, while in Riudarenes the females had higher growth rates than the males, in Jementrie the males were the ones that grew faster.

Table 3
Analysis of covariance (ANCOVA) for *D. pictus* size as log(10)-transformed SVL.

Source	Square sum	Df	Mean sum of squares	*F*-ratio	*P*-values
Covariates					
age [Log10(LAGs)]	0.0350182	1	0.0350182	6.92	0.010
Main effects					
population	0.767648	1	0.767648	151.76	< 0.0001
sex	0.0879539	1	0.0879539	17.39	0.0001
Interactions					
population × sex	0.0000174216	2	0.000174216	0.00	0.953
Residuals	0.359134	71	0.00505823		
Total (corrected)	1.35533	75			

4. Discussion

Body size and reproductive life history are important evolutionary and ecological traits that can vary geographically in amphibians (e.g., Morrison and Hero, 2003; Sinsch et al., 2010; Oromi et al., 2012; Sinsch, 2015). In the present study, we found clear differences in the adult body size among populations of *D. pictus*, without an effect of their origin (native or invasive). Geographical variation in anuran body size is often attributed to climatic conditions (e.g., Rosso et al., 2004: *Hyla intermedia;* Schäuble, 2004: *Limnodynastes tasmaniensis, Limnodynastes peronei*). However, geographical size variation in amphibians seems to have multiple causes (e.g., *Bufo calamita*: Sinsch et al., 2010), which may differ from the ecological, physiological and evolutionary explanations previously proposed for other tetrapods (Olalla-Tarraga and Rodriguez, 2007; Adams and Church, 2008; Gaston, 2008; Green and Middleton, 2013). For example, body size variation in Fowler's toads (*Anaxyrus fowleri*) is related to density-dependent resource availability for growth during the terrestrial stage (Green and Middleton, 2013). In our case, the data show an important influence of mean annual precipitation on the SVL of *D. pictus* (Fig. 3), resulting in bigger specimens in the populations inhabiting areas with higher annual precipitation (Riudarenes, Vidreres and Mas Pla). In contrast, temperature variables or the geographic origin of the specimens (invasive vs. native) did not significantly improve the models explaining SVL. Population also had a significant effect on *D. pictus* adult size, indicating that other local or microhabitat effects can influence the final size of individuals.

Overall, although this relationship may not be causal, geographic patterns of SVL variation seem to relate only to annual precipitation and local environmental attributes. *D. pictus*, as other anurans, is active during rainy and humid nights (García-Paris et al., 2004), and thus possibly can forage more often in areas with higher precipitation. However, we should not discard the possibility that larval life history traits can have long-lasting effects on the postmetamorphic and adult life (Gomez-Mestre and Buchholz, 2006; Johansson and Richter-Boix, 2013). Remarkably, larval studies with this species show that several factors, such as predation, resource availability and hydroperiod, influence the size of the metamorphs. For example, *D. pictus* metamorphs became larger after having been under predation risk as larvae (Pujol-Buxó et al., 2013). On the other hand, the species usually breeds in ephemeral ponds and has a plastic ability to accelerate development (Enriquez-Urzelai et al., 2013), resulting in smaller metamorphs. In natural environments of *D. pictus* there is an inverse relationship between the risks of predation and desiccation (Richter-Boix et al., 2007), which could increase metamorphic – and possibly adult – size as the pond hydroperiod increases (Richter-Boix et al., 2007).

Several other factors can plastically determine the adult body size of amphibians, such as variations in maturity age and the associated variation of growth rate, food availability and annual growth period (e.g., Jørgensen, 1992; Gramapurohit et al., 2004; Sinsch et al., 2010). Although in our study we analysed the age–size relationship of two populations (a native one and an invasive one) of *D. pictus* that were probably influenced by their microhabitat conditions, the variations found in these traits were similar to those found in other anuran species (e.g., in *Bufo calamita*: Leskovar et al., 2006). In the present study, the age structure differed significantly between small-sized (Jementrie) and large-sized (Riudarenes) populations. It is known that there is a positive correlation between female body size and clutch size in anurans (e.g., Prado and Haddad, 2005; Leskovar et al., 2006). Therefore, the larger females with short reproductive periods in Riudarenes and the smaller females with longer reproductive lifespans in Jementrie might result in relatively similar reproductive outputs. In addition, the growth of amphibians slows down after attaining sexual maturity (e.g., Sinsch, 2015). Therefore, a delay of one year in sexual maturity in the Riudarenes population may result in a larger adult size due to a longer subadult growth period. Despite the fact that the active periods of growth can explain the differences in body size, these variations could also have a genetic component. Restricted gene flow and a divergence in selection pressures among local environments can promote the evolution of populations in response to specific ecological conditions, leading to local adaptation. Therefore, it remains unclear whether the observed variability of life history traits is due to phenotypic plasticity alone or in combination with genetic adaptation.

In 90% of anuran species males are smaller than females (Shine, 1979), but in *D. pictus* the sexual size dimorphism is inversed (García-Paris et al., 2004; this study). The variation in size dimorphism has been proposed to be due to differences in the age structure between sexes (Monnet and Cherry, 2002). In fact, in all species analysed by Monnet and Cherry (2002), in which females exhibit smaller sizes, males are the older sex (*Bufo achalensis, Rana cascadae* and *Hylarana nigrovittata*, with the exception of *Scaphiopus couchii*), suggesting that delayed maturity in males in these cases is related to size dimorphism. However, as in *S. couchii*, we did not find differences in the age structure between the sexes in *D. pictus*. Increased male body size is an important determinant of male mating success (Wells, 2007). In the breeding sites of *D. pictus*, the competition for females is intense and large males may be more likely to reproduce than small ones. However, the mating tactics of *D. pictus* are not well studied and other factors might explain the sexual size dimorphism in this species.

Favourable climatic conditions and event-level factors are reported as the main drivers of establishment and invasion in anurans (Rago et al., 2012), but there are some traits – such as larger adult size or clutch size – identified as candidate promoters of range expansion abilities in bufonids (Van Bocxlaer et al., 2010). Similarly, some studies suggest that the invasive capacity of *D. pictus* depends mainly on favorable abiotic conditions (Escoriza et al., 2014), but previous studies using larval stages have highlighted the plasticity of this species, indicating its remarkable ability to react to native predators (Pujol-Buxó et al., 2013) and to perform better under interspecific competition than its native competitors (Richter-Boix et al., 2013; San Sebastián et al., 2015). In the present study, we have found a variation in life history traits of *D. pictus* related to differences in mean annual rainfall. These results suggest that the higher mean annual precipitation could promote faster growth rates and larger adult body size that could facilitate the successful establishment of an invasive population.

In summary, we report significant geographic variations in the body size of adults of *D. pictus* without a clear effect of their native or invasive origin. The bigger size found in the invasive population may be a consequence of a higher mean annual precipitation. The variation of life history traits of *D. pictus* probably is also influenced by microhabitat conditions, but it remains unclear whether the observed variability of these traits is due to phenotypic plasticity (and its consequences) or a combination of this with genetic adaptation. Our results suggest that the plasticity and adaptability of this species could be a main driver of its invasive capacity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.zool.2016.02.003.

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Supplementary Tables

Table S1. Mean age (years) and standard deviation (SD) for each group.

Population	**Sex**	**Mean**	**SD**
Jementrie	Female	2.54	1.36
Jementrie	Male	2.86	0.65
Riudarenes	Female	3.08	0.88
Riudarenes	Male	3.00	0.57

Table S2. Pairwise Euclidean distances of mean age between groups.

Population.Sex	Jementrie.Male	Riudarenes.Female	Riudarenes.Male
Jementrie.Female	0.318	0.537	0.454
Jementrie.Male		0.219	0.136
Riudarenes.Female			0.083

Table S3. Pairwise p-values for the differences in mean age between groups.

Population.Sex	Jementrie.Male	Riudarenes.Female	Riudarenes.Male
Jementrie.Female	0.272	0.062	0.178
Jementrie.Male		0.358	0.634
Riudarenes.Female			0.757

Table S4. Pairwise Euclidean distances of standard deviation of age between groups.

Population.Sex	Jementrie.Male	Riudarenes.Female	Riudarenes.Male
Jementrie.Female	0.715	0.479	0.791
Jementrie.Male		0.235	0.076
Riudarenes.Female			0.311

Table S5. Pairwise p-values for the differences in standard deviation of age between groups.

Population.Sex	Jementrie.Male	Riudarenes.Female	Riudarenes.Male
Jementrie.Female	0.136	0.017	0.007
Jementrie.Male		0.351	0.211
Riudarenes.Female			0.760

PART A

Intraspecific variation along the invasive range of *Discoglossus pictus*

Chapter 2

A genomics study of population structure within the invasion range of the Mediterranean Painted Frog (*Discoglossus pictus*)

[in prep.]

Range expansions are an intrinsic part of the biogeography of most species. Among them, some invasive species possibly represent a paradigmatic case in which discrete introduction points are followed by a rapid expansion in several directions. Theoretically – and empirically, according to recent studies –, expanding populations create a recursive founder effect, which should lead to stochastic changes in allele frequency and a sustained loss of genetic diversity.

Here, we use population genomics to study the genetic effects of an ongoing range expansion in the invasive populations of the Mediterranean Painted Frog (*Discoglossus pictus*) in Europe. Although the frog has a few translocated isolated populations, nearly all its invasive range corresponds to its self-sustained expansion from a single introduction point, making a very suitable system to study the genetics of range expansions. To this end, we identified and genotyped a large panel of loci using genotyping-by-sequencing (GBS) in several populations along the two main invasion directions – northwards and southwards – of the invasive frog.

As expected due to previous theoretical and empirical works, we find evidence for the loss of genetic diversity with increasing distance from the original point of introduction. This was observed both north and southwards, and regardless of the measure used. Concerning population structure, it is interesting to note that the first axis from the multidimensional scaling (MDS) coincides perfectly with a latitudinal gradient, and that recursive clustering and Fst values grouped sampling locations always according to latitude as well – *i.e.* north and south expansions. The origin area and the nearby location of La Jonquera – in this last case, even though being located at the southern side of the Pyrenees – grouped with northern populations. The observed diversity patterns and genetic structure may serve to confirm that, as expected, the two expansion directions represent independent processes of loss of genetic diversity, and therefore different cases of range expansion ready for further examinations.

PART A

Variació intraespecífica al llarg de la distribució invasora de la granota pintada (*Discoglossus pictus*)

Capítol 2

Estudi genòmic de l'estructura de poblacions a dins de la distribució invasora de la granota pintada (*Discoglossus pictus*).

[en preparació]

Les expansions – de la mateixa manera que les retraccions – són una part intrínseca de la biogeografia de la majoria d'espècies. D'entre tots els possibles casos d'expansió geogràfica, algunes espècies invasores en representen un cas paradigmàtic, en el qual un o pocs punts d'introducció són l'origen d'una ràpida expansió en diverses direccions. En teoria – i també demostrat empíricament ja en alguns casos, d'acord amb estudis recents –, les poblacions en expansió estan sota un efecte fundador recursiu o constant, que hauria de conduir a canvis estocàstics en freqüència dels al·lels i una pèrdua sostinguda de la diversitat genètica.

En aquest estudi, fem servir la genòmica de poblacions per estudiar els efectes genètics d'una expansió geogràfica en curs a les poblacions invasores de la granota pintada (*Discoglossus pictus*) a Europa. Tot i que la granota té algunes poblacions aïllades translocades, gairebé tota la seva distribució invasora correspon a la seva expansió autosostinguda des d'un únic punt d'introducció original – Banyuls de la Marenda, a la Catalunya Nord –, de manera que representa un sistema molt adient per estudiar la genètica d'expansions. En aquest cas, vam identificar i genotipar un gran nombre de loci mitjançant la tècnica del genotipat per seqüenciació (Genotyping by sequencing, GBS) aplicada a mostres d'uns vint adults per població, situades aquestes expressament al llarg de les dues direccions principals d'invasió - cap al nord i cap al sud - de la granota.

Com era d'esperar segons els treballs teòrics i empírics precedents, hem trobat que la pèrdua de diversitat genètica augmenta d'acord amb la distància des del punt d'introducció. Això s'observa tant al cap nord com cap al sud, i amb independència de la mesura de diversitat genètica utilitzada. Pel que fa a l'estructura de poblacions, és interessant observar que el primer eix de l'escalament multidimensional (multidimensional scaling, MDS) coincideix perfectament amb un gradient latitudinal, i que tant els valors de l'índex de fixació (FST) com un agrupament no jeràrquic recursiu agrupen els llocs de mostreig sempre d'acord amb la latitud, és a dir, a grans trets, agrupant les expansions nord i sud. Curiosament, tant l'àrea d'origen de la invasió – Banyuls de la Marenda – com la ubicació propera de la Jonquera – en aquest cas, tot i estar al vessant del sud dels Pirineus – s'agrupen amb les poblacions de l'expansió nord. Els patrons de diversitat i estructura genètica observats poden servir per confirmar que, com era d'esperar, les dues direccions d'expansió representen processos evolutius i independents de pèrdua de la diversitat genètica, i per tant, diferents casos d'expansió geogràfica per a examinacions addicionals.

A genomics study of population structure within the invasion range of the Mediterranean Painted Frog (*Discoglossus pictus*)

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Abstract

Range expansions are an intrinsic part of the biogeography of most species. Among them, some invasive species represent an paradigmatic case in which discrete introduction points are followed by a rapid expansion in several directions. Theoretically – and empirically, according to recent studies –, expanding populations create a recursive founder effect, which should lead to stochastic changes in allele frequency and a sustained loss of genetic diversity. Here, we use population genomics to study the genetic effects of an ongoing range expansion in the invasive populations of the Mediterranean Painted Frog (*Discoglossus pictus*) in Europe. Although the frog has a few translocated isolated populations, nearly all its invasive range corresponds to its self-sustained expansion from a single introduction point, making a very suitable system to study the genetics of range expansions. We identify and genotype a large panel of loci using genotyping-by-sequencing (GBS) in several populations along the two main invasion directions – northwards and southwards – of the invasive frog and, as expected due to previous theoretical and empirical works, we find evidence for the loss of genetic diversity with increasing distance from the point of introduction. This was observed both north and southwards, and regardless of the measure used. Concerning population structure, it is interesting to note that the first axis from the multidimensional scaling (MDS) coincides perfectly with a latitudinal gradient, and that recursive clustering and Fst values grouped sampling locations always according to latitude – *i.e.* north and south expansions. The origin area and the nearby location of La Jonquera – even though being from the southern side of the Pyrenees – grouped with northern populations. The observed diversity patterns and genetic structure may serve to confirm that, as expected, the two expansion directions represent independent processes of loss of genetic diversity *i.e.* different cases of range expansion ready for further examinations.

Keywords. genotyping-by-sequencing, GBS, population genomics, RAD, Invasive species, Range expansion, genetic diversity.

Introduction

The distributional ranges of most species along time are far from static, alternating naturally periods of growth and contraction (Hewitt 2000, Davis & Shaw 2001, Taberlet & Cheddadi 2002). Thus, range expansions are intrinsic and common part of the biogeography of species, but despite their frequency, broad interest in the genetic effects of range expansions – either at neutral or functional loci – is rather recent (Hewitt 2000, Prugnolle *et al.* 2005, Biek *et al.* 2007, Besold *et al.* 2008; Buckley *et al.* 2012; Velo-Antón *et al.* 2012, Waters *et al.* 2012, for a review see Excoffier *et al.* 2009). Some of these studies describe the genetic effects of the historical expansion from glacial refugia (Hewitt 2000, Besold *et al.* 2008) or the spread of pathogens during disease epidemics (Biek *et al.* 2007; Velo-Antón *et al.* 2012). Cases related to human-mediated dynamics have been also studied, for example in the recovery of species after persecution or overexploitation (Fabbri *et al.* 2014, Hagen *et al.* 2015), or poleward expansions linked to global warming (Parmesan & Yohe 2003, Buckley *et al.* 2012).

Theoretically, an expanding population is under a continuous founder effect, which normally should lead to stochastic changes in allele frequency and a sustained loss of genetic diversity (Slatkin & Excoffier 2012). Concerning mutations arising in the expansion front, Edmonds *et al.* (2004) already detected stochastic changes in allele frequency, a process which was lately also observed by Klopfstein *et al.* (2006), which coined the term “allele surfing” for mutations which increase its frequency thanks to the expansion process itself. Thus, neutral mutations which arise on the expansion front can sometimes ‘surf’ on the wave of the advance and maintain clearly higher frequencies in the range margin than would be expected in a population at equilibrium, possibly leading to increased rates of evolution in the expanding populations (Klopfstein *et al.* 2006, Excoffier & Ray 2008). Regarding genetic diversity, the continuous founder effect commonly translates to dropping allelic richness and heterozygosity along the axis of expansion, *i.e.* going from the origin area to the expansion front (Velo-Antón *et al.* 2012; White *et al.* 2013). Interestingly, both this processes can have negative impacts in the populations from the expansion front. First, the inbreeding associated with reduced genetic diversity is expected to negatively impact the fitness of a population *per se* but, in addition, simulation models show that deleterious mutations can indeed also surf to high frequencies at expanding range margins (Travis *et al.* 2007). Despite a lot of theoretical work however, a reduced number of empirical studies have indeed tested these predictions, and therefore the population structure of expanding species remains poorly investigated.

Among all possibilities, invasive species can represent an extreme and paradigmatic case in which introduction points are followed by a rapid expansions in several directions. This cases however, pose an additional interesting dilemma, which has been called the “genetic paradox” (for reviews see Roman & Darling 2007, Dlugosch & Parker 2008): how do introduced populations cope with low genetic diversity and low evolutionary potential to overcome all possible barriers to become invasive? In this sense, the increase in the number of species introduced worldwide, of which many become invasive (Vilà *et al.* 2010, Richardson & Ricciardi 2013), poses the deep study of the genetic effects of invasion dynamics both as a need and a opportunity. Several cases have recently been explored, with varied results. Even though there is an amazing phenotypic variation among expanding and long-established Australian populations of *Rhinella marina* (Phillips *et al.* 2006, Shine *et al.* 2011, Rollins *et al.* 2015), Estoup *et al.* (2001, 2004) detected low interpopulation genetic differences within each expansion front – but with important differences among both fronts –, possibly due to significant migratory exchanges among expansion and rear front populations. On the other hand, Rollins *et al.* (2009) found much poorer genetic connectivity and significantly lower genetic diversity in the recently established populations – compared to ancient invaders – of *Sturnus vulgaris* in Southern Australia. Using the invasion of the bank vole (*Myodes glareolus*) in Ireland as a study system, White *et al.* (2013) found declines in genetic diversity along three studied transects, with no evidence of accumulation of deleterious mutations in the expansion front. In this case, a series of loci possibly related to immunological and behavioural genes possibly under selection were also found. It seems therefore that even though losses in genetic diversity are common in expanding species, neither great genetic isolation among populations is required for great phenotypic change nor high genetic diversity is required for a successful invasion (Rollins *et al.* 2013, Rollins *et al.* 2015). Anyhow, the study of the genetic connectivity and population structure is a totally necessary step to better assess the possible geographic differences in the impacts or dynamics of an invasive species, mainly when it has more than one expanding direction (Estoup *et al.* 2001, 2004).

Here, we report the first results of a population genomics study of an ongoing range expansion of an invasive species. More concretely, we use genotyping-by-sequencing (GBS) to identify and genotype a large panel of SNPs for the Mediterranean Painted Frog (*Discoglossus pictus*) in Europe. This species is native to North Africa and Sicily, and it was accidentally introduced from Algerian specimens in Banyuls de la Marenda – hereafter Banyuls, SE France – approximately 110 years ago (Wintrebert, 1908, Zangari *et al.* 2006). The introduced population became invasive and is currently present in a wide coastal strip approximately from Montpellier – S France – to Sant Celoni – Catalonia, NE Spain. Its distribution is constantly increasing (Geniez &

Cheylan 2012, Llorente *et al.* 2015) while further traslocations outside its range of expansion have been described along the Spanish and possibly the French Mediterranean coast (Fradet & Geniez 2004, Franch *et al.* 2007, Geniez & Cheylan 2012, Llorente *et al.* 2015). Phenotypic differences across populations of the invasive frog have already been detected (Oromi *et al.* 2016), but genetic explorations of diversity within the invasion range have not been made yet. In this study, we explore the genetic structure of several populations of the invasive frog, describing changes in genetic diversity over the course of the two main directions of range expansion – north and southwards. In addition, we identify a latitudinal genetic structure largely resulting in three main genetic groups with different levels of structure within them, possibly describing different evolutionary histories for the northern and southern expansion fronts.

Materials and Methods

Sampling, DNA extraction and genotyping

During spring and autumn from 2013 to 2016, we sampled from 20 to 24 adult painted frogs from nine populations in north-eastern Spain and Southern France, totalling 198 individuals. Populations (exact locations in Table 1) were chosen to cover the two main directions of expansion of the species, going from the original location of introduction to both present expansion fronts. Sampling consisted in the amputation of a finger of the hindlimb, posteriorly conserved in an Eppendorf tube with 95% ethanol. To avoid biases in the estimations of genetic diversity across populations, all frogs from each location were sampled from a single pond.

Population	**Expansion**	**Latitude**	**Longitude**	**Distance to origin (km*)**	**n**	**Sampling years**	**A_{rich}**	**H_e**
Jacou	North	43.662	3.904	165	22	2013	1.1504	0.1507
Vendres	North	43.270	3.217	95	22	2013	1.2335	0.2345
Millars	North	42.696	2.697	43	22	2013	1.2283	0.2293
Banyuls	Origin	42.480	3.130	0	24	2013	1.2770	0.2786
Jonquera	South	42.403	2.901	21	23	2014	1.2512	0.2524
Girona	South	41.999	2.844	58	21	2015-2016	1.2079	0.2091
Vidreres	South	41.788	2.765	83	20	2013	1.2377	0.2390
Hostalric	South	41.736	2.601	94	21	2015-2016	1.2143	0.2154
Sant Celoni	South	41.714	2.541	99	23	2015-2016	1.2146	0.2157

** Lineal distances. When lineal distances were unrealistic – due to the presence of the Mediterranean sea – we calculated the minimum distance bordering the sea.*

Table 1. Sampling information and and two genetic diversity parameters (A_{rich} = Allelic richness, H_e= expected heterozigosity) for each population

Genomic DNA was extracted using the DNeasy kit from Qiagen, and sent to the Centre Nacional d'Anàlisi Genòmica (CNAG, Barcelona, Spain) to conduct genotyping-by-sequencing (GBS). GBS (Elshire *et al.* 2011) is a similar technique to RAD sequencing (Hohenlohe *et al.* 2010) which constructs reduced representation libraries for Illumina. Digestion – in this case using the restriction enzyme PstI (CTGCAG) – and ligation – to a series of appropriate individually barcoded and common adaptors with sticky ends – are initially carried separately for each individual in a 96-well plate. After ligation, the wells are pooled, cleaned and subjected to a PCR for fragment enrichment using long primers that match the barcoded and common adaptors. After PCR, the library was paired-end sequenced for 2*100 bp fragments in an Illumina HiSeq 2000 plattform (Illumina, San Diego, California, USA) including 96 individuals in each lane of a flow cell. Each individual was sequenced two or three times in order to increase sequence depth.

We converted Illumina sequences to genotypes using the GIbPSs toolkit (Hapke & Thiele 2016), which is explicitly designed to deal with paired GBS libraries of non-model organisms – e.g. variable fragment lengths, indels, heterogeneous sequencing depths across individuals and loci – and is able to deal correctly with reverse complement clustering of paired-end reads. First, all sequences were trimmed to 115 bp – to discard the last bases with lower quality – and had the 5' restriction sites – 4bp – removed. This makes 222 bp the maximum length of loci after assembling both paired-end sequences. Minimum overlap of 5 bp was the requisite to assemble forward and reverse sequences, while loci containing N's, shorter than 32 bp, or with a *phred* quality score lower than 22 in a sliding window of 5 bp, had been discarded beforehand. When two restriction targets are close, loci shorter than the maximum sequencing length are generated, in which forward and reverse reads correspond to exactly the same fragment. In some cases – depending on the length of the fragment – the initial 115 bp trimming might fail to completely cut off the 3' restriction enzyme sequence. As a consequence, the program was is unable to overlap the two reads and appends them instead. We identified those duplicates and kept only the forward read for further analyses.

Firstly, we carried out the locus search separately for each individual. Identical sequences were grouped in the same cluster – *svar,* in GIbPSs –, and these clusters were grouped into loci using the default GIbPSs distance settings. We deemed as valid for subsequent analysis loci with at least 5 sequences from the same or different cluster, and we defined as alleles those clusters representing more than 30% of the total number of reads in a locus. Once this process was done for each individual, we stored the individual genotypes in a database and then we filtered them according to quality and coverage. Thus, we discarded all loci with more than two alleles in any individual or potentially including indels, and we used the median scalar depth sequencing of 0.2 to detect and discard deeply sequenced loci that might represent paralogous DNA regions. Finally, all loci present in less than 129 – approximately 70% – of the individuals were discarded as well.

Genetic diversity and population structure

Mean expected heterozygosity (H_e), mean observed heteorzigosity (H_o) and mean allelic richness (A_{rich}) were calculated for each population using the function *basic.stats* from the package *genetics* (Warnes *et al.* 2013) and were then regressed onto the lineal distance – in km – between the sampling locality and the origin area – *i.e.* the original point of introduction, Banyuls. Due to low number of cases regressions were made and tested using permutation ANOVAs implemented in *lmPerm* (Wheeler & Torchiano 2016). In the case of northernmost populations, we had to calculate the shortest path by land bordering the Mediterranean Sea.

To explored patterns of genetic structure across the whole set of individuals, we run a sequential k-means clustering (Hartigan & Wong 1979) implemented in the *find.clusters* from the package *adegenet* (Jombart & Ahmed, 2011). This consists in running a k-means clustering, after scaling the influence of variables – *i.e.* loci –, and repeat the process while increasing the number of clusters, in this case from one to nine. Then, the Bayesian Information Criterion (BIC, Schwarz 1978) is used as an approximate measure to assess the most supported number of clusters, and the results of the k-means to explore each assignation. We plotted the genomic differences among individuals by using a multidimensional scaling on the individual pairwise genetic distances – using the prevosti method implemented in the *diss.dist* function from the package poppr (Kamvar *et al.* 2014). We calculated the fixation index (Fst) among populations using the *genet.dist* from the package *hierfstat (*Goudet & Jombart 2015). We tested isolation by distance among individuals and populations using the mantel test procedures in the *mantel.randtest* function available in the package *adegenet*. We also repeated the mantel test separatedly for three clusters of populations (based on previous results, Table 2): the central group of populations, the whole southern expansion group, and the three populations embodying the southern tip of the expansion. We compared the obtained pairwise Fst with the geographical pairwise distances and and explored the residuals to detect possible outlyers. All statistical analyses were done in R (R Core Team, 2015).

Results

Data quality and coverage

Illumina sequencing of 186 individuals on three lanes initially resulted in 975 303 loci, with a mean coverage of 19.5 reads per candidate locus. Once discarded all loci with more than two alleles in some individual, only 473 682 loci remained, and the suppression of loci with possible indels cut this number in half leaving 223 119 possible loci. Of them, only 6987 were shared by at least 129 individuals. Filtering of possibly paralogous regions detected 181 loci that had to be eliminated, leaving 6806 loci. After eliminating 97 monomorphic loci, we finally had 6709 loci that could be confidently called in at least 70% of individuals.

Genetic diversity and population structure

Genetic diversity significantly diminished with distance from the origin area – Banyuls –, regardless of the measure used ($p[A_{rich}]= 0.0018$; $p[H_e]= 0.0034$; $p[H_o] = 0.0054$; Table 1, Fig 1 & Fig S1). According to the sequential K-means clustering, the optimal number of clusters ranged from 3 to 6 (Fig. S2). Individuals were clustered initially – two clusters – largely according to north or south expansions (Table 2), falling the origin area and La Jonquera with northern populations. Further examination with more clusters signalled the extreme north expansion front – Jacou – as a clearly separated population (Table 2) and the southern tip of the expansion front (Sant Celoni, Hostalric, Vidreres, and partly Girona) as very homogeneous genetically. An individual from Banyuls clustered with la Jonquera, and with increasing number of clusters three individuals from Banyuls formed an independent cluster.

	number of clusters in K-means						
population	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Jacou	B	B	B	B	B	B	B
Vendres	B	C	D	D	D	D	D
Millars	B	C	C	E	E	E	E
Banyuls	B	C	C	C	F(21) C(1)	F(21) C(1)	F(21) C(1) H(3)
Jonquera	B	C	C	C	C	C	C
Girona	A	A	A	A	A	G	G
Vidreres	A	A	A	A	A	A	A
Hostalric	A	A	A	A	A	A	A
StCeloni	A	A	A	A	A	A	A

Table 2. Results of a sequential k-means clustering from two to eight clusters. Numbers in parenthesis indicate how many individuals are classified in each cluster when the populations is divided. If the population is classified as a whole intro a cluster, a letter without any numbers indicates its classification.

Fst values denote a similar pattern (Fig. 3, Table S1), clustering together the southern expansion front (from Girona to Sant Celoni) and keeping Jacou separately while grouping together the rest of the expansion front, the origin area, and La Jonquera. These pairwise Fst largely coincide with the expected by geographic distance (Fig. 4, Tables S2 & S3), with the notable exception of Jacou-Vendres (0.146 larger than the expected).

Mantel tests for isolation by distance among individuals or populations were largely significant for the overall dataset (p=0.001 in both cases). Isolation by distance among individuals from the central group of populations (La Jonquera to Vendres) was also significant (p=0.001), as it was among the southern expansion populations (Girona to Sant Celoni, p=0.001). However, when we tested isolation by distance among the tip of the expansion (Vidreres to Sant Celoni) the significance disappeared (p=0.282).

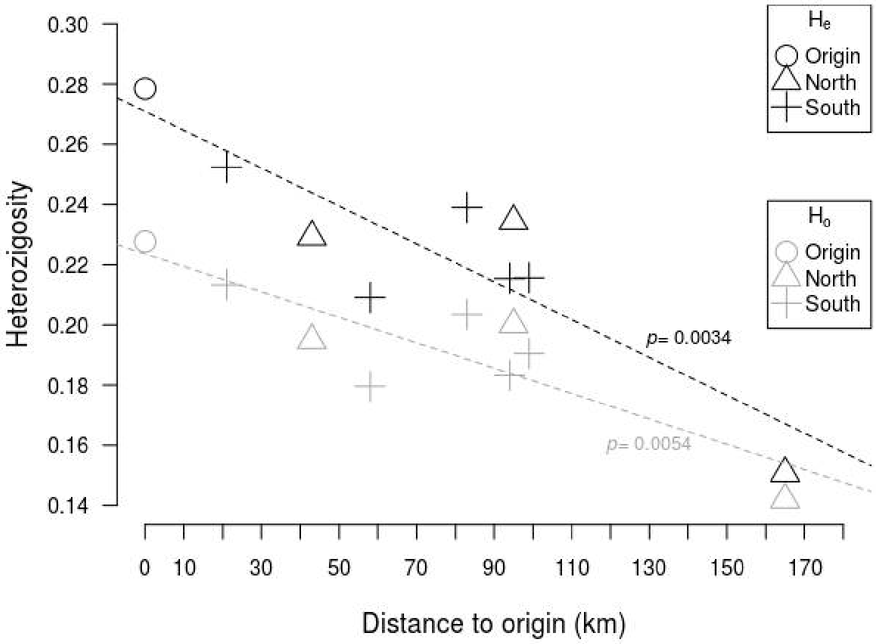

Figure 1. Mean observed and expected heterozigosity in relation to the distance from the original point of introduction – *i. e.* Banyuls – of the frog.

Discussion

We here use a genome-wide approach to track changes in genetic diversity and population structure across a well-characterized range expansion. As expected due to previous theoretical (Slatkin & Excoffier 2012) and empirical works (e.g. Estoup *et al.* 2004, Velo-Antón *et al.* 2012, White *et al.* 2013), we found evidence for the loss of genetic diversity with increasing distance from the point of introduction. This was observed in both directions of expansion – northwards or southwards from the origin area – and regardless of the measure used (Allelic richness, Expected or

Observed heterozigosity, Table 1, Fig 1 & Fig S1), and the direction of expansion. Thus, the general prediction of stochastic loss of alleles as an effect of a recurrent or serial founder effect caused by the range expansions (Slatkin & Excoffier 2012) in this case holds as well. Among them however, low values of genetic diversity are markedly low compared to the expected in a lineal prediction (Fig. 1) for the Girona population. This might suggest a lower connectivity for this population with the surrounding areas due to, for example, lower quality of the terrestrial matrix for the dispersal and connectivity of painted frog populations. In this sense, the incorporation of environmental variables to better assess the resistance to invasion – or dispersal among populations – should be incorporated to improve the study of genetic diversity of the frog.

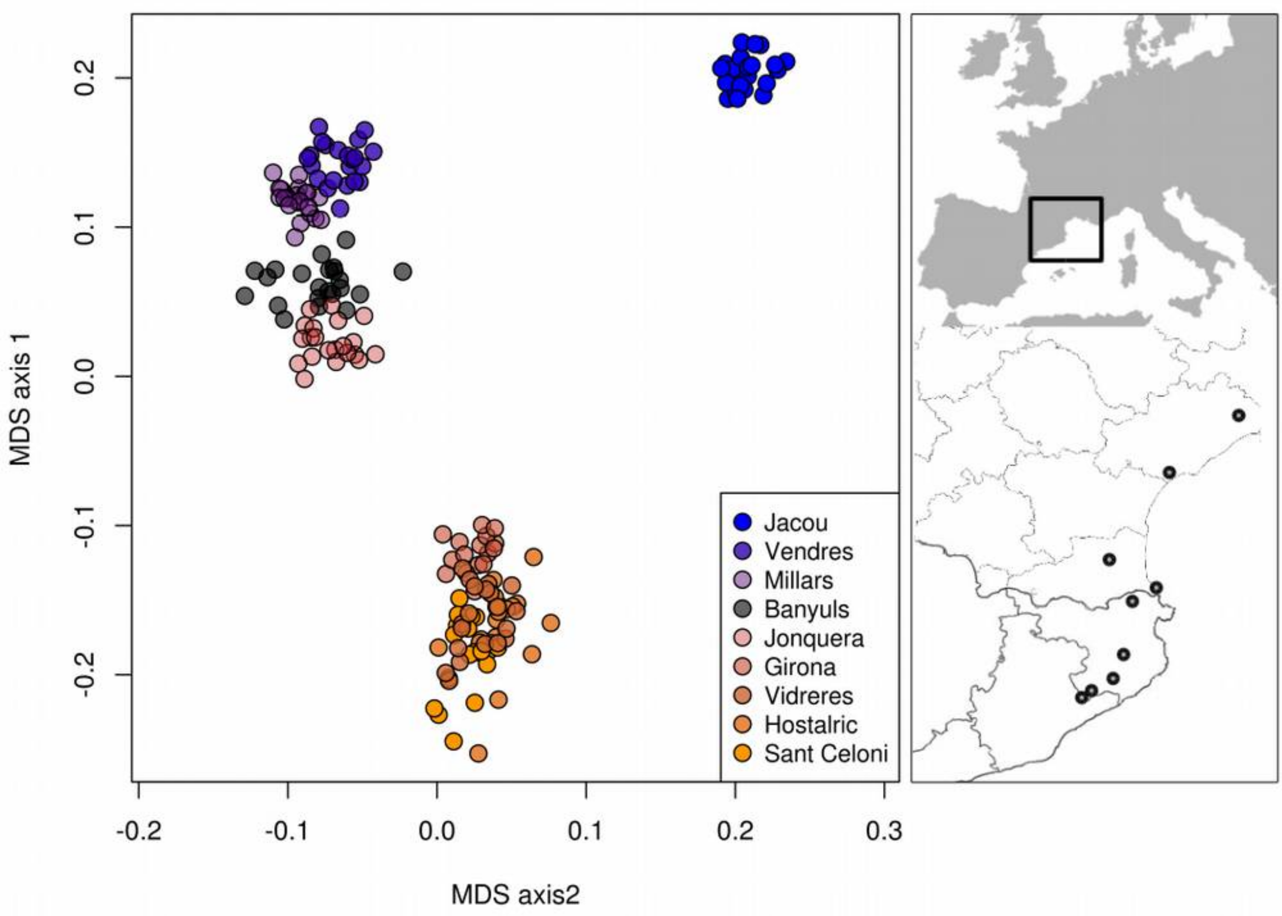

Figure 2. Two first axes from multidimensional scaling (MDS, left) and map showing the approximate locations of the samplings (right). Locations coincide in latitudinal order with the legend and the MDS axis 1.

Concerning genetic similarities and possible connectivity among other sampling locations, it is interesting to note that the first axis from the multidimensional scaling (MDS) coincides perfectly with a latitudinal gradient (Fig. 2). This, along with Fst values (Fig. 3) may serve to confirm that, as expected, the two expansion directions – north and southwards – arguably represent independent processes. Fixation index (Fst) values were highly variable, ranging from 0.03 to 0.36, but in general terms were much higher than those reported for the Cane Toad's invasion (Estoup *et al.* 2004: mean Fst in southward transect= 0.08; mean Fst for westward transect = 0.01). Although there is no use in direct comparisons of Fst between studies using different types of genetic data, in general terms it is useful to note that they signal different expansion histories for both anurans. Generally higher and more variable values could for example point at poorer population connectivity in the painted frog here studied, perhaps due to intrinsically lower dispersal abilities compared to the Bufonid *Rhinella marina,* which might be evolutionarily pre-adapted for a fast invasion (Van Bocxlaer *et al.* 2010).

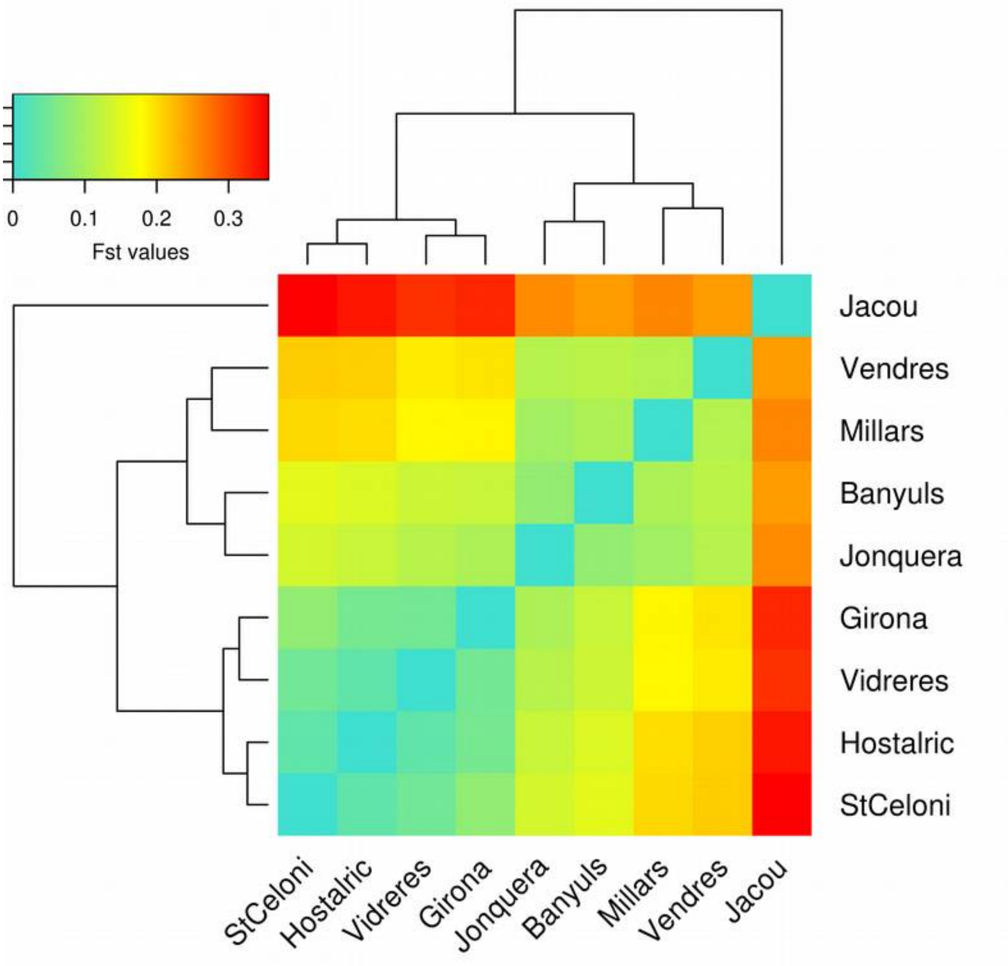

Figure 3. Dendrograms for interpopulation similarities and heat diagram for Fst values.

Regarding geographic structure, populations were clustered initially – in the case of two clusters – approximately according to north or south expansions (Table 2). Thus, genetic drift and loss of genetic diversity is much probably occurring in both directions independently, serving as two different examples of range expansion for further examinations (Hallatschek *et al.* 2007). It is interesting to note as well that even though until recent times both expansions were considered to show similar expansion rates (Montori *et al.* 2007), they have now created a distributional pattern in which the northern expansion nearly doubles the southern expansion in the geographic distance dispersed from the origin area. Thus, either by continuous expansion along a landscape with lower resistance to invasion or, most probably, by mid- and long-distance dispersal events (Fradet & Geniez 2004, Geniez & Cheylan 2012, further discussed below), the northern populations have arrived twice further from the origin area than their southern counterparts. This is also clearly apparent in the genetic diversity of populations in both expansion fronts, which are in absolute numbers much lower in the northern distributional limit, Jacou (Fig. 1). Going further, although pairwise genetic differences among locations (Fig. 4) largely correspond with pairwise geographic distances, the genetic distance between Jacou and Vendres is the only one that does not visually correspond to the lineally expected (the outlyer of Fig. 4). Thus, even though there is no clear geographical barrier between these two populations lying at the northern expansion front, they have higher than expected genetic divergence. Accordingly, Fst values (Fig. 3) and recursive clusterings done from with as few as three groups always flag individuals from Jacou as a clearly separated cluster encompassing only this population (Table 2). This might signal the persistence of the genetic effects of a possible mid-distance dispersal or translocation event (Fradet & Geniez 2004, Geniez & Cheylan 2012) which created a genetically separated population in what is now the northern limit of the distribution. Hence, despite having been pictured repeatedly with a more or less continuous invasion range (Bosch et al. 2009, Oromi *et al.* 2016), our data supports that the apparent discontinuity between Vendres and Jacou (Geniez and Cheylan 2012) represents a real absence of connection between both areas. On the other hand, the major similarity of Jacou to the second northernmost location in absolute terms – and generally northern populations – might also mean that the long-distance dispersion or translocation included individuals from northern tip of the range at that time (Fig. 2). Interestingly, this particular expansion history of the northern expansion has been recently matched, as an increasing number of populations are found near Barcelona outside of the continuous range of expansion of the species (Franch *et al.* 2007, Llorente *et al.* 2015), most probably embodying similar cases to that already commented.

Concerning population structure, there are other interesting results that need to be commented. Firstly, even though La Jonquera is located on the southern slopes of the Pyrenees, its genetic resemblance is with the origin area and the northwards populations up to Vendres (Fig. 2 & 3, Table 2), and thus totally according with lineal geographic distance, regardless of the presence of a mountainous ridge among them. This may confirm that, as some authors have previously suggested (Montori *et al.* 2007, Llorente *et al.* 2015), the easternmost tip of the Pyrenees does not represent a dispersal barrier to the invasive frog neither through elevation or climate. This is also confirmed with the lack of markedly higher genetic differentiations among north- and south-side populations within the invasion range. Finally, at the southern tip of the expansion front (Fig. 2: Girona, Vidreres, Hostalric and Sant Celoni) populations are very homogeneous genetically (Table 2), even appearing largely mixed together in the MDS. Although it is very important to remark that southern expansion populations are sampled much more closely than in the northern expansion – and thus genetic homogeneity would approximately correspond to the expected by geographic distance –, it is in fact remarkable that increasing the number of k-means clusters divides the Banyuls – origin area – population in several clusters instead of separating the three southermost locations, which are spread along 20km (Table 2). In addition, testing isolation by distance among the three southern tip populations (from Vidreres to Sant Celoni) did not give significant results. This possibly signals that the most recent colonizations – 20 years at most – indeed create genetically homogeneous areas which may be as extensive as from 20 to 40 km long. Further samplings in the northern expansion front would be necessary to detect if these patterns hold as well in that area.

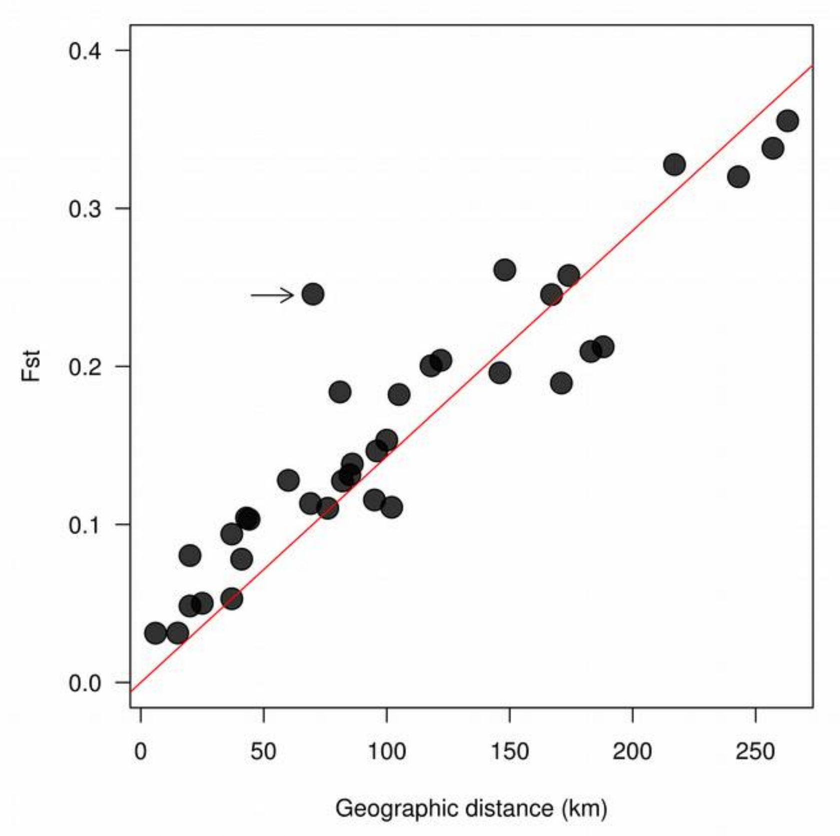

Figure 4. Pairwise Fst distances in relation to pairwise geographic distance among populations. The outlyer with greater genetic distance than expected (arrow) is the Fst distance among Vendres and Jacou.

The Mediterranean Painted Frog in Europe might become a very good system to study the effects of recursive translocations and self-sustained range expansions. A single introduction point dated more than a century ago was followed by a range expansion which is, due to human-mediated or intrinsic dynamics, accelerating nowadays. The opposite direction of the two main directions of expansion – north and southwards along the Mediterranean Coast – largely impedes genetic connectivity between them, and creates two largely independent systems in which explore evolutionary consequences of range expansions.

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Supplementary Material for:

Pujol-Buxó E., Garcia-Cisneros A. & Llorente, G.A.: A genomics study of population structure within the invasion range of the Mediterranean Painted Frog (*Discoglossus pictus*)

Fig S1. Allelic richness in relation to distance from the original point of introduction of the frog.

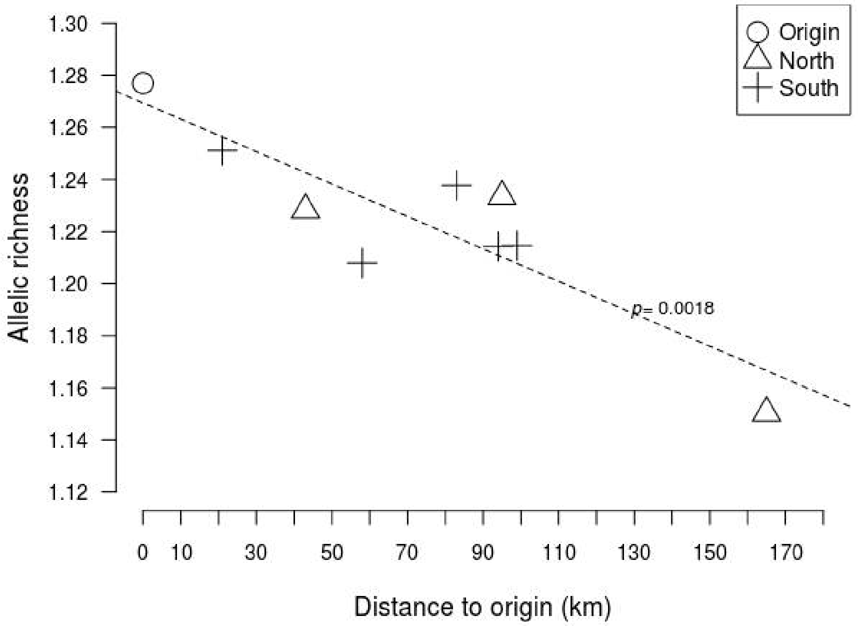

Fig S2. BIC versus number of k-means clusters:

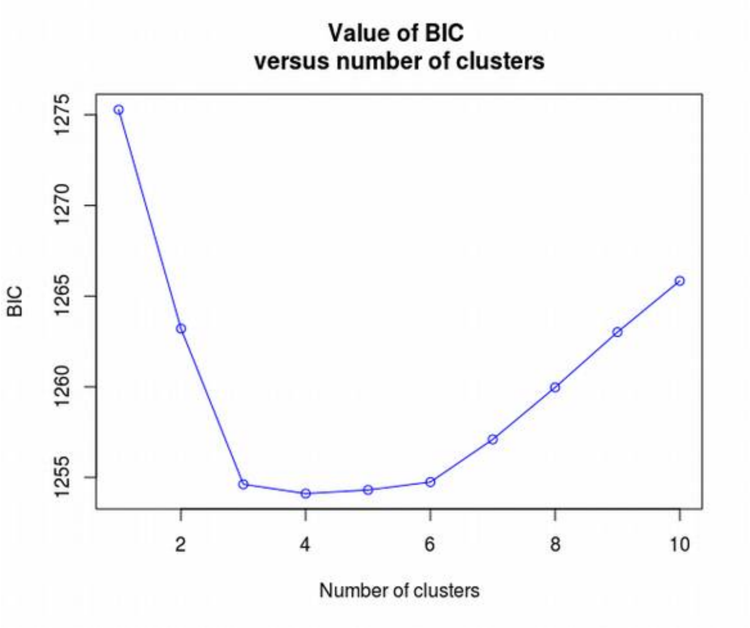

Table S1. Pairwise fixation indexes (Fsts) among all sampled populations.

	Jacou	Vendres	Millars	Banyuls	Jonquera	Girona	Vidreres	Hostalric	StCeloni
Jacou	0	0.246	0.261	0.245	0.258	0.328	0.320	0.338	0.355
Vendres	0.246	0	0.110	0.115	0.111	0.196	0.189	0.209	0.212
Millars	0.261	0.110	0	0.104	0.094	0.184	0.182	0.200	0.204
Banyuls	0.245	0.115	0.104	0	0.080	0.128	0.131	0.146	0.153
Jonquera	0.258	0.111	0.094	0.080	0	0.103	0.113	0.127	0.138
Girona	0.328	0.196	0.184	0.128	0.103	0	0.050	0.053	0.078
Vidreres	0.320	0.189	0.182	0.131	0.113	0.050	0	0.031	0.048
Hostalric	0.338	0.209	0.200	0.146	0.127	0.053	0.031	0	0.031
StCeloni	0.355	0.212	0.204	0.153	0.138	0.078	0.048	0.031	0

Table S2. Pairwise geographic distances among all sampled populations.

	Jacou	Vendres	Millars	Banyuls	Jonquera	Girona	Vidreres	Hostalric	StCeloni
Jacou	0	70	148	167	174	217	243	257	263
Vendres	70	0	76	95	102	146	171	183	188
Millars	148	76	0	43	37	81	105	118	122
Banyuls	167	95	43	0	20	60	85	96	100
Jonquera	174	102	37	20	0	44	69	82	86
Girona	217	146	81	60	44	0	25	37	41
Vidreres	243	171	105	85	69	25	0	15	20
Hostalric	257	183	118	96	82	37	15	0	6
StCeloni	263	188	122	100	86	41	20	6	0

Table S3. Residuals of a regression of Fsts on geographic distances for all sampled populations.

	Jacou	Vendres	Millars	Banyuls	Jonquera	Girona	Vidreres	Hostalric	StCeloni
Jacou	0	0.1457	0.0494	0.0066	0.0087	0.0174	-0.0275	-0.0294	-0.0207
Vendres	0.1457	0	0.0016	-0.0204	-0.035	-0.0128	-0.0551	-0.0522	-0.0564
Millars	0.0494	0.0016	0	0.0426	0.041	0.068	0.032	0.0316	0.0294
Banyuls	0.0066	-0.0204	0.0426	0	0.0517	0.0421	0.0099	0.0092	0.0103
Jonquera	0.0087	-0.035	0.041	0.0517	0	0.0403	0.0146	0.0102	0.0153
Girona	0.0174	-0.0128	0.068	0.0421	0.0403	0	0.0143	0.0001	0.0194
Vidreres	-0.0275	-0.0551	0.032	0.0099	0.0146	0.0143	0	0.0098	0.0198
Hostalric	-0.0294	-0.0522	0.0316	0.0092	0.0102	0.0001	0.0098	0	0.0226
StCeloni	-0.0207	-0.0564	0.0294	0.0103	0.0153	0.0194	0.0198	0.0226	0

PART B

Intraguild competition in tadpoles of *Discoglossus pictus*

PART B

Intraguild competition in tadpoles of *Discoglossus pictus.*

Chapter 3

Growth strategies of tadpoles along the pond permanency gradient

[published in *Evolutionary Ecology*]

The preference for particular features of water bodies for reproduction is one of the most important aspects of anuran ecology, affecting key aspects of both tadpole and adult life. The use by each species of only certain habitats along the pond permanency gradient has been already studied, noting conflicting selective pressures from predation and desiccation risk. Here, we aim to discover physiological patterns related with this gradient. As a study system, we used the full anuran community of the NE Iberian Peninsula. We quantified growth rate, consumption rate, food assimilation and the proportion of energy allocated to growth, as well as gut length, for all species.

Food consumption rate and growth allocation were the variables that defined tadpole growth, while food assimilation abilities and gut length seem to have a secondary or cryptic role in growth. More interestingly however, our data suggests a labile continuum of consumption-based versus allocation/assimilation-based growth strategies differentiating species. Differences among species follow predictions of adaptation to the pond permanency gradient selective pressures. Thus, species from ephemeral ponds are more prone to use consumption-related growth tactics while species inhabiting ponds with longer hydroperiod are more efficient retaining and allocating energy into growth. Nevertheless, results seem also partly shaped by strong interspecific competition, leaving the door open for further analysis.

Concerning the invasive species studied here in this thesis, the Mediterranean Painted Frog, it physiologically represents a unique addition to the local tadpole guild. Its growth is very rapid and is based mainly on an increased consumption, representing an extreme tactic in this sense, at least as compared to all the rest of local tadpole species. As a final remark, physiological differences in growth and the usage of the assimilated energy could be an additional factor to understand how tadpoles adapt to the features of ponds they inhabit, as well as how they compete and coexist.

PART B

Competència intragremi en capgrossos de la granota pintada, *Discoglossus pictus.*

Capítol 3

Estratègies de creixement dels capgrossos segons el gradient de permanència de basses.

[publicat a la revista *Evolutionary Ecology*]

La preferència per característiques particulars dels punts d'aigua per escollir-los com a punts de reproducció és un dels aspectes més importants de l'ecologia dels amfibis anurs, ja que afecta a aspectes clau de la vida tant del capgròs i com de l'adult. Així doncs, l'ús per part de les diferents espècies de només una part dels diferents tipus d'hàbitats aquàtics al llarg del gradient de permanència ja ha estat repetidament estudiat, assenyalant l'existència de pressions selectives contradictòries entre el risc de depredació i el risc de dessecació de la bassa. En aquest estudi, el nostre objectiu és descobrir si també existeixen patrons fisiològics relacionats amb aquest gradient. Com a sistema d'estudi, hem fet servir la comunitat completa de capgrossos del nord-est de la Península Ibèrica. Mitjançant experiments de laboratori amb alimentació controlada, vam quantificar-ne les taxes de creixement, taxes de consum i d'assimilació d'aliments, i també la proporció d'energia obtinguda que és assignada al creixement, així com la longitud de l'intestí dels capgrossos, per a totes les espècies de la zona d'estudi.

Entre totes, la taxa de consum d'aliments i la proporció d'energia assignada al creixement van ser les variables que més clarament semblen definir les taxes de creixement d'un capgròs, mentre que, curiosament, la capacitat d'assimilació dels aliments i la longitud de l'intestí semblen tenir-hi un paper molt més secundari o críptic. No obstant això, potser és més interessant fer notar que les nostres dades suggereixen un continu làbil des de creixements basats en grans taxes de consum fins a creixements basats en l'eficiència en l'assignació / l'assimilació de l'energia obtinguda. A més a més, les diferències entre les espècies semblen seguir les prediccions d'adaptació a les pressions selectives de depredació i dessecació del gradient de permanència de basses. Les espècies de basses efímeres i temporals són més propenses a fer servir tàctiques de creixement relacionades amb el consum mentre que les espècies que habiten estanys i basses amb hidroperiodes llargs estan més sovint lligades a creixements basats en la retenció més eficient i l'assignació de l'energia al creixement. Tot i això, els resultats semblen en part determinats també per la forta competència interespecífica, deixant els qüestions obertes per futures exploracions.

Quant a l'espècie invasora objecte d'aquesta tesi, aquesta representa una addició única al gremi local de capgrossos. El seu creixement és molt ràpid i es basa principalment en un gran consum alimentari, representant l'espècie una tàctica extrema en aquest sentit, almenys en comparació amb la resta de les espècies de capgrossos locals. Les diferències fisiològiques en el creixement i en l'ús de l'energia assimilada podrien ser un factor important a tenir en compte per entendre com els capgrossos s'adapten a les característiques dels estanys en què habiten, així com la forma en què competeixen i coexisteixen.

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ORIGINAL PAPER

Growth strategies of tadpoles along the pond permanency gradient

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Abstract The preference for particular features of water bodies for reproduction is one of the most important aspects of anuran ecology, affecting key aspects of both tadpole and adult life. The use by species of different habitats along the pond permanency gradient has been already studied, noting conflicting selective pressures from predation and desiccation risk. Here, we aim to discover physiological patterns related with this gradient. As a study system, we used the full anuran community of the NE Iberian Peninsula. We quantified growth rate, consumption rate, food assimilation and the proportion of energy allocated to growth, as well as gut length, for all species. Food consumption rate and growth allocation were the variables that defined tadpole growth, while food assimilation abilities and gut length seem to have a secondary or cryptic role in growth. More interestingly however, our data suggests a labile continuum of consumption-based versus allocation/assimilation-based growth strategies differentiating species. Differences among species follow predictions of adaptation to the pond permanency gradient selective pressures. Species from ephemeral ponds are more prone to use consumption-related growth tactics while species inhabiting ponds with longer hydroperiods are more efficient retaining and allocating energy into growth, although results seem partly shaped by strong interspecific competition. Physiological differences in growth and the usage of the assimilated energy could be an additional factor to understand how tadpoles adapt to the features of ponds they inhabit, as well as how they compete and coexist.

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Keywords Predation risk · Energy allocation · Consumption rate · Growth rate · Resource exploitation

Introduction

Certain features of freshwater bodies determine the biology of the entire biota that they host. Each kind of water mass is linked to a desiccation risk and is comprised of different types of predators and competitors, among other parameters, such as water quality or available food resources (Wellborn et al. 1996; Williams 2005). Accordingly, anuran species typically prefer reproducing in water bodies with a particular set of characteristics (Altig and McDiarmid 1999b). For each species, the repeated use of some kinds of water masses over evolutionary time has shaped aspects of larval biology, such as rates of development, behaviour, morphology, and phenotypic plasticity (Altig and McDiarmid 1999b; Van Buskirk 2009; Strauß et al. 2010; Richter-Boix et al. 2011). The choice of site for reproduction will have clear effects not only on larval life, but consequently on juvenile morphology and fitness (Tejedo et al. 2010; Richter-Boix et al. 2011). All these cascade effects have been demonstrated to influence the evolution of functional (Strauß et al. 2010; Van Bocxlaer et al. 2010) and morphological diversification of anurans (Gomez-Mestre and Buchholz 2006; Johansson and Richter-Boix 2013; Van Buskirk 2014).

Anuran communities from semiarid environments are a good system to study trade-offs linked to the choice of a reproduction site (e.g. da Silva Vieira et al. 2009; Dayton and Fitzgerald 2001). More precisely, anuran communities of the Mediterranean basin comprise generally well-studied species inhabiting the same area but with different preferences along the pond permanency gradient (Richter-Boix et al. 2006b, 2007a). This gradient is defined by each pond's tendency to dry out (ranging from merely a month of mean continuous water presence to permanent year-round water), but it also correlates with predation risk (Wellborn et al. 1996). Desiccation of ponds can lead to a total obliteration of a full cohort of an amphibian population, and thus desiccation risk is possibly the most important selective pressure for larvae (Semlitsch 1987; Degani and Kaplan 1999; Jakob et al. 2003; Richter-Boix et al. 2006b). Anuran species adapted to breed in ephemeral ponds, virtually free of predators, have fast developing tadpoles plus plastic abilities that enable them to accelerate development if water levels drop dramatically (Richter-Boix et al. 2006b). Desiccation risk in temporary ponds is lower, but more arthropod predators can breed in these ponds since they keep water several months. Permanent ponds, with no risk of desiccation, comprise even fish as top predator. Therefore, there is a counter-gradient between predation and desiccation risks, implying a trade-off between choosing a pond with a high number of predators or with high probabilities of drying out (Richter-Boix et al. 2006b). Although evolutionary effects of these gradients have been studied in terms of general biology and ecology in different freshwater organisms, including amphibians (e.g. De Meester et al. 2005; Serrano and Fahd 2005; Richter-Boix 2005; Gascón et al. 2008), studies trying to relate physiological traits of tadpole species with this pond permanency gradient are generally lacking (but see Richardson 2002). As a consequence, how growth-related physiological parameters (consumption rate, assimilation, proportion of energy invested in growth) of each species are related to its particular use of the pond permanency gradient is still largely unexplored. In this study, we obtained physiological data on the resource exploitation and growth physiology of the full community of tadpole species present in the NE Iberian Mediterranean coast to explore whether physiological traits, such as consumption rate, or assimilation and growth allocation, can represent an adaptive dimension for tadpoles.

We base our predictions on the interactions among predation risk, foraging activity and physiology. As a general rule, active tadpoles can increase harvesting rate and thus develop and grow faster, becoming superior competitors (Dayton and Fitzgerald 2001; Smith et al. 2004), but increased activity can also result in increased predation risk (Morin 1983; Polo-Cavia and Gomez-Mestre 2013). Therefore, tadpoles inhabiting ponds with high predation risk are usually found to be less active than tadpoles from ponds with low predator presence (e.g. Van Buskirk and Arioli 2005; Richter-Boix et al. 2007b). Accordingly, we predict that physiological traits enabling tadpoles to grow with low foraging rates—like high food assimilation or high energy allocation in growth—should be common in species usually inhabiting ponds with high numbers of predators, that is, long hydroperiod ponds. In these species, features positively interacting with foraging activity—like high consumption rate abilities—relate weakly to enhancing energy acquisition and therefore should have a mild link to individual fitness and positive selection. Instead, all features allowing tadpoles to grow using fewer resources are expected to be under selection in order to compensate for the lack of foraging activity (similarly as found in Thaler et al. 2012). On the other hand, species inhabiting short hydroperiod ponds that comprise fewer predators have fewer constraints on foraging rates, but must cope with desiccation risk instead. Previous experiments demonstrate that species from temporary ponds have higher activity rates both in absence and presence of predators in comparison to permanent pond breeders (Richter-Boix et al. 2007b). In the absence of activity constraints, all physiological traits that enhance growth rates could be selected for, assuming that there are no trade-offs among physiological traits. Concretely, the possibility of profiting from abundant resources (Wassersug 1975; Diaz-Paniagua 1989) through high consumption rates should be specially selected for. In summary, we expected to find that species inhabiting long hydroperiod ponds should be adapted to grow under foraging constraints, and therefore display higher assimilation and growth allocation abilities compared to species inhabiting short hydroperiod ponds. Species inhabiting short hydroperiod ponds, in turn, are usually exempt of foraging constraints and should have higher consumptive abilities compared to species from long hydroperiod ponds.

Materials and methods

Study system

Our study area is based in the NE Iberian Peninsula, which is mainly a typical Mediterranean region with hot and dry summers, mild winters and two rainy seasons in spring and autumn (Martín-Vide and Olcina 2001), but also with some areas displaying an Eurosiberian climate. The entire anuran community in this area is comprised of nine species (Pleguezuelos et al. 2002; Montori et al. 2007): *Alytes obstetricans* (Laurenti 1768), *Pelodytes punctatus* (Daudin 1802), *Pelobates cultripes* (Cuvier 1829), *Bufo calamita* (Laurenti 1768), *Bufo spinosus* Daudin 1803, *Hyla meridionalis* Boettger 1874, *Pelophylax perezi* (López Seoane 1885), *Rana temporaria* Linnaeus 1758, and the introduced *Discoglossus pictus* (Otth 1837). Mouth and gut morphology of these tadpoles are similar, fitting the traditional classification of tadpoles as filter-feeding herbivores (Altig and

McDiarmid 1999a). They feed mostly on suspended or deposited matter (mainly detritus, algae and phanerogams), and their diets are usually interpreted as simply coupled to the availability of resources (Wassersug 1975; Diaz-Paniagua 1985, 1989; Campeny 2001; Caut et al. 2012; but see Richter-Boix et al. 2007c). Some studies report negative effects of competition among species included in our study (Banks and Beebee 1987; Richter-Boix et al. 2004, 2007a), implying a role of interspecific competition in the population dynamics of the guild. However, as previously stated, physiological traits of these species potentially enhancing or interacting with competitive abilities have been largely overlooked hitherto.

In our study area, the continuous pond permanency gradient used by species can be classified into three major categories (Richter-Boix et al. 2013): (1) ephemeral pools that dry up within weeks, containing water for <2 months, with few or no predators. The anuran community of ephemeral pools is composed of *D. pictus*, *B. calamita*, and *P. punctatus;* (2) temporary ponds that flood in the spring and autumn rainfall. These ponds dry out in summer and occasionally in winter, usually containing water for more than 2 consecutive months, and comprise invertebrate predators. Species present in these ponds are *D. pictus*, *B. calamita*, *P. punctatus, A. obstetricans*, *P. cultripes*, *H. meridionalis*, *R. temporaria,* and *B. spinosus;* (3) permanent ponds containing water all year round, with many invertebrate predators or even fish as the top predators, comprising *P. cultripes*, *A. obstetricans*, *H. meridionalis*, *P. perezi*, and *B. spinosus*.

Experimental settings

During March to June of 2007, we conducted a set of laboratory experiments under light and temperature control (12D:12L, 22–24 °C) to study the differential exploitation of a standardized food resource by the nine tadpole species from the study area. In order to minimize the possibility of genetic particularities, we collected three egg masses from three different and separated (>3 km at least) locations for each species in the surroundings of both Garraf and Montseny Natural Parks. As *A. obstetricans* males perform parental care of their eggs, we had to directly collect tadpoles of similar sizes and Gosner Stage 25—instead of egg masses—from three different ponds within Garraf NP. All eggs—or tadpoles—of each species were collected synchronously (within 2 days) from the most typical breeding habitats in each case, excluding spawns or tadpoles from pond typologies uncommon to the species. Once collected, we transported eggs or tadpoles to the laboratory in the University of Barcelona, where we mixed and reared them in 100 × 60 × 30 cm tanks in low densities until tadpoles hatched and reached Gosner stage 25 (Gosner 1960). Then we randomly took 30 individuals from each species and raised them individually in the experimental unit containers. Experimental units consisted of two circular 1-L plastic containers (diameter 12.5 cm, depth 10 cm), one inside the other. We removed the bottom of the inner container and we added a rigid mesh (mesh size 4 mm), which was 2–3 cm away from the bottom of the other container, to prevent tadpoles from eating their own faeces. In order to familiarize tadpoles with the food used in the experiments, each tadpole was fed small pieces of Sera® Spirulina tabs placed in a small Petri dish on the mesh for 2 weeks. Despite possible differences in the ability to assimilate Spirulina among species (Altig and McDearman 1975; Steinwascher and Travis 1983), the similarities among the diets and feeding of the studied species (plus 2 weeks of familiarization) allow us to discard important biases due to the food chosen. Moreover, it was necessary to standardize the food type in order to allow interspecies comparisons. After the 2 weeks of familiarization, we starved tadpoles for 1 day to ensure that their guts were empty (Savage 1952), and we weighed them to the nearest 0.1 mg (balance: Mettler Toledo AG204 DeltaRange).

We then placed tadpoles back individually into the same containers, where they were kept for 5 days. During this period, all tadpoles were fed Spirulina Tabs ad libitum according to tadpole size every second day. Spirulina Tabs were weighed previously to incorporation into individual containers to correctly ensure the ad libitum conditions without dirtying the water unnecessarily. Every 2 days, water was changed and any uneaten food was removed carefully and placed on a small pre-weighed glass Petri dish, then replaced with a new weighed piece of Spirulina Tab. When tadpole faeces were detected in the Petri dish of the food, they were carefully removed by pipetting to ensure that the tadpoles only consumed Spirulina. After the fifth day, any uneaten food was removed and the tadpoles starved for 1 day before being weighed again. Given the reported relationships among relative gut length, growth rate, predation risk and competition (Relyea and Auld 2004) we decided to measure gut length of tadpoles. Therefore, tadpoles were then photographed and euthanized using tricaine methane sulfonate (MS-222), before their guts were extracted, unfolded and photographed. Body size and gut length were measured using SigmaScan Pro 5.0. All tadpoles used for the experiments were between Gosner stages 26 and 30. We had to reduce the number of individuals of *R. temporaria* and *P. cultripes* prior to the experiment due to previous mortality. Survival throughout all the experimental process was high in most species (>80 %), except for *B. calamita* (50 %) and *B. spinosus* (37 %). As previous researchers have noted (Richardson 2002), *Bufo* species sometimes grow poorly when isolated, advising caution with the interpretation of these data.

Faeces (FE) and uneaten food were dried (12 h at 60 °C) and weighed to determine the dry mass of food ingested (FI = (dry mass of food given) − (dry mass of food uneaten)). Wet body mass was converted into dry body mass using the following equation (Feder 1981): Dry mass = $0.047^{1.06} \times$ Wet mass. The increase in dry weight (IDW) was calculated by subtracting Dry Weight 1 (DW1, weight at the start of the experiment) from Dry Weight 2 (DW2, weight at the end of experiment). We then calculated daily growth rate (referred to as growth rate, GR = IDW/[days × DW1]), the daily consumption rate (referred to as consumption rate, CR = FI/[days × DW1]), the assimilation efficiency (ASSM = (FI − FE)/FI)) and the growth allocation (GA = IDW/(FI − FE)) for each tadpole (Richardson 2002). Gut length was corrected by body length to give the relative gut length (RGL).

Statistical analyses: growth physiology

During data exploration, we detected two clearly different physiological groups o*f A. obstetrican*s (Figs. S1 to S6). Once these differences were statistically confirmed (see Supporting Information A, Figs. S1 to S6) through a k-means clustering analysis (Hartigan and Wong 1979), we interpreted these two groups as overwintering versus non-overwintering cohorts of *A. obstetricans* (García-París et al. 2004; Salvador and García-París 2001), and subsequently included each cohort separately into interspecific analyses. We evaluated interspecific differences among species/cohorts using one-way ANOVAs followed by TukeyHSD post hoc tests (Supporting Information A, Table S1). We also explored possible physiologic patterns related to size and possible variable correlations testing linear regressions for each combination and adjusting the α level following the Bonferroni correction (Bonferroni 1936) (Supporting information A).

After data exploration, we aimed to (1) elucidate which variables better define growth rate (GR) from an intra- and interspecific point of view, (2) elucidate if physiological variables are correlated, and (3) detect possible relationships among relative gut length (RGL) and physiological traits.

To visually explore which physiological variables could better define the growth rate of tadpoles, we first tested linear regressions for CR, GA and ASSM against GR separately for each species (Figs. S7 to S9). To statistically determine which variables better define GR for each species, we fitted a linear mixed model (LMMs) per species. These LMMs included as fixed effects all above-mentioned physiological variables, plus individual as a random effect in order to cope with overdispersion (Zuur et al. 2009). We tested each fixed term using a likelihood ratio test (LRT) between the full model and a simplified model without the variable of interest.

We also fit global LMMs (all species together) including "species" as an extra fixed effect (cohorts of *A. obstetricans* were treated separately). We then selected the best global LMM defining GR of tadpoles using AIC, AICc, BIC and LRTs. We only allowed for interactions among species and the other variables, and we included individual as a random effect again in order to cope with overdispersion. We fitted all LMMs using the function *lme* in the package nlme (Pinheiro et al. 2016).

To visually explore which physiological variables could be related with the amount of energy invested in growth for each species, or if physiological variables are correlated, we ran and tested linear regressions of CR, GA and ASSM against each other separately for each species (Figs. S10 to S12). We tried to detect possible relationships of all studied variables with RGL using the same methodologies (Figs. S13 to S16).

Statistical analyses: evolutionary and ecological analyses

The aim of these analyses was to: (1) detect evolutionary patterns in the mean values of physiological parameters of each species (i.e. phylogenetic signal, possible constraints), (2) relate these variables with the use of the pond permanency gradient of each species and (3) explore other interspecific patterns which may explain each species particular physiology.

For phylogenetic analyses, we worked using the ape package in R (Paradis et al. 2004) using the most comprehensive amphibian dated phylogeny to date (Gomez-Mestre et al. 2012), which includes all species in our study. In order not to distort results, we only used data from spring-born tadpoles in all phylogenetic analyses, thus excluding overwintering *Alytes obstetricans* tadpoles (Fig. S17).

First, we needed to evaluate which evolutionary model best fit the studied traits. We used "fitContinuous" in geiger (Harmon et al. 2008) to fit a Brownian motion (BM) and an Ornstein–Uhlenbeck (OU) model for each variable (i.e. GR, CR, GA, ASSM, RGL). We then tested the simpler model (i.e. BM) against the more complex model (i.e. OU) using likelihood ratio tests. If OU models were not significantly better than BM models, we kept the simpler model. Once we determined that BM was the best evolutionary model for all our variables (See "Results" section), we proceeded to test their phylogenetic signal.

Since the tests for phylogenetic signal are poor with a small number of species, we tested for phylogenetic signal using the four most used indices (Münkemüller et al. 2012), all suitable for BM evolutionary patterns. Thus, we estimated and tested the significance of Moran's I (Moran 1950; Gittleman and Kot 1990) and Abouheif's C mean (Abouheif 1999) using the function *abouheif.moran* in the package adephylo (Jombart et al. 2010) and using *patristic* and *oriAbouheif* methods for the proximity matrices, respectively. Pagel (1999) λ and Blomberg et al. (2003) were estimated using *phylosig* in the package phytools (Revell 2012), which allows the user to incorporate individual variation (following Ives et al. 2007). Randomization tests (5000 iterations) were used to determine the significance level of the latter two indices. Given that some variables gave values near to phylogenetic relatedness and CR had a significant phylogenetic signal (see "Results" section), interspecific patterns were analyzed and discussed both regarding and disregarding phylogeny.

To detect interspecific patterns, we summarized the information in a 2D plane by conducting a principal components analysis (PCA) with GR and the variables defining it (GA and CR, see "Results" section).

In order to test if interspecific patterns are related with the pond permanency gradient and co-occurrence in ponds, we used as additional data a presence/absence data matrix (partially used in Richter-Boix et al. 2013) on 274 ponds in the studied area (Table S4). Thus, for each species we had a mean pond permanency gradient value for ponds it inhabited, and a mean value for each trait studied. We studied the relationship between the gradient and the traits of interest by means of both linear models (LM) and phylogenetic generalized least squares (PGLS) regressions. We also applied a partial mantel test (correcting for phylogenetic distance) among a matrix representing physiological dissimilarity (defined by CR, ASSM and GA using Euclidean distances) and a matrix of pond permanency gradient distance or a matrix dissimilarity of pond co-occurrence. Both matrices of ecological data were defined from the presence/absence matrix of 274 ponds (Table S4), using Bray-Curtis dissimilarity among species for co-occurrence, and linear distance among pond preference mean (1 = ephemeral, 2 = temporary, 3 = permanent) for pond permanency preference dissimilarity matrix.

All statistical analyses were performed using R (R Development Core Team 2014).

Results

Linear regressions revealed a positive relationship of both consumption rate (CR) and growth allocation (GA) with growth rate (GR) for all species. According to LMMs, these two variables had a significant role defining GR in all cases (Table S2). On the other hand, results for assimilation (ASSM) were inconsistent throughout the set of species studied (Fig. S8; Table S2). Accordingly, the best global model (all species together) explaining GR included only species (SP), CR and GA as explanatory variables (Table 1), leaving out

Table 1 Summary of the most representative linear mixed models possibly defining individual growth rate included in model selection procedures

Model	*df*	AIC	BIC	logLik	*p* > X2 versus best model
gr ~ cr + ga	5	−671.11	−655.06	340.55	0.0053
gr ~ sp + cr	12	−403.88	−365.37	213.94	<0.0001
gr ~ sp + ga	12	−565.56	−527.05	294.78	<0.0001
*gr ~ sp + cr + ga	13	−676.89	−635.16	351.44	(−)
gr ~ sp + cr + ga + assm	14	−676.09	−631.16	352.05	0.2729
gr ~ sp + cr + ga + rgl	14	−677.65	−632.72	352.83	0.0965
gr ~ sp + cr + ga + assm + rgl	15	−676.76	−628.62	353.38	0.1442

Models are ordered by increasing number of degrees of freedom. Log-likelihood ratio tests are done always against the best model in order to test the significance of each term. Best model is marked with an asterisk. All models include individual as a random effect (not shown in the table)

gr Growth rate, *sp.* species, *ga* growth allocation, *cr* consumption rate, *assm* assimilation efficiency, *rgl* relative gut length

ASSM. According to the criteria used and likelihood ratio tests, among the three selected explanatory variables CR and GA surpassed SP in importance.

The physiological variables CR, GA and ASSM are essentially unrelated at the intraspecific level (Figs. S10 to S12): only in 3 out of 30 regressions among these variables did we found a significant relationship. A negative relationship between GA and ASSM is seen in both bufonids (which could indicate particularities in the way the assimilated energy is invested), and a negative relationship between GA and CR occurs in overwintering tadpoles of *A. obstetricans*. The relative gut length (RGL) of tadpoles is generally unrelated to any of the physiological variables studied (Figs. S13 to S16). We found significant relationships between RGL and the other variables in only 5 out of 40 regressions tested. Interestingly, these five significant relationships were all found in only two species: *D. pictus* and *P. punctatus*. RGL of *D. pictus* related with GA, GR and CR, while RGL of *P. punctatus* related with GR and GA.

All traits were better fitted with a brownian motion model (Table S3) and among them only one trait studied, namely CR, had a significant phylogenetic signal in some tests (Table 2).

The PCA on GR, CR and GA summarized 98 % of the available information in a 2-dimensional plane. The first principal component (PC1, 64 % of variance) correlated positively with all three variables, mainly GR. Second axis (PC2, 38 % of variance) was not correlated at all with GR, instead having a clear positive relationship with CR and a negative correlation with GA. Species have significantly different values for all these individual variables, and therefore are distributed heterogeneously in this plane, defining different tactics to attain a certain GR (Fig. 1).

We did not find any significant regression or phylogenetic generalized least squares (PGLS) regression results of variables against the pond permanency gradient (Fig. 2). Species GR seems to be unrelated with its distribution across the gradient (Linear model: $F_{1,7} = 0.235$, $p = 0.630$, PGLS: $F_{1,7} = 0.144$, $p = 0.715$), but the way this growth is attained tends to be more consumption-focused in ponds with shorter hydroperiod, and more allocation- or assimilation-based in ponds with longer hydroperiod. Thus, CR is mildly higher—but far from being statistically significant—in species inhabiting ponds with short hydroperiod (Linear model: $F_{1,7} = 0.746$, $p = 0.416$, PGLS: $F_{1,7} = 1.886$, $p = 0.212$), while ASSM (Linear model: $F_{1,7} = 2.597$, $p = 0.151$, PGLS: $F_{1,7} = 3.880$, $p = 0.089$) tends to increase along with hydroperiod. On the other hand, GA (Linear model: $F_{1,7} = 0.037$, $p = 0.854$, PGLS: $F_{1,7} = 0.224$, $p = 0.650$) and RGL (Linear

Table 2 Phylogenetic signal of studied traits according to the four chosen indices

	GR	CR	ASSM	GA	RGL
Mean (Bloomberg's K)	1.0171	1.2854	0.8123	1.0278	1.0807
Mean (Pagel's L)	0.5085	0.8466	0.4568	0.4723	0.5331
p (Bloomberg's K)	0.0982	**0.0300**	0.2709	0.2116	0.0750
p (Pagel's L)	0.5773	0.1439	0.8221	0.7688	0.4496
p (Moran's I)	0.1115	0.0501	0.2927	0.2450	0.1651
p (Abouheif's C)	0.1823	**0.0113**	0.2894	0.1937	0.0646

GR growth rate, *CR* consumption rate, *ASSM* assimilation efficiency, *GA* growth allocation, *RGL* relative gut length

Significant values ($p < 0.05$) are in bold

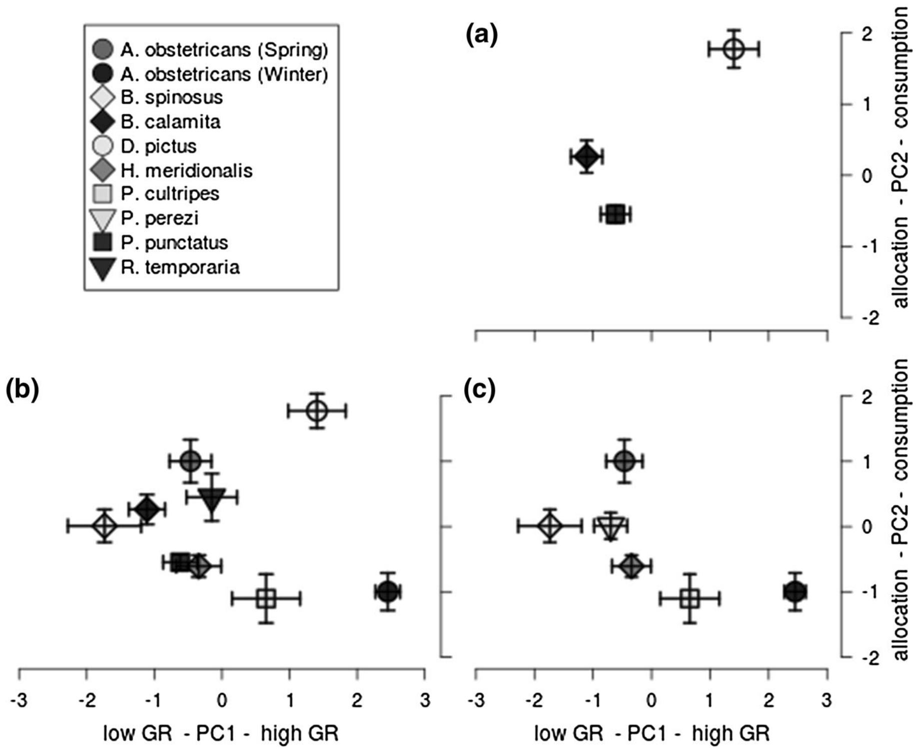


Fig. 1 Species mean ± SE on the two first axes of a PCA using GR (= growth rate), GA (= growth allocation) and CR (= consumption rate) for all studied specimens, representing 98 % of data variability. Species are represented in all pond types where they usually breed. PC1 is positively correlated with growth rate (GR). Values in PC2 increase as tadpoles rely more on consumption and less in allocation to grow. **a** Ephemeral ponds. **b** Temporary Ponds. **c** Permanent ponds

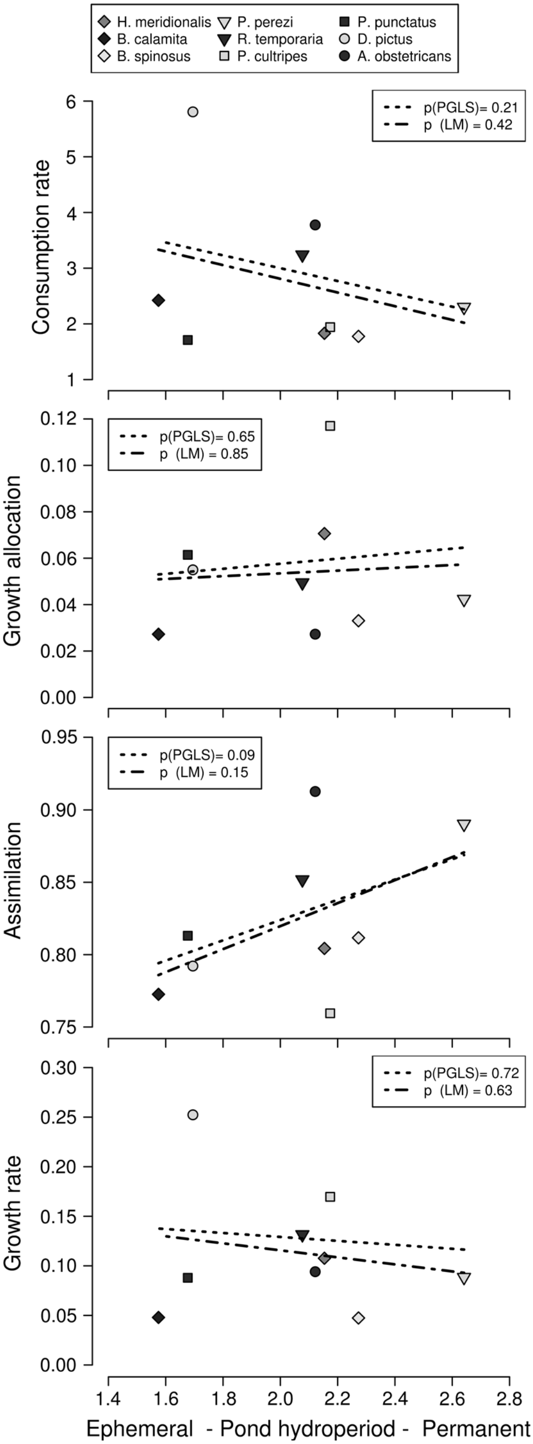
model: $F_{1,7} = 0.013$, $p = 0.913$, PGLS: $F_{1,7} = 0.385$, $p = 0.555$) showed no visible tendencies. Finally, partial Mantel tests point to a lack of a clear relationship among physiology and use of pond permanency gradient ($r = -0.00022$, $p = 0.406$) or among physiology and co-occurrence in ponds ($r = 0.00933$, $p = 0.431$).

Discussion

All physiological variables used in this study are generally uncorrelated, thus clearly representing different facets of the physiological abilities of tadpoles. But more importantly, physiological abilities represented by these variables were not randomly distributed across species. Each species has a particular physiology (Fig. 1), and significant differences among species were widespread in all features (Table S1).

Growth rates of tadpoles could depend on (1) food consumption rates, (2) the efficiency of assimilation of the ingested food and (3) energetic allocation preferences (i.e. investment of energy in growth). According to our results both at intra- and interspecific levels, only food consumption rates and the proportion of energy invested in growth visibly influence tadpole growth rates at individual level. Although they must be important for

Fig. 2 Species-level relationship among preferred pond type (*horizontal axis*: higher values indicate longer hydroperiods) and consumption rate, growth allocation, assimilation and growth rate. *Dots* indicate mean species value. *PGLS* phylogenetic generalized least squares, *LM* linear model



other purposes (like increasing the amount of energy available to allocate), food assimilation abilities lacked a direct relationship with growth rates. Within our array of species, the growth rate of a tadpole is defined mainly by the amount of food consumed and the capacity to allocate the energy into new somatic tissues. According to interspecific models these two factors surpass species (that is, species-specific features) in importance (Table 1).

Interestingly, the relative importance of consumption and allocation to defining growth rate is not the same for all species. Species in our study exhibit a continuum between a "consumer strategy" and an "efficient strategy": while the growth rate of the species following the consumer strategy is based chiefly on high food consumption, species using the efficient strategy base their growth more on the ability to increase assimilation and convert the assimilated energy into growth. As prominent examples, *D. pictus* showed the highest consumer strategy, while overwintering cohorts of *A. obstetricans* and *P. cultripes* use high energetic investments in growth to produce large tadpoles.

What makes a species evolve towards one or the other strategy? At least in our array of species (with comparable results to Richardson 2002), phylogenetic relationships do not seem to be an important factor to take in consideration. We found only limited evidence of phylogenetic signal in the consumption rate of species. The value of Bloomberg's was 1.29, higher than 1, indicating values more similar than expected phylogenetically. This could possibly reflect a constraint to evolving larger ingestion abilities without changing the general body plan of the tadpole, which is the same for all species in our study. In general terms, negative results of phylogenetic signal, in addition to the existence of discrete physiological groups within a species (*A. obstetricans*), suggest a degree of evolutionary lability in the studied traits.

From an ecological point of view, although the use of the consumer-efficient continuum seems related to the use of the pond permanency gradient by each species (Fig. 2), statistical results are not conclusive in this study. Similarly, as we hypothesized, species from ephemeral ponds tend to base growth more on consumptive abilities, while species usually inhabiting ponds with longer hydroperiods show an allocation-based tactic for growth, at the same time having higher assimilation rates (Fig. 2). These tendencies, although non-significant, coincide with the predictions of predation risk and activity levels along the pond permanency gradient. In ephemeral ponds with few predators, tadpoles should be able to base their growth in more consumptive tactics because increased activity might not be linked to a survival cost. In contrast, in permanent or long hydroperiod ponds with more abundant and complex predator communities, the need to maintain lower activity levels must be compensated for with a higher assimilation of ingested food and a higher allocation of obtained energy in growth. From another point of view, if we focus on pairs of related species or cohorts, the inhabitants of shorter hydroperiod ponds also tend to be the most consumptive. *R. temporaria* has a more flexible use of ponds and also a more consumptive physiology than the other ranid in the area (*P. perezi*), which is mainly restricted to permanent ponds. In another case, despite similarities in most biological and morphological features of the larval phase, *B. spinosus* tadpoles allocate more energy in growth than *B. calamita* (more common in ephemeral ponds). Finally, overwintering cohorts of *A. obstetricans* (mostly found in permanent ponds) also have a more allocation-based growth tactic than spring cohorts (more consumptive). Moreover, the enhanced allocation abilities of overwintering tadpoles could be useful for growth using scarcer resources during cold season (Begon et al. 1996; Bennion and Smith 2000). In this case, specific studies would be required to evaluate the ecological and evolutionary importance of these physiological differences between cohorts.

The inconclusive results for all of these tendencies could be primarily due to the relatively low number of anuran species inhabiting the area, meaning very few data for interspecific tests (n = 9). Moreover, only one of these species has clear preferences for permanent ponds, further impeding a powerful and reliable statistical assessment of physiological tendencies along the pond permanency gradient. On the other hand however, we cannot discard coevolutionary pressures to use different physiological tactics: efficient species may be forced to allocate even more energy to growth if they have to overcome competition from highly consumptive competitors, and vice versa. Thus, the effects of similar ecological preferences could be a dissimilarity among species instead, i.e. an overdispersion of species traits (Astor et al. 2014). Therefore, a diversification of physiological tactics among usually coexisting species cannot be disregarded. Competitively, all tadpoles present in the NW Mediterranean basin fit the traditional classification of tadpoles as filter-feeding herbivores (Altig and McDiarmid 1999a). Dietary studies yield similar results for all species, feeding on detritus, algae and phanerogams, complemented with small amounts of fungi, bacteria and small animals or their carcasses (Diaz-Paniagua 1985, 1989; Campeny 2001; Caut et al. 2012). Although a different phenology and use of the pond gradient seem to relax competition among our array of species (Jakob et al. 2003; Richter-Boix et al. 2006a, 2007a), complex larval guilds with high niche overlap between species can be easily found (Diaz-Paniagua 1985, 1989; Richter-Boix et al. 2013). Some experiments report negative fitness effects due to competitive resource depletion using species included in our study (Banks and Beebee 1987; Richter-Boix et al. 2004, 2007a). Thus, although a high availability of feeding resources seems to allow a certain niche overlap (Wassersug 1975; Diaz-Paniagua 1989), species coexisting with resource-depleting competitors like *D. pictus* (San Sebastián et al. 2015a, b) could become forced to allocate more energy in growth to overcome negative competitive effects. These or similar competitive interactions could easily blur the patterns and tendencies related to the pond permanency gradient in our data, making species that inhabit similar ponds diverge instead of converging in their physiology (Fig. 1).

Although relative gut length is a plastic feature mainly reported to change in order to enhance assimilation and growth rates (Noble 1931; Altig and Kelly 1974; Horiuchi and Koshida 1989; Relyea and Auld 2004), in this study gut length was generally unrelated to assimilation or any other variables. The only exceptions are found in *P. punctatus* and *D. pictus*, both common inhabitants of Mediterranean ephemeral ponds. Species inhabiting these ephemeral ponds are all forced to breed simultaneously when ponds fill in raining season to rapidly grow and develop before desiccation (Diaz-Paniagua 1990). The fact that significant relationships of relative gut length with growth-related features are restricted to species from these habitats (*P. punctatus* and the invasive *D. pictus*) might signal that food processing is especially important for these taxa.

Of all species, *D. pictus* showed the most extreme consumer strategy. In this particular case, this could be related with the superior ability to exploit resources described in some invasive populations (Petren and Case 1996; Kupferberg 1997; Holway 1999). Studies using native specimens of *D. pictus* would be needed to test possible physiological changes related to invasion. According to our results, although species found in ephemeral pools (*D. pictus, P. punctatus* and *B. calamita*) assimilate food similarly, native competitors rely less in consumption, showing a more allocative tactic. This employment of a more allocation-based strategy could be, to our knowledge, a first hypothesis explaining the apparent lack of competitive exclusion of natives *P. punctatus and B. calamita* by the resource-depleting invasive *D. pictus*. On the other hand however, mild physiological differences between both bufonids seem insufficient as a mechanism to avoid competitive exclusion:

studies report clear negative effects on tadpoles of *B. calamita* under competition with *B. bufo* larvae (Bardsley and Beebee 2000; Richter-Boix et al. 2007a). Further studies should disentangle if physiological factors are really important in these competitive systems.

Summing up, physiological differences among species generally follow the ecological predictions for the pond permanency gradient, but results are not clear enough to be conclusive. Despite mild interspecific physiological tendencies along the gradient (Fig. 2), once selected the list of species according to pond type, usual competitors appear rather separated (Fig. 1). Similarly as seen in Richardson (2002), phenotypic differences between species seem to persist in the same habitat, suggesting that species can display different evolutionary solutions to the same problems. The absence of convincing results may also indicate contrasting selective pressures of both competition with other tadpole species and different ecological conditions along the predation—pond desiccation risk gradient. The relative importance of competitive pressure and predation/desiccation risk shaping the physiology of tadpoles remains to be further studied.

Conclusions

Food consumption rates and the proportion of energy invested in growth (i.e. growth allocation) define tadpole growth rate, while assimilation abilities or other features like gut length seem not directly related with it. Our data suggests a labile continuum of physiological tactics from consumption-based to allocation-based growth along which species differentiate. Differences among species in this continuum generally follow predictions of adaptation to the ecological conditions along the pond permanency gradient, but also to strong competition in confined systems like ponds. The relative importance of these two selective pressures remains to be further studied. Physiological traits of each species could be an interesting field of study to understand how larval anurans adapt to the ecology of ponds, and how they compete and coexist.

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Chapter 3: Supplementary Information

Statistical analyses: preliminary analyses

The aim of preliminary analyses was to: (1) Ensure that data from our species form homogeneous groups (determine if there are different intraspecific groups), (2) determine if there are interspecific differences among species.

During data exploration we visually detected two clearly different physiologic groups of *A. obstetricans*. To statistically confirm the existence of this intraspecific division, we conducted a k-means clustering analysis (Hartigan and Wong 1979) using the information of the scaled (in order to give each variable the same importance) physiologic variables. We set the number of random starts to 10 and the maximum number of iterations at 100, values at which divergence of results after different runs were totally undetectable. We conducted clusterings with different numbers of clusters from 1 to 6, and the rate among each total sums of squares (TSS) and within groups sums of squares (WSS) was used to assess the optimal number of clusters (Fig. S1). Finally, we run ANOVAs on all studied variables to detect significant differences among tadpole groups (see Results). No other species displayed non-homogeneous data or signs of subgroups. In order not to distort the interpretation of the *A. obstetricans* data, intraspecific groups (see Results and Discussion) were treated separately in all analyses, and in some analyses overwintering tadpoles were excluded.

For each variable, we determined if there were interspecific differences among species using one-way ANOVAs followed by TukeyHSD post-hoc tests. Both overwintering and spring tadpoles (see Results and Discussion) of *A. obstetricans* were included in the analyses, but treated separately.
We explored possible physiologic patterns related to the size of tadpoles by fitting and testing linear regressions for each variable and each species separately. Given that we conducted ten regressions (9 species, but 2 cohorts for *A. obstetricans*) for each hypothesis, we adjusted the α level to 0.005 following the Bonferroni correction (Bonferroni 1936). Again, both overwintering and spring tadpoles of A. obstetricans were included but treated separately.

Results: Preliminary Analyses

The clustering analysis pointed at the existence of two physiologic groups in *A. obstetricans*, as the drop in the ratio of WSS to TSS was greatest at $n = 2$ (Fig S1). Moreover, this clustering coincided perfectly with tadpole size and GR, even though these variables were not included in the clustering analysis. Thus, *A. obstetricans* tadpoles with a dry weight lower than 9.7 mg displayed low growth rates and low GA, while those with a dry weight greater than 9.7 mg displayed high growth rates and high values of GA. According to ANOVAs, these two groups differed in GR ($F_{1,26}$=269.4, p<0.0001), CR ($F_{1,26}$=10.47, p=0.0033), GA ($F_{1,26}$= 141.6, p<0.0001), ASSM ($F_{1,26}$=13.27, p=0.0011), but not in RGL ($F_{1,26}$=0.58, p= 0.452). Most probably, this grouping corresponds to spring and overwintering cohorts, a fact long known for this species in the area of study (García-París *et al.*2004; Salvador and García- París 2001). Thus, the unintentional collection of tadpoles born before and after winter could explain the presence of a non-homogenous data sample of *A. obstetricans* and a total coincidence between physiological clustering and size of tadpoles.

There were differences among species in all variables studied (Fig.1): DryWeight1 ($F_{8,158}$=84.15, p<0.0001), Growth Rate ($F_{8,158}$=20.57, p<0.0001), Consumption Rate ($F_{8,158}$=64.35, p<0.0001), Assimilation ($F_{8,158}$=10.44, p<0.0001), Growth Allocation ($F_{8,158}$=14.01,p<0.0001), and Relative Gut Length ($F_{8,158}$=20.46, p<0.0001). TukeyHSD results are summarized in Table S1.

Only two variables studied seem to be related to size of tadpoles (results summarized in Figs S2 to S6): GR and CR tend to decrease with increasing tadpole size in all species, giving significant results in 5 and 4 out of 10 regressions, respectively. On the other hand, GA has a clearly milder downward trend while ASSM and RGL do not show any visual tendency of a relationship with size, giving significant results in 0, 0 and 1 out of 10 regressions respectively.

Table S1: TukeyHSD Post-hoc p-values for all variables studied. Variables: DW1= initial dry weight, GR= growth rate, CR= consumption rate, ASSM= assimilation, GA= growth allocation, RGL = relative gut length. Species: AobS: *Alytes obstetricans* (non-overwintering), AobW: *Alytes obstetricans* (overwintering), Dpi: *Discoglossus pictus*, Ppu: *Pelodytes punctatus*, Pcu: *Pelobates cultripes*, Rte: *Rana temporaria*, Ppe: *Pelophylax perezi*, Bsp: *Bufo spinosus*, Bca: *Bufo calamita*, Hme: *Hyla meridionalis*.

COMPARISON	DW1	GR	CR	ASSM	GA	RGL
Bca-Hme	0.5784	0.0893	0.2938	0.7928	0.0001	1.0000
Bsp-Hme	0.0620	0.0041	0.9945	0.8529	0.0000	0.0036
Ppe-Hme	0.0541	0.9755	0.3307	0.0000	0.0045	0.9999
Rte-Hme	1.0000	0.9941	0.0000	0.1894	0.2108	0.8841
Pcu-Hme	0.0000	0.2311	1.0000	0.6049	0.0012	0.0000
Ppu-Hme	0.1714	0.9766	1.0000	1.0000	0.9570	0.0037
Dpi-Hme	0.0388	0.0000	0.0000	0.9988	0.5394	0.4884
AobS-Hme	1.0000	0.9999	0.0000	0.0005	0.0049	0.6901
AobW-Hme	0.5712	0.0000	0.0003	0.0308	0.0000	0.8202
Bsp-Bca	0.9768	0.9798	0.1368	0.1516	0.8532	0.0054
Ppe-Bca	0.0003	0.5687	1.0000	0.0000	0.7940	1.0000
Rte-Bca	0.8930	0.0244	0.1302	0.0070	0.6070	0.8572
Pcu-Bca	0.0000	0.0003	0.8965	1.0000	0.0000	0.0000
Ppu-Bca	0.0013	0.6239	0.1258	0.5411	0.0086	0.0711
Dpi-Bca	0.9986	0.0000	0.0000	0.9924	0.0873	0.5309
AobS-Bca	0.9720	0.8051	0.0033	0.0000	1.0000	0.6578
AobW-Bca	0.0134	0.0000	0.8347	0.0006	0.0000	0.8054
Ppe-Bsp	0.0000	0.0637	0.1706	0.3066	0.0365	0.0009
Rte-Bsp	0.2561	0.0011	0.0000	0.9993	0.0265	0.2346
Pcu-Bsp	0.0000	0.0000	0.9842	0.1019	0.0000	0.0000
Ppu-Bsp	0.0000	0.0810	0.9999	0.9732	0.0000	0.0000
Dpi-Bsp	0.9999	0.0000	0.0000	0.5062	0.0006	0.2560
AobS-Bsp	0.5149	0.2463	0.0000	0.1979	0.9385	0.7214
AobW-Bsp	0.0004	0.0000	0.0006	0.9831	0.0000	0.1628
Rte-Ppe	0.1041	0.6485	0.0078	0.7317	0.9999	0.6142
Pcu-Ppe	0.0000	0.0277	0.9667	0.0000	0.0000	0.0000
Ppu-Ppe	1.0000	1.0000	0.1235	0.0001	0.2548	0.0190
Dpi-Ppe	0.0000	0.0000	0.0000	0.0000	0.8242	0.1734
AobS-Ppe	0.3409	1.0000	0.0001	0.9948	0.9403	0.4299
AobW-Ppe	0.9984	0.0000	0.2910	0.8741	0.0000	0.4770
Pcu-Rte	0.0000	0.8373	0.0044	0.0068	0.0000	0.0000
Ppu-Rte	0.2348	0.6616	0.0000	0.4499	0.8842	0.0002
Dpi-Rte	0.3006	0.0000	0.0000	0.0445	0.9986	1.0000
AobS-Rte	1.0000	0.9729	0.8218	0.4880	0.8167	0.9999
AobW-Rte	0.5784	0.0000	0.9208	1.0000	0.0000	1.0000
Ppu-Pcu	0.0000	0.0309	0.9988	0.3853	0.0000	0.0000
Dpi-Pcu	0.0000	0.0303	0.0000	0.9272	0.0000	0.0000
AobS-Pcu	0.0000	0.2878	0.0001	0.0000	0.0000	0.0000
AobW-Pcu	0.0000	0.0000	0.1020	0.0010	0.1260	0.0000
Dpi-Ppu	0.0000	0.0000	0.0000	0.9658	0.9983	0.0000
AobS-Ppu	0.5142	1.0000	0.0000	0.0023	0.0850	0.0004
AobW-Ppu	1.0000	0.0000	0.0001	0.1315	0.0000	0.0000
AobS-Dpi	0.6512	0.0000	0.0000	0.0001	0.3211	1.0000
AobW-Dpi	0.0001	0.0000	0.0000	0.0044	0.0000	1.0000
AobW-AobS	0.7886	0.0000	0.1186	0.6283	0.0000	0.9998

Table S2: Results from likelihood ratio tests for each variable, explaining growth rate of each species. To test variables we extracted them from a full linear mixed model which included individual as a random effect (growth rate ~ growth allocation + consumption rate + assimilation + (individual) + residuals). Then the reduced model (= model without the variable) was likelihood ratio tested against the full model in order to obtain a p-value for the isolated (dropped) variable. If the p - value is not significant, the simpler model (brownian motion) should be selected. Variables: GA = growth allocation, CR = consumption rate, ASSM = assimilation. LR= likelihood ratio. Species: AobW: *Alytes obstetricans* (overwintering), AobS: *Alytes obstetricans* (non-overwintering), Bsp: *Bufo spinosus*, Bca: *Bufo calamita*, Dpi: *Discoglossus pictus*, Hme: *Hyla meridionalis,* Pcu: *Pelobates cultripes,* Ppe: *Pelophylax perezi*, Ppu: *Pelodytes punctatus*, Rte: *Rana temporaria.*

	GA		**CR**		**ASSM**	
	LR	**p value**	**LR**	**p value**	**LR**	**p value**
AobW	53.34	< *0.0001*	55.24	< *0.0001*	22.47	< *0.0001*
AobS	35.76	< *0.0001*	31.33	< *0.0001*	6.63	*0.0100*
Bsp	20.00	< *0.0001*	13.77	< *0.0001*	1.02	0.3122
Bca	34.75	< *0.0001*	20.40	< *0.0001*	0.40	0.5262
Dpi	84.47	< *0.0001*	60.66	< *0.0001*	29.90	< *0.0001*
Hme	98.59	< *0.0001*	48.51	< *0.0001*	4.12	*0.0424*
Pcu	32.76	< *0.0001*	23.39	< *0.0001*	2.87	0.0900
Ppe	82.45	< *0.0001*	43.76	< *0.0001*	7.71	*0.0055*
Ppu	93.92	< *0.0001*	67.03	< *0.0001*	10.24	*0.0014*
Rte	37.65	< *0.0001*	27.84	< *0.0001*	5.19	*0.0227*

Table S3: Corrected Akaike information criterion (AICc) values and likelihood ratio tests (LRT) for both models of continuous trait evolution for each variable. Variables: GR= growth rate, CR= consumption rate, ASSM= assimilation, GA= growth allocation, RGL = relative gut length. Rows: AICc (BM) = AICc value for a browninan motion evolutionary model. AICc (OU) = AICc value for a Ornstein–Uhlenbeck evolutionary model. LR = likelihood ratio among two models. LRT p = p - value for the likelihood ratio test among two models. If the p-value is not significant, the simpler model (brownian motion) should be selected.

	GR	**CR**	**ASSM**	**GA**	**RGL**
AICc (BM)	-19.5309	32.6535	-21.9365	-34.5092	36.2712
AICc (OU)	-15.2641	37.4535	-17.7693	-30.0566	41.0712
LR	0.5332	0.0000	0.6328	0.3474	0.0000
LRT p	0.4653	0.9974	0.4263	0.5556	0.9998

Table S4: Presence (1) / absence (0) matrix data for the studied anuran species in the ponds surveyed in the area used for the study. Pond type: 1 = ephemeral pond, 2 = temporary pond, 3 = permanent pond. <u>Species:</u> Dpi: *Discoglossus pictus*, Bca: *Bufo calamita*, Bsp: *Bufo spinosus*, Ppu: *Pelodytes punctatus*, Aob: *Alytes obstetricans*, Pcu: *Pelobates cultripes*, Hme: *Hyla meridionalis,* Ppe: *Pelophylax perezi*, Rte: *Rana temporaria.*

Region	Site	pond type	Dpi	Bca	Bsp	Ppu	Aob	Pcu	Hme	Ppe	Rte
Barcelona	L156	1	0	0	0	0	1	0	0	0	0
Barcelona	L269	1	0	1	0	1	1	0	0	0	0
Barcelona	L246	1	0	1	1	1	1	0	0	0	0
Barcelona	L253	1	0	1	0	1	1	0	1	0	0
Barcelona	L259	1	0	1	0	1	1	0	1	0	0
Barcelona	L284	1	0	1	0	1	1	0	1	0	0
Barcelona	L272	1	0	1	0	0	0	0	0	0	0
Barcelona	L236	1	0	1	0	1	1	0	0	0	0
Barcelona	L267	1	0	1	0	0	1	0	0	0	0
Barcelona	L260	1	0	1	0	0	0	0	0	0	0
Barcelona	L261	1	0	1	1	1	0	0	0	0	0
Barcelona	L262	1	0	1	1	0	0	0	0	0	0
Barcelona	L153	1	0	1	0	0	0	0	0	0	0
Barcelona	L161	1	0	0	0	0	0	0	1	0	0
Barcelona	L177	1	0	0	0	0	1	0	0	0	0
Barcelona	L222	1	0	1	0	0	0	0	0	0	0
Barcelona	L157	1	0	0	0	0	1	0	0	0	0
Barcelona	L175	1	0	0	0	0	1	0	0	0	0
Barcelona	L244	1	0	1	0	0	1	0	0	0	0
Barcelona	L242	1	0	0	0	0	1	0	0	0	0
Barcelona	L266	1	0	0	0	0	1	0	0	0	0
Barcelona	L169	1	0	0	0	0	1	0	0	0	0
Barcelona	L178	1	0	0	0	0	1	0	0	0	0
Barcelona	L215	1	0	0	0	0	1	0	0	0	0
Barcelona	L214	1	0	0	0	0	1	0	0	0	0
Barcelona	L238	1	0	0	0	1	1	0	0	0	0
Barcelona	L237	1	0	0	0	0	1	0	1	0	0
Barcelona	L254	1	0	0	0	1	0	0	0	0	0
Barcelona	L130	1	0	0	1	1	0	0	0	0	0

Barcelona	L122	1	0	1	0	1	0	0	0	0	0
Barcelona	L123	1	0	1	0	1	0	0	0	0	0
Barcelona	L124	1	0	0	0	1	0	0	0	0	0
Barcelona	L125	1	0	0	0	1	0	0	0	0	0
Barcelona	L126	1	0	1	0	1	0	0	0	0	0
Barcelona	L127	1	0	1	0	0	0	0	0	0	0
Barcelona	L166	1	0	1	0	1	1	0	0	0	0
Barcelona	L255	1	0	1	0	1	1	0	1	0	0
Barcelona	L288	1	1	0	0	0	0	0	0	0	0
Barcelona	L289	1	1	0	0	0	0	0	0	0	0
Barcelona	L197	1	0	0	0	0	1	0	0	0	0
Barcelona	L198	1	0	0	0	0	1	0	0	0	0
Barcelona	L208	1	0	0	0	0	1	0	0	0	0
Barcelona	L194	1	0	0	1	0	0	0	0	0	0
Barcelona	L196	1	0	1	0	0	0	0	0	0	0
Barcelona	L154	1	0	0	0	0	1	0	0	0	0
Barcelona	L206	1	0	0	0	0	1	0	0	0	0
Barcelona	L211	1	0	0	1	0	1	0	0	0	0
Barcelona	L274	1	0	1	0	0	0	0	0	0	0
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Barcelona	L277	2	0	1	0	1	1	0	0	0	0
Barcelona	L283	2	0	0	1	0	0	0	0	0	0
Barcelona	L256	2	0	1	1	1	1	0	0	0	0
Barcelona	L273	2	0	1	1	1	0	0	0	0	0
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Barcelona	L293	2	0	0	0	1	0	0	0	0	1
Barcelona	L294	2	0	0	0	1	0	0	1	0	1
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Barcelona	L121	2	0	0	0	0	0	0	0	1	0
Barcelona	L165	2	0	0	0	0	1	0	0	0	0
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Barcelona	L282	2	0	0	0	0	1	0	0	0	0
Barcelona	L276	2	0	0	0	0	1	0	0	0	0
Barcelona	L167	2	0	0	0	0	1	0	0	0	0
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Barcelona	L210	2	0	0	1	0	0	0	0	0	0
Barcelona	L199	2	0	0	0	0	1	0	0	0	0
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Barcelona	L221	2	0	1	0	1	0	0	0	0	0
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Barcelona	L245	3	0	1	0	1	1	0	0	0	0
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Barcelona	L229	3	0	0	0	0	1	0	0	0	0
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Girona	L58	2	1	0	0	0	0	0	0	0	0
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Girona	L306	2	0	0	1	0	0	0	0	0	1
Girona	L310	2	0	0	0	0	1	0	0	0	1
Girona	L311	2	0	0	0	0	0	0	0	0	1
Girona	L103	2	0	0	1	0	0	0	0	0	0
Girona	L6	2	1	1	1	1	0	1	1	1	0
Girona	L74	2	1	0	0	0	0	0	0	0	0
Girona	L83	2	1	1	0	0	0	1	0	0	0
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Girona	L81	2	1	0	0	0	0	0	0	0	0
Girona	L82	2	1	1	0	0	0	0	0	0	0
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Girona	L69	2	1	1	0	0	0	0	0	0	0
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Girona	L313	3	0	0	0	0	1	0	0	0	0
Girona	L10	3	1	1	1	1	0	1	1	1	0
Girona	L11	3	1	1	1	0	0	0	1	1	0
Girona	L93	3	1	0	1	0	0	1	1	1	0
Girona	L96	3	0	0	1	0	1	1	1	1	0
Girona	L298	3	0	0	1	0	1	0	0	0	0
Girona	L299	3	0	0	1	0	1	0	1	0	0
Lleida	L315	2	0	0	0	0	0	0	0	0	1
Lleida	L316	2	0	0	0	0	0	0	0	0	1
Lleida	L317	2	0	0	0	0	0	0	0	0	1
Lleida	L318	2	0	0	1	0	0	0	0	0	1
Lleida	L319	2	0	0	1	0	0	0	0	0	1
Lleida	L320	2	0	0	0	0	0	0	0	0	1
Lleida	L314	3	0	0	1	0	1	0	0	0	1
Lleida	L321	3	0	0	0	0	1	0	0	0	1

Figure S1: Ratio between within-groups sums of squares (withinSS) and total sums of squares (totalSS) using a different number of clusters for K-means clustering. Clustering was performed using *Alytes obstetricans* standardized physiologic variables: Consumption Rate, Growth Allocation, Assimilation Efficiency.

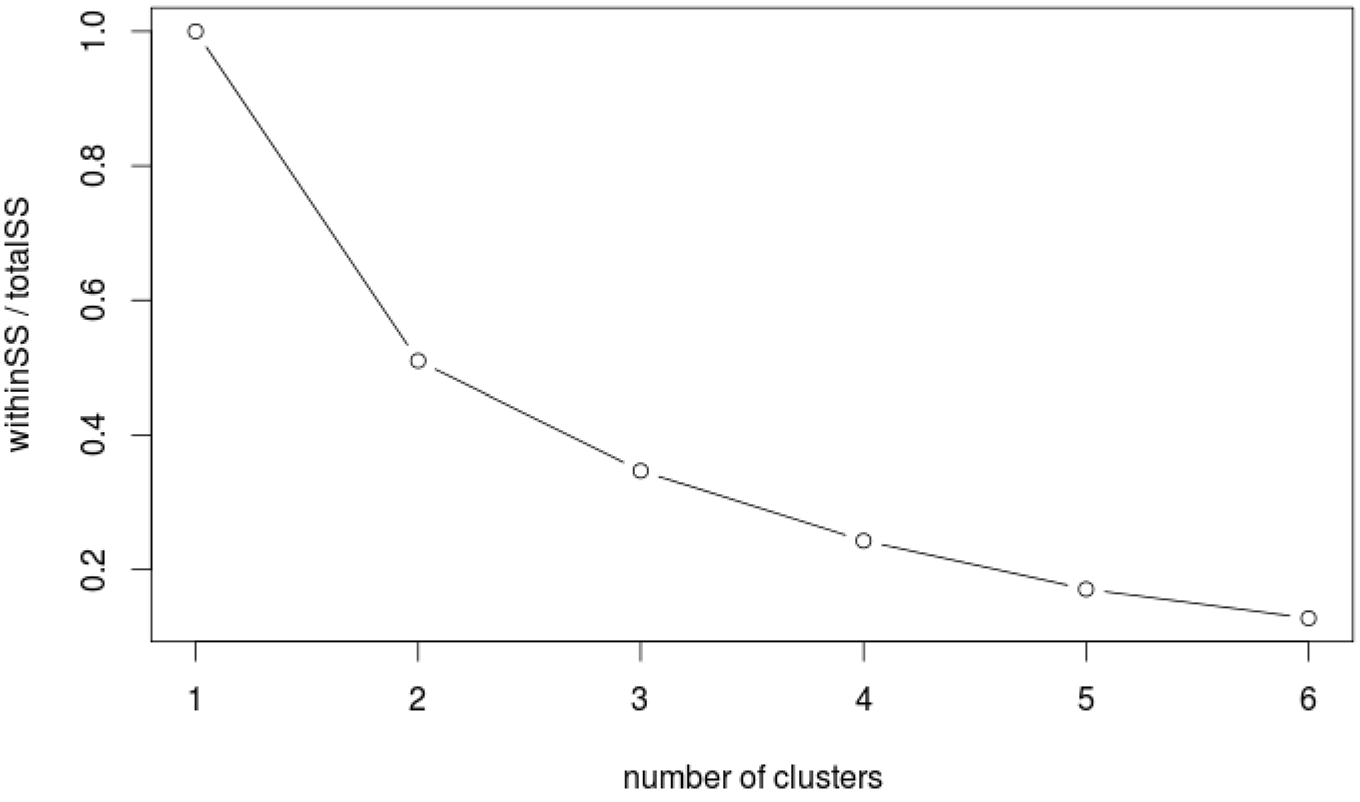


Figure S2: Regressions of growth rate (GR) against dry weight (DW1), for each species. Cohorts of *Alytes obstetricans* are represented in different colors: spring (red), and overwintering (blue). Presence of solid line indicates significant regression.

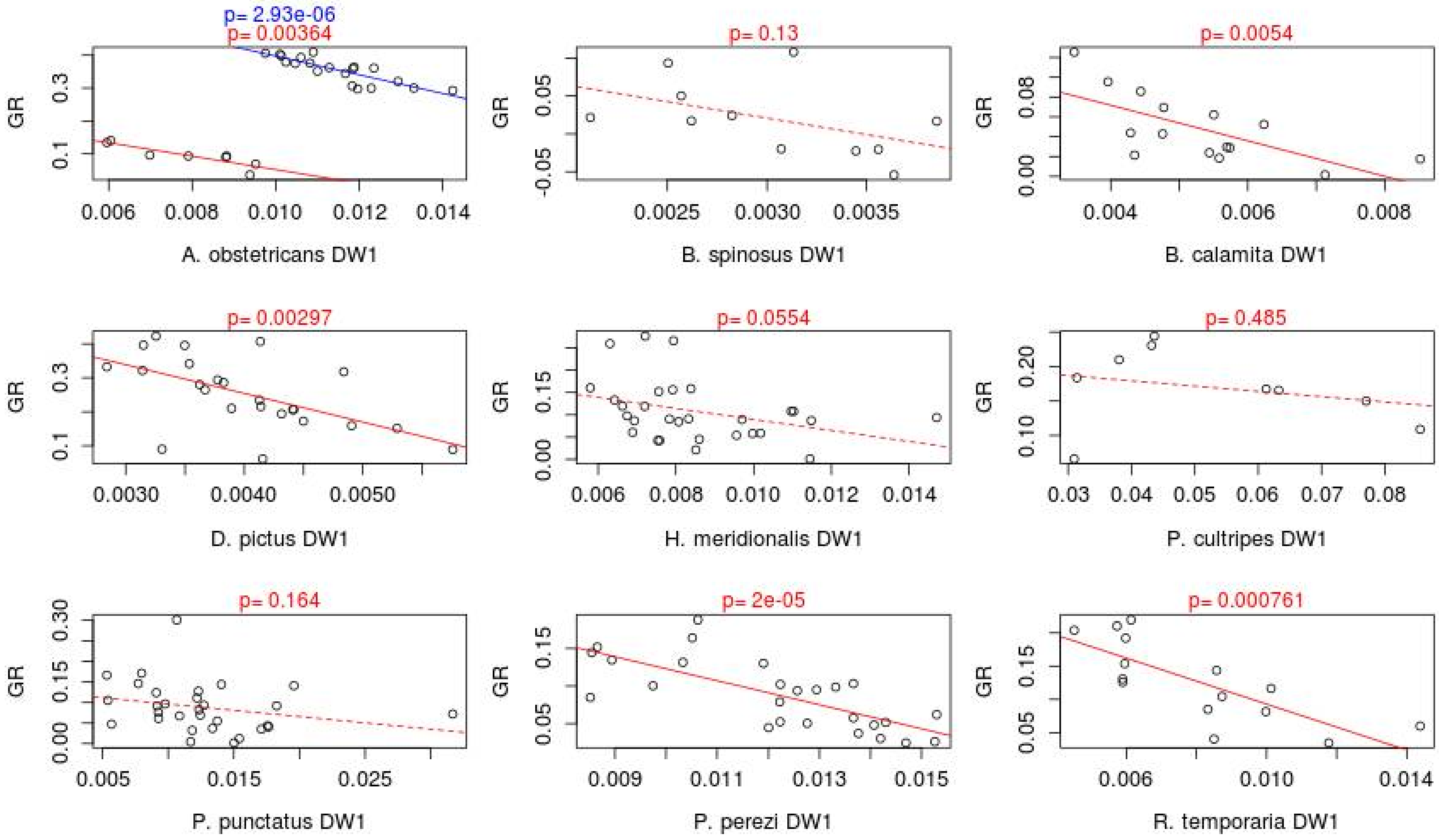


Figure S3: Regressions of consumption rate (CR) against dry weight (DW1), for each species. Cohorts of *Alytes obstetricans* are represented in different colors: spring (red), and overwintering (blue). Presence of solid line indicates significant regression.

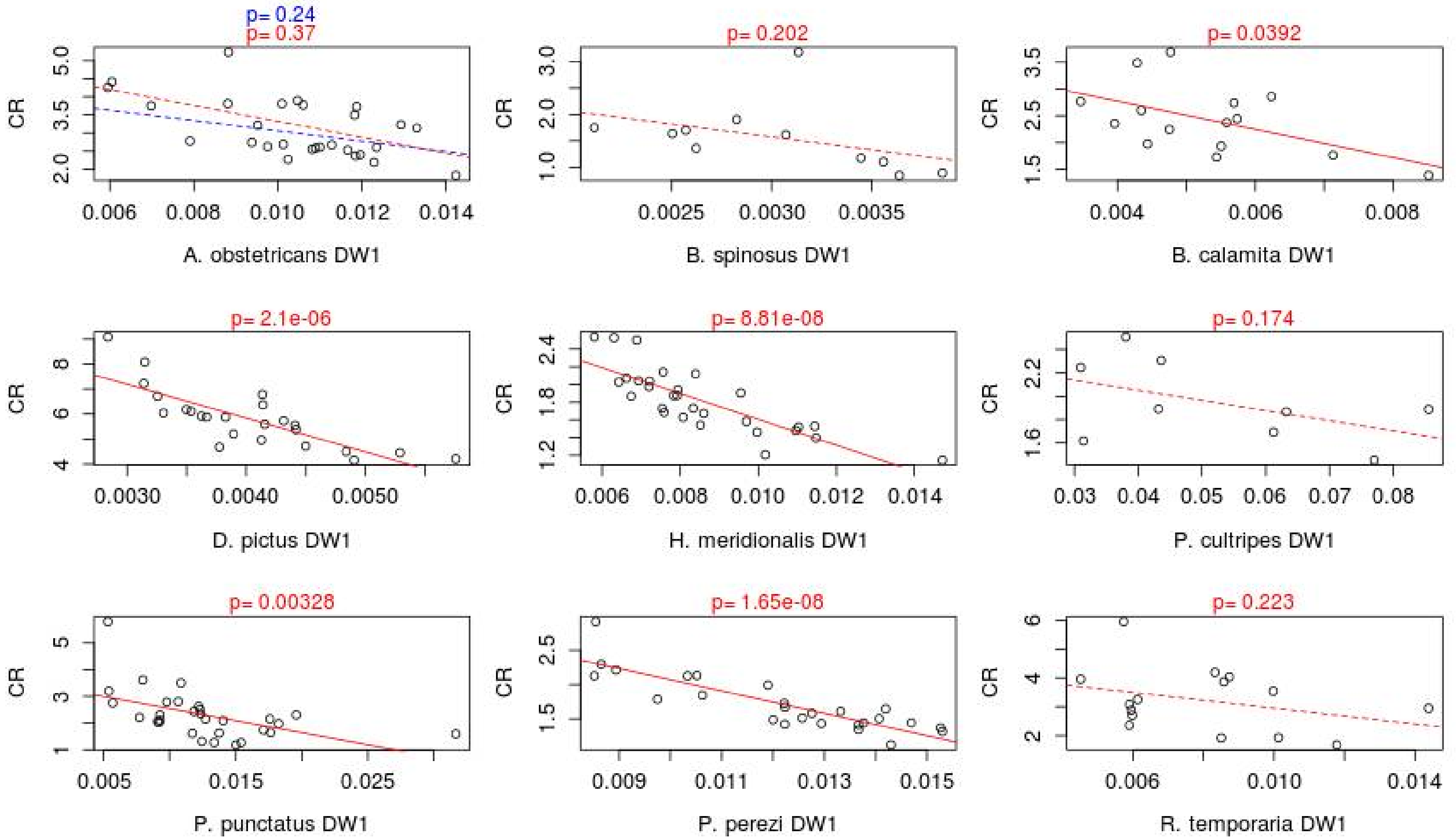


Figure S4: Regressions of growth allocation (GA) against dry weight (DW1), for each species. Cohorts of *Alytes obstetricans* are represented in different colors: spring (red), and overwintering (blue). Presence of solid line indicates significant regression.

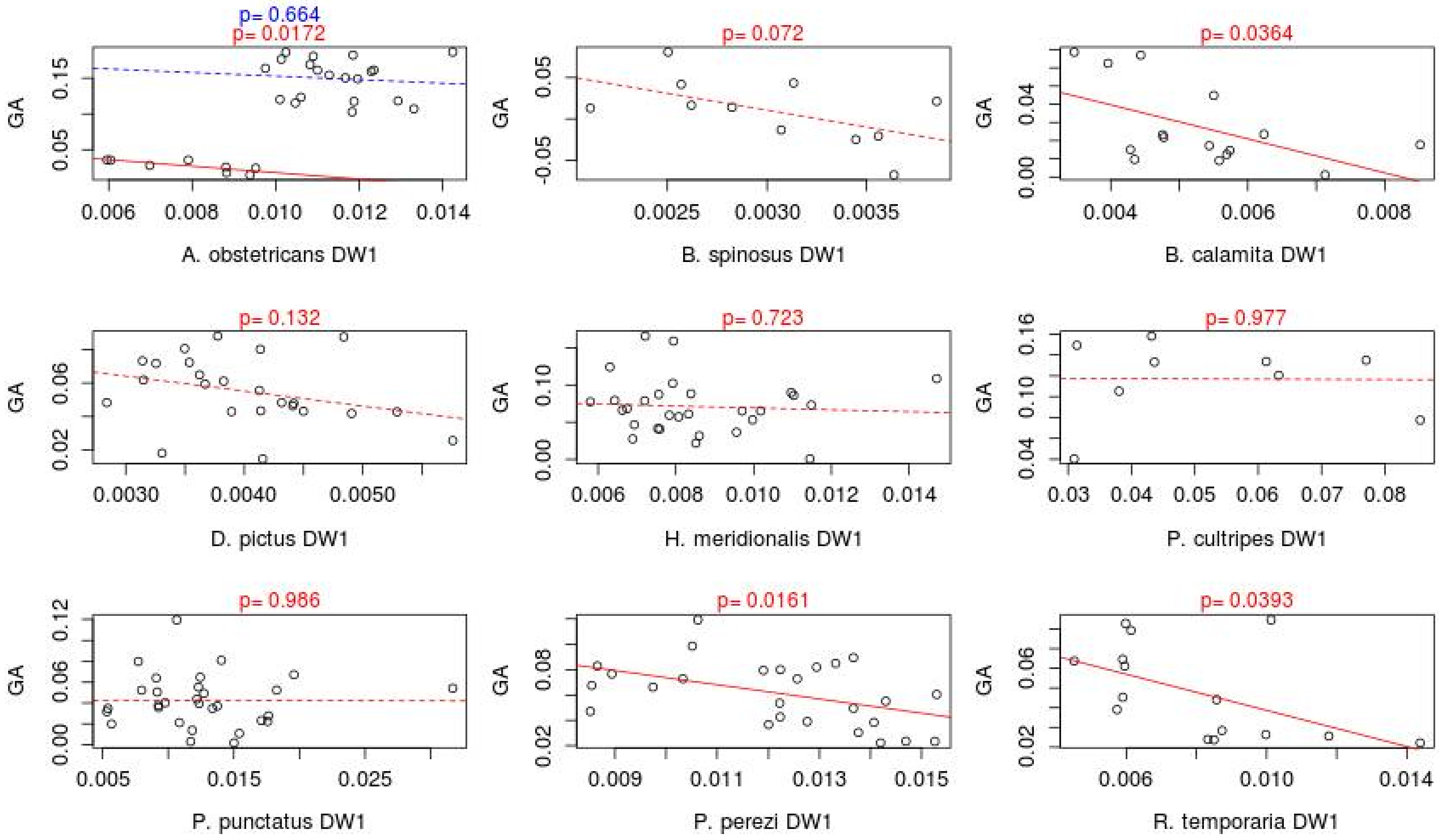


Figure S5: Regressions of assimilation efficiency (ASSM) against dry weight (DW1), for each species. Cohorts of *Alytes obstetricans* are represented in different colors: spring (red), and overwintering (blue). Presence of solid line indicates significant regression.

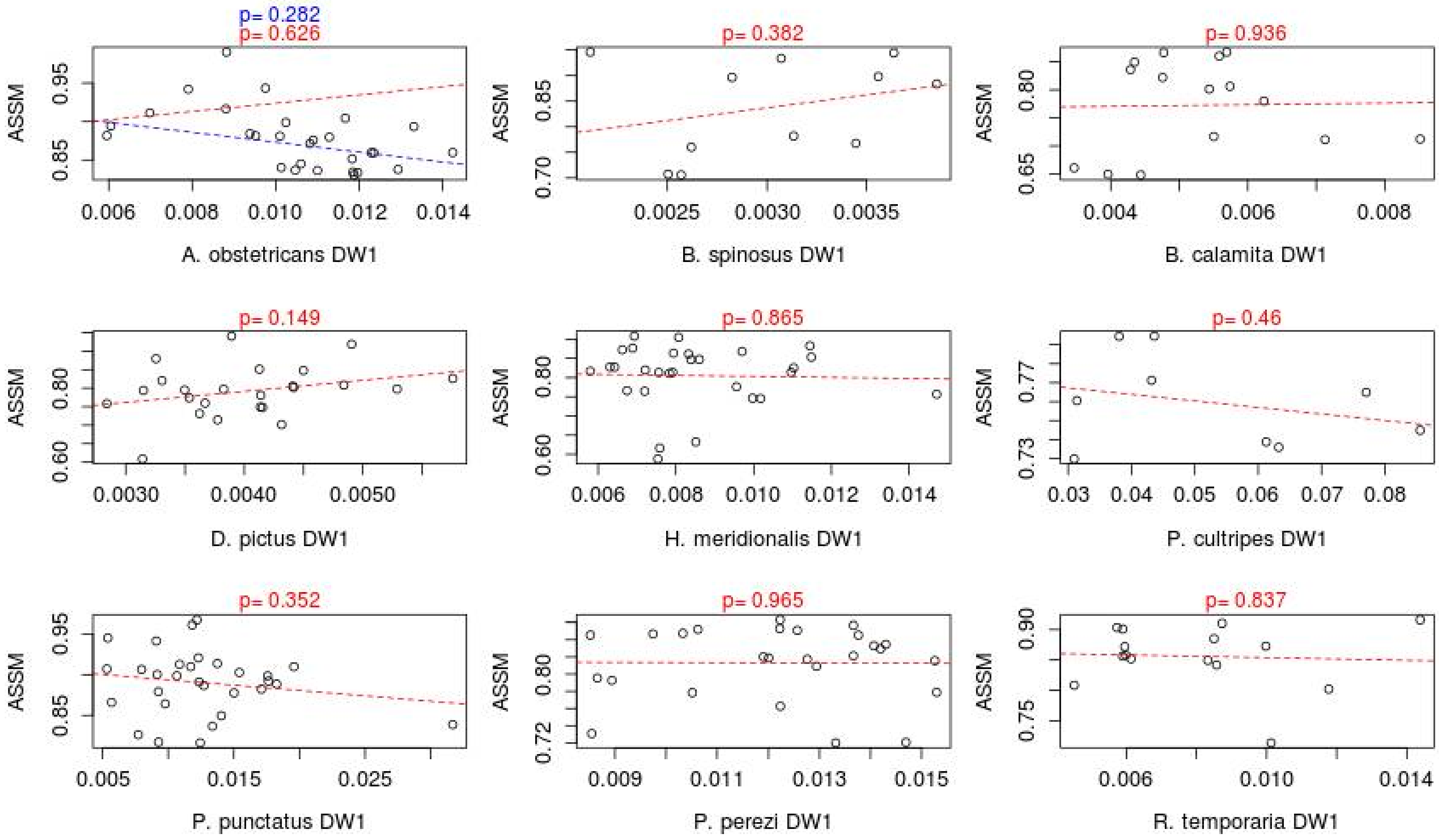


Figure S6: Regressions of relative gut length (RGL) against dry weight (DW1), for each species. Cohorts of *Alytes obstetricans* are represented in different colors: spring (red), and overwintering (blue). Presence of solid line indicates significant regression.

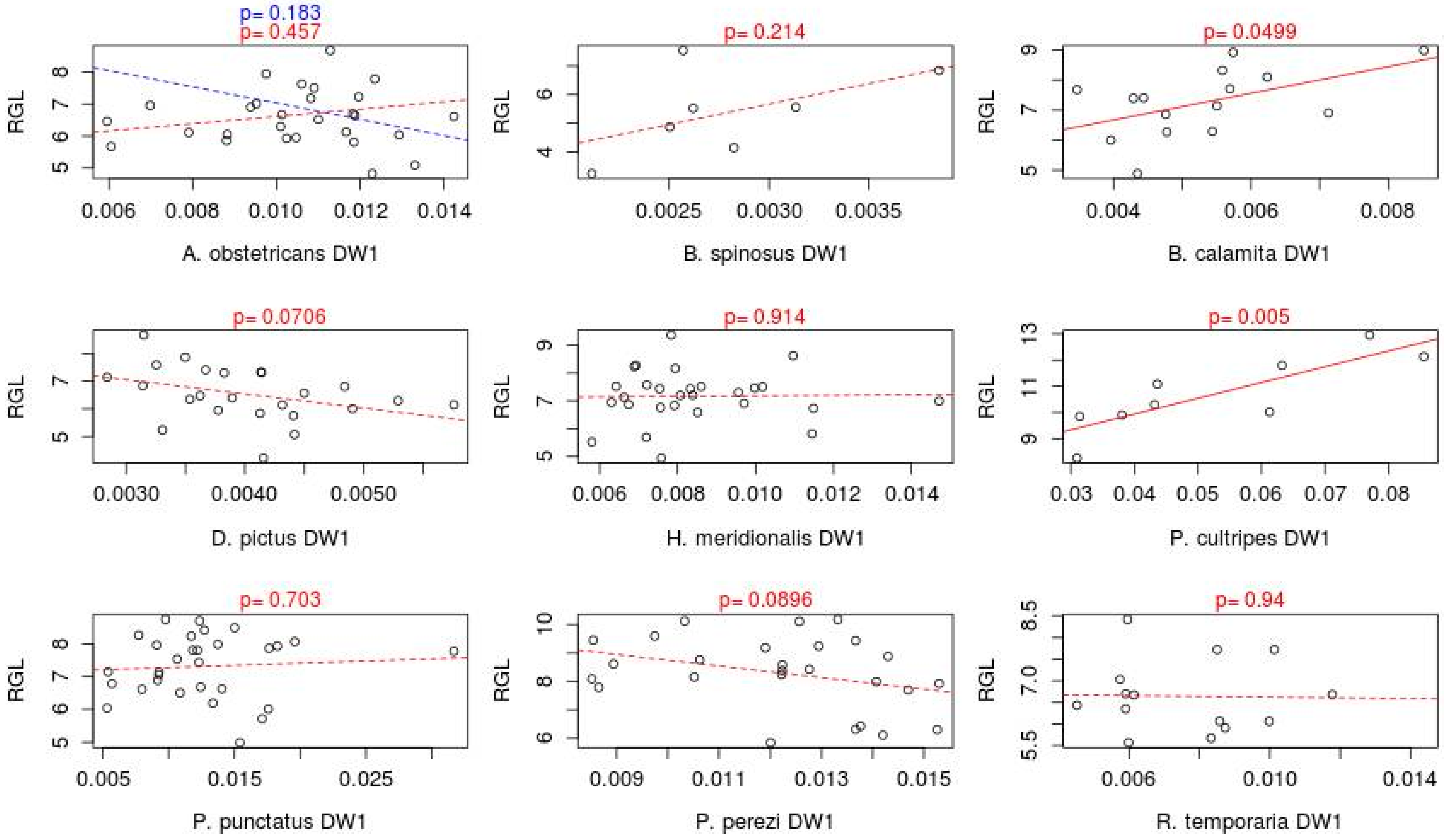


Figure S7: Regressions of GR against CR, for each species. Cohorts of *Alytes obstetricans* are represented in different colors: spring (red), and overwintering (blue). Presence of solid line indicates significant regression.

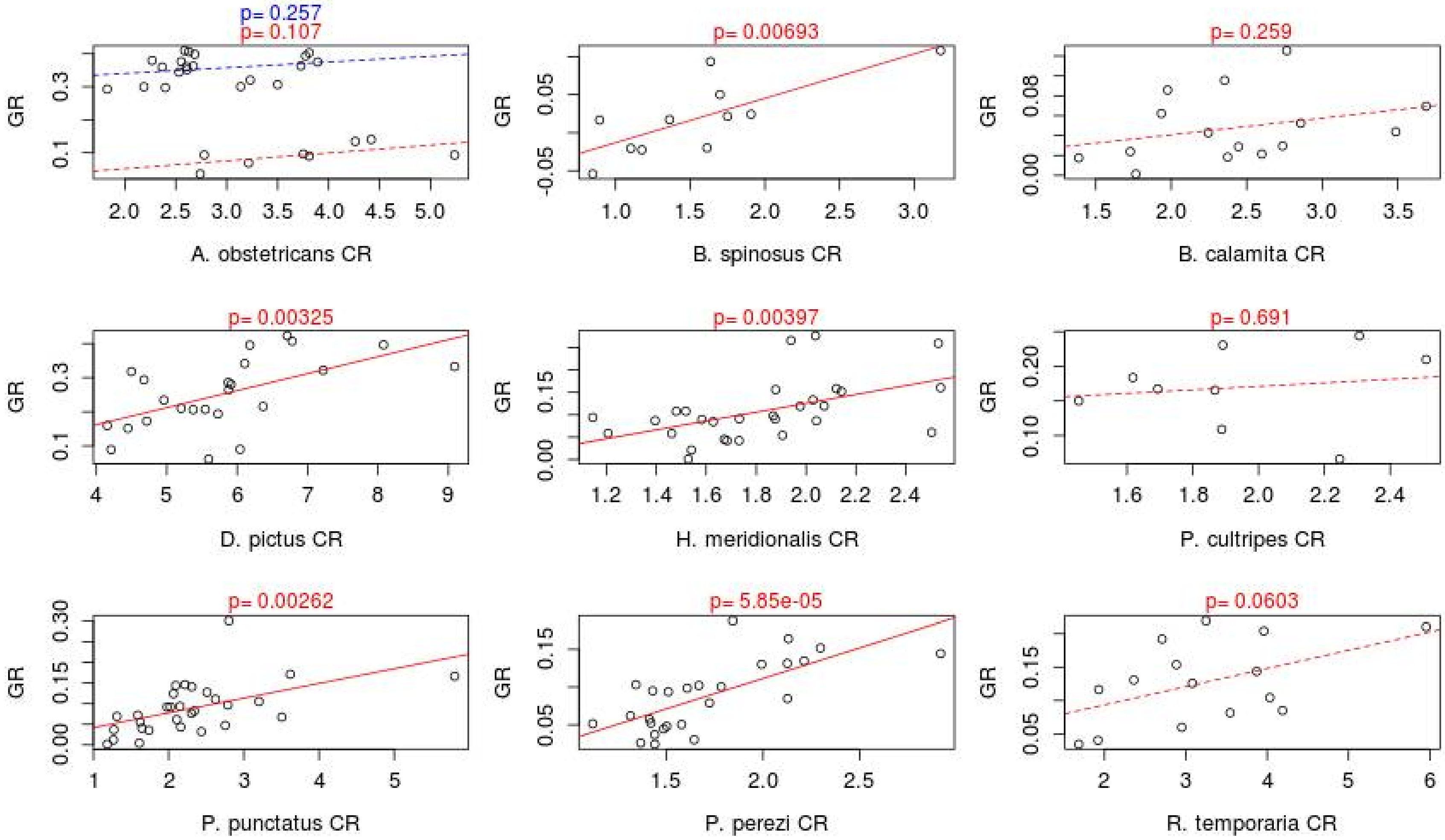


Figure S8: Regressions of GR against ASSM, for each species. Cohorts of *Alytes obstetricans* are represented in different colors: spring (red), and overwintering (blue). Presence of solid line indicates significant regression.

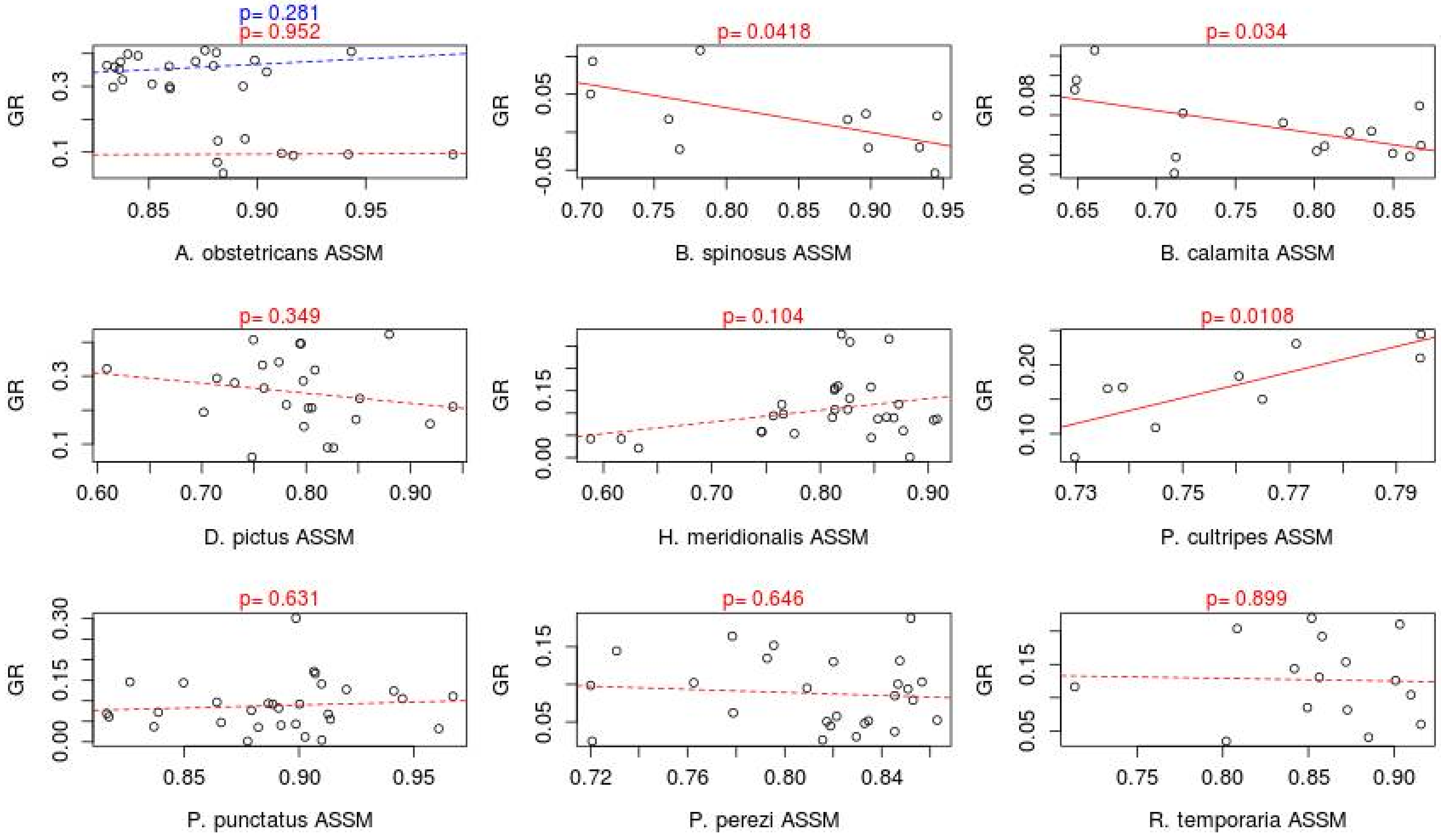


Figure S9: Regressions of GR against GA, for each species. Cohorts of *Alytes obstetricans* are represented in different colors: spring (red), and overwintering (blue). Presence of solid line indicates significant regression.

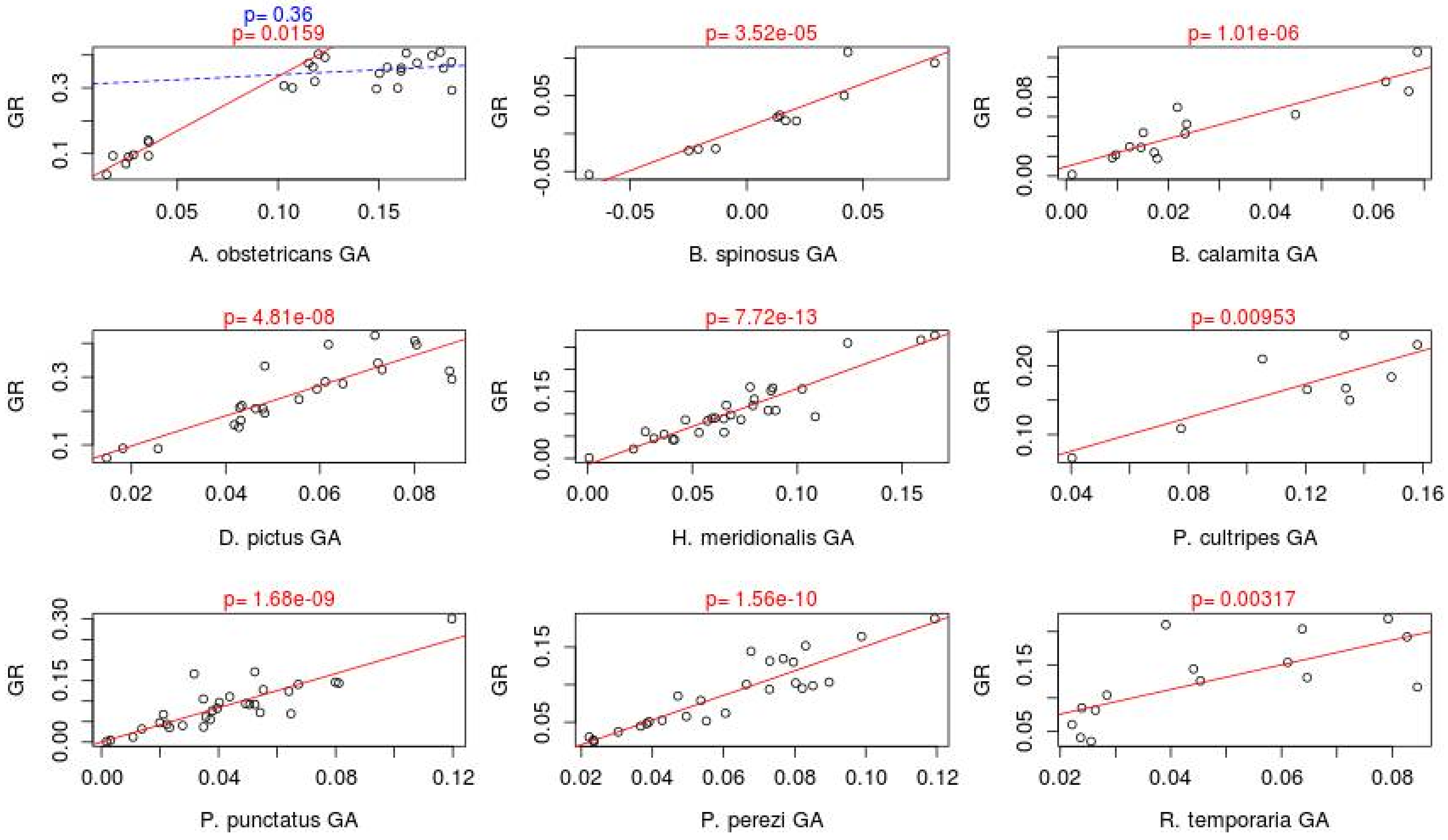


Figure S10: Regressions of CR against GA, for each species. Cohorts of *Alytes obstetricans* are represented in different colors: spring (red), and overwintering (blue). Presence of solid line indicates significant regression.

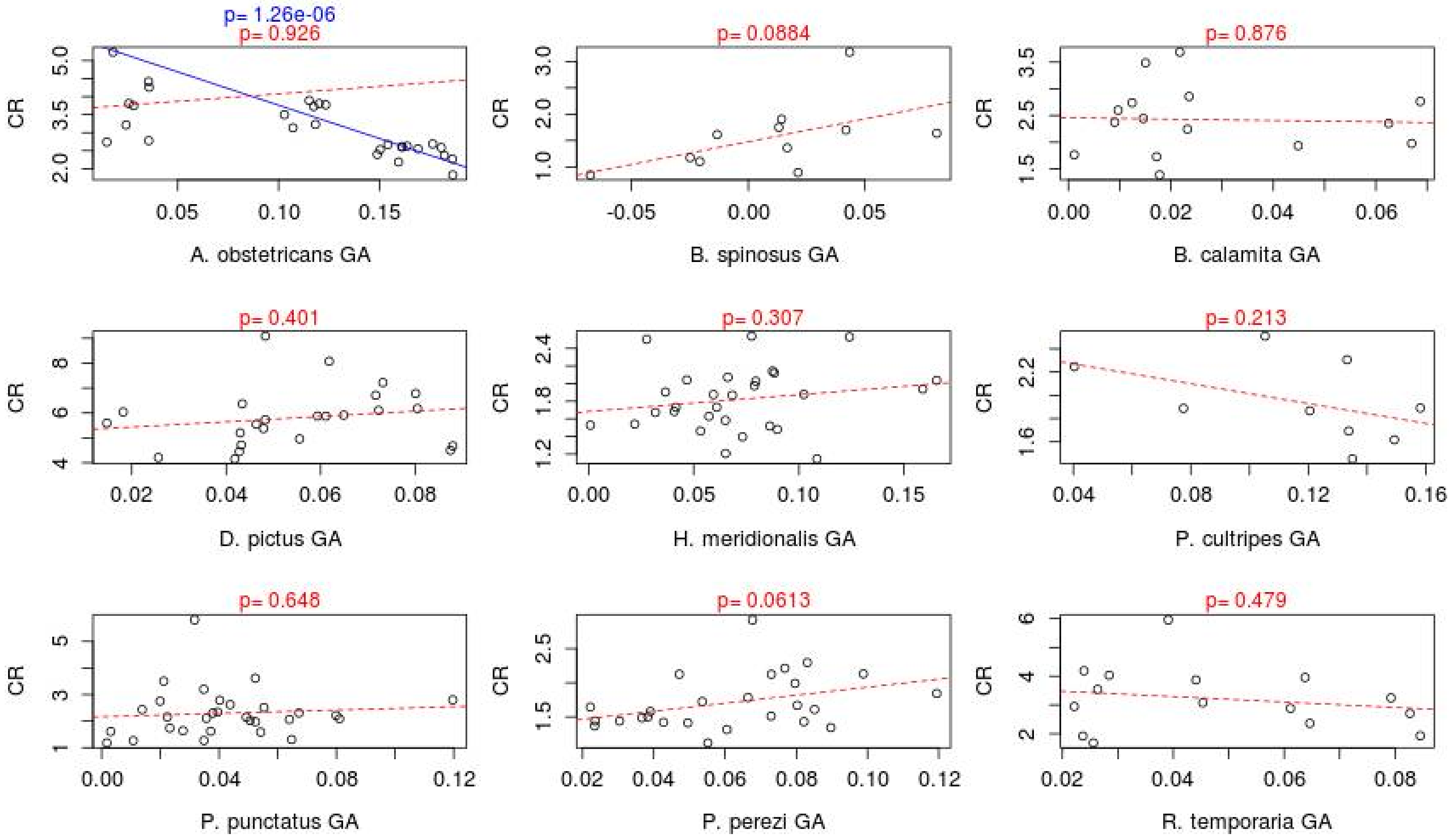


Figure S11: Regressions of ASSM against GA, for each species. Cohorts of *Alytes obstetricans* are represented in different colors: spring (red), and overwintering (blue). Presence of solid lines indicates significant regression.

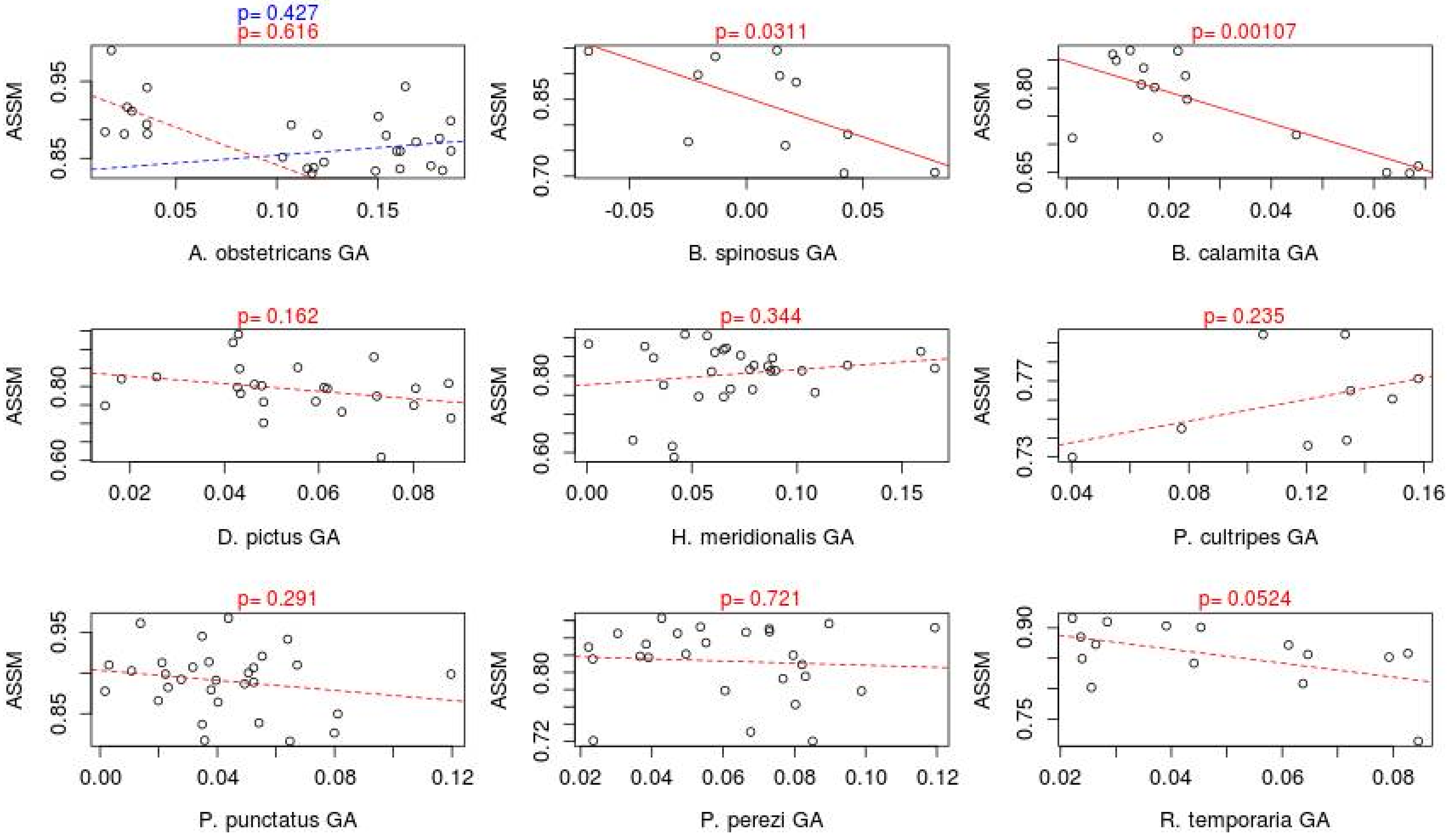


Figure S12: Regressions of ASSM against CR, for each species. Cohorts of *Alytes obstetricans* are represented in different colors: spring (red), and overwintering (blue). Presence of lines indicates significant regressions.

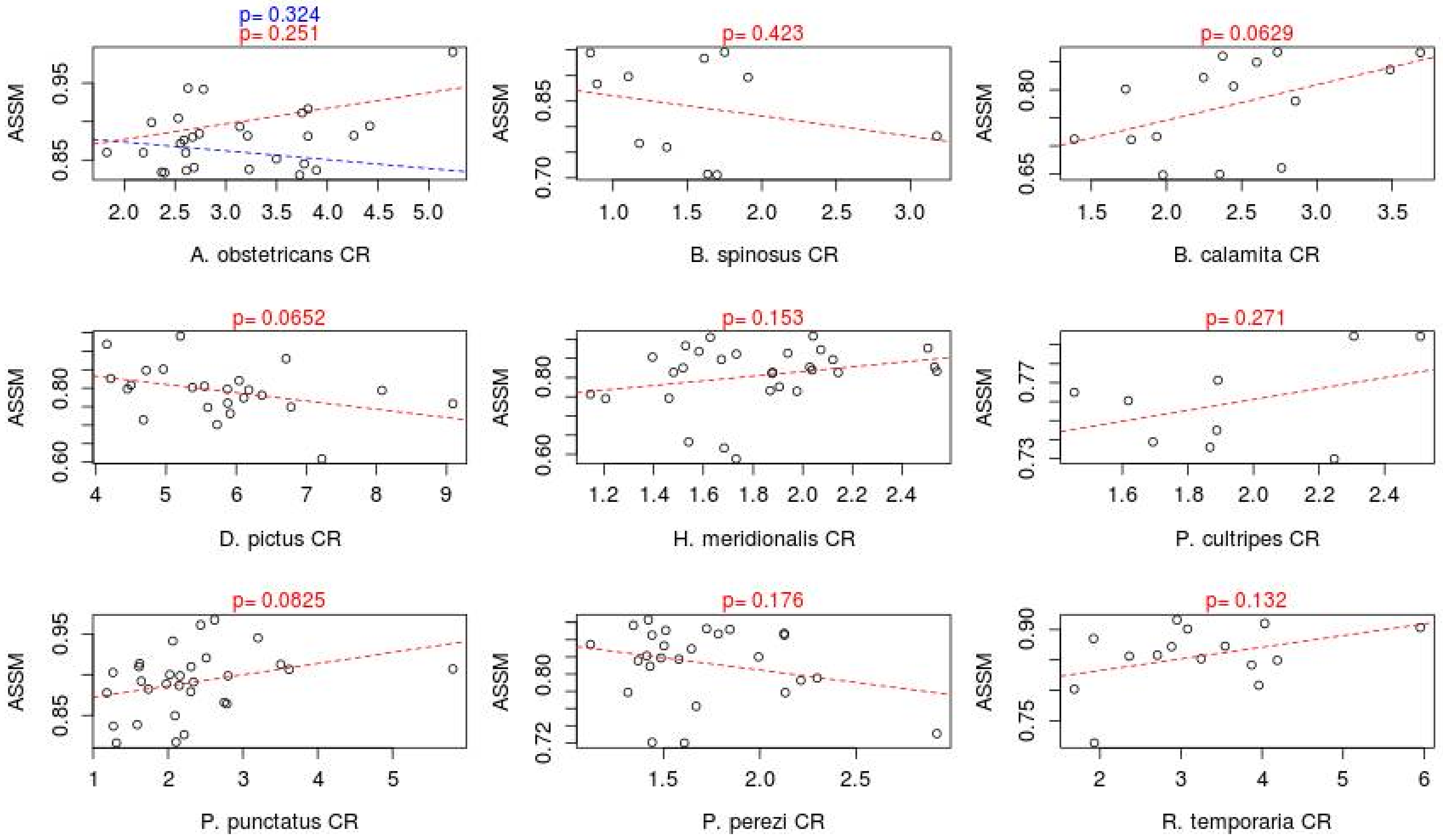


Figure 13: Regressions of GR against RGL, for each species. Cohorts of *Alytes obstetricans* are represented in different colors: spring (red), and overwintering (blue). Presence of solid lines indicates significant regression.

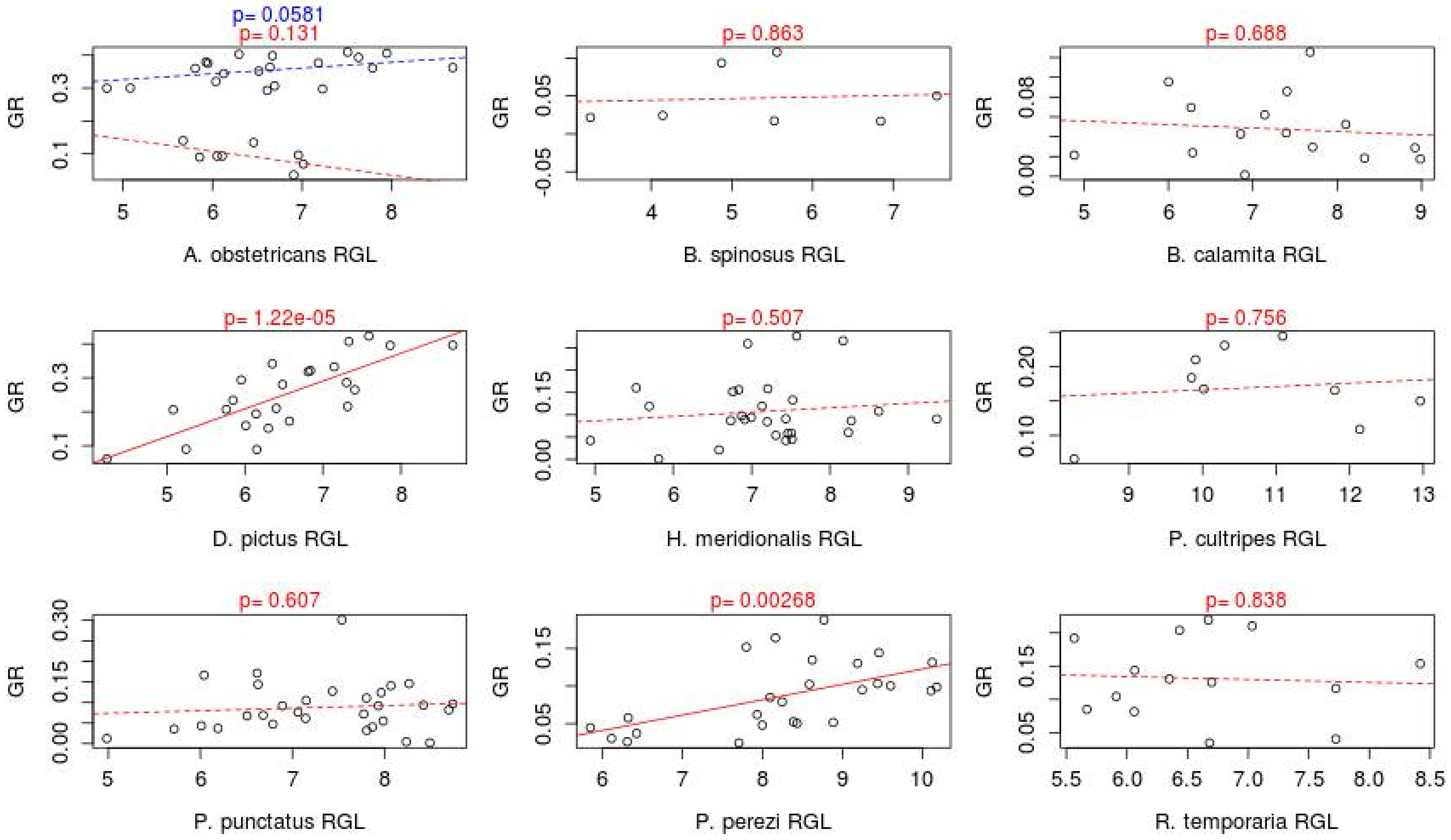


Figure 14: Regressions of CR against RGL, for each species. Cohorts of *Alytes obstetricans* are represented in different colors: spring (red), and overwintering (blue). Presence of line indicates significant regression.

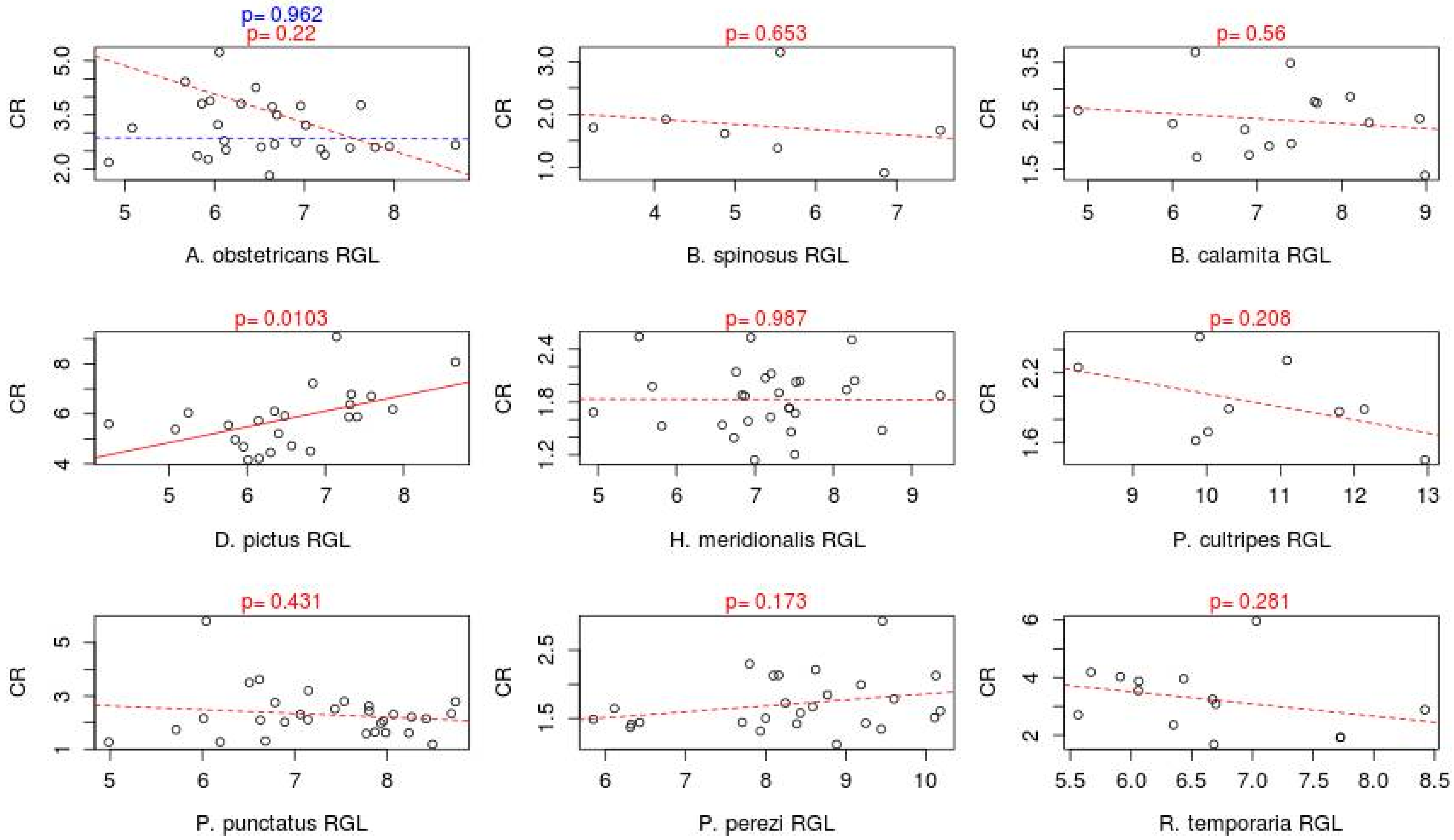


Figure S15: Regressions of GA against RGL, for each species. Cohorts of *Alytes obstetricans* are represented in different colors: spring (red), and overwintering (blue). Presence of line indicates significant regression.

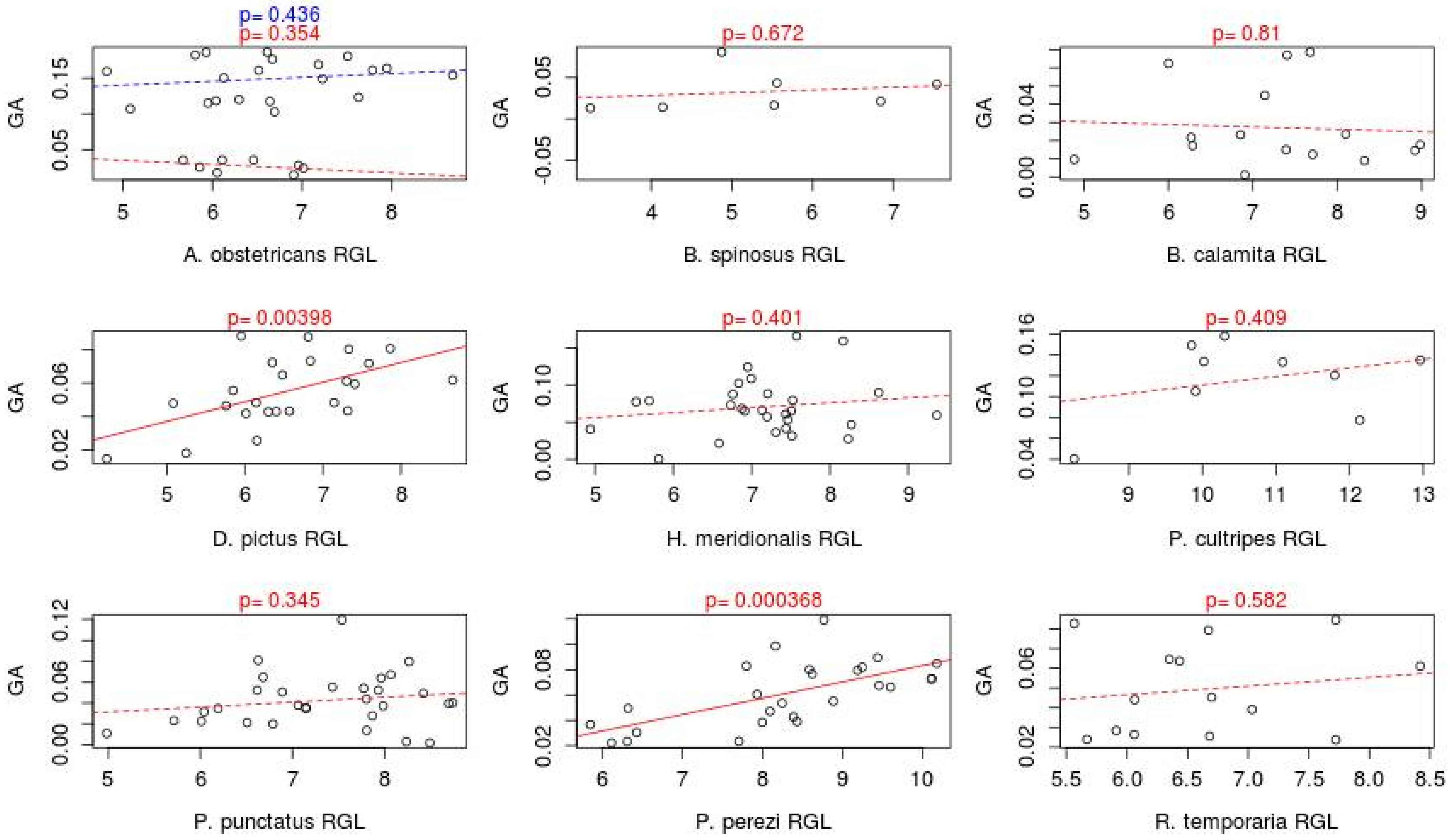


Figure S16: Regressions of ASSM against RGL, for each species. Cohorts of *Alytes obstetricans* are represented in different colors: spring (red), and overwintering (blue). Presence of line indicates significant regression.

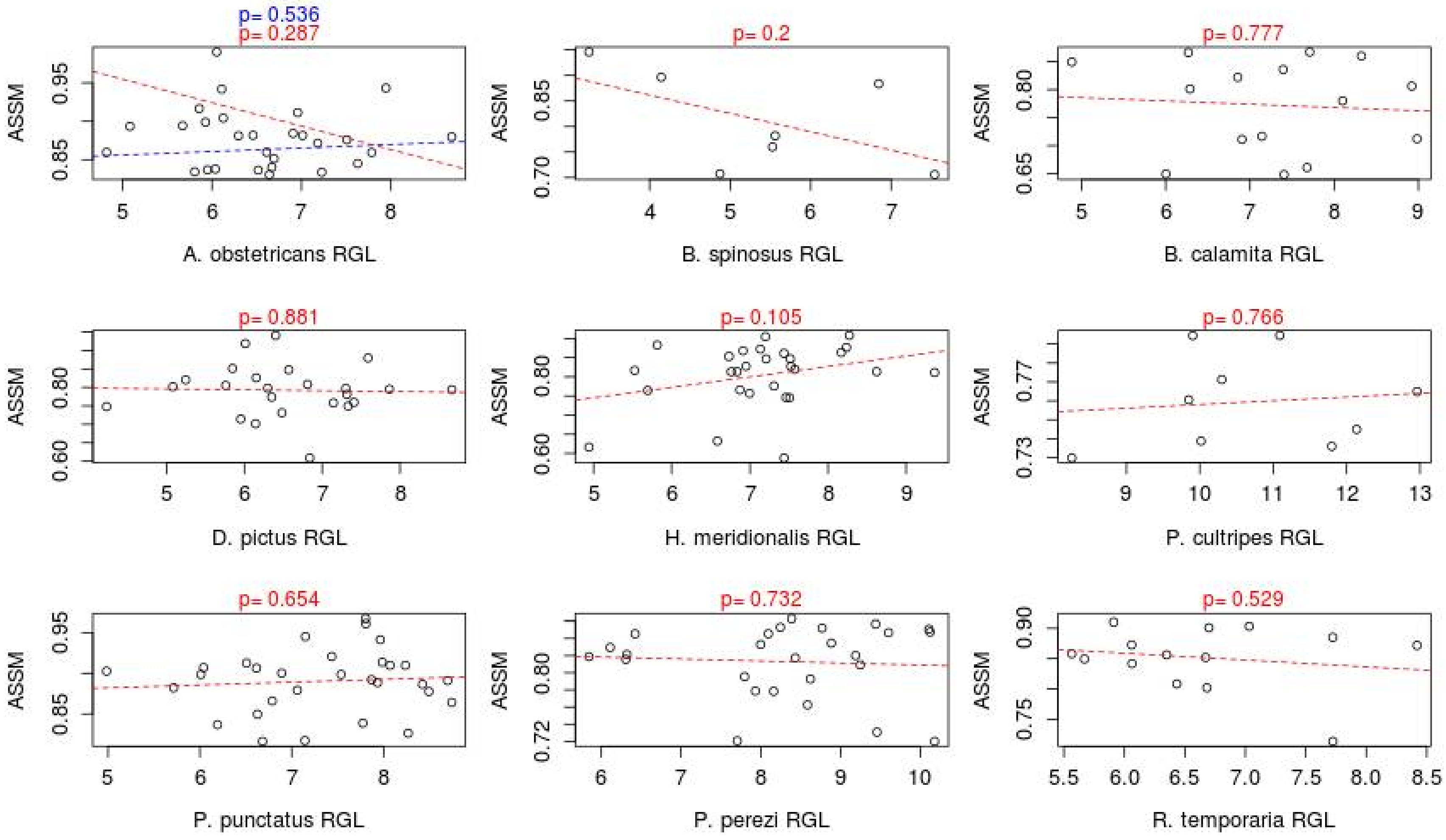
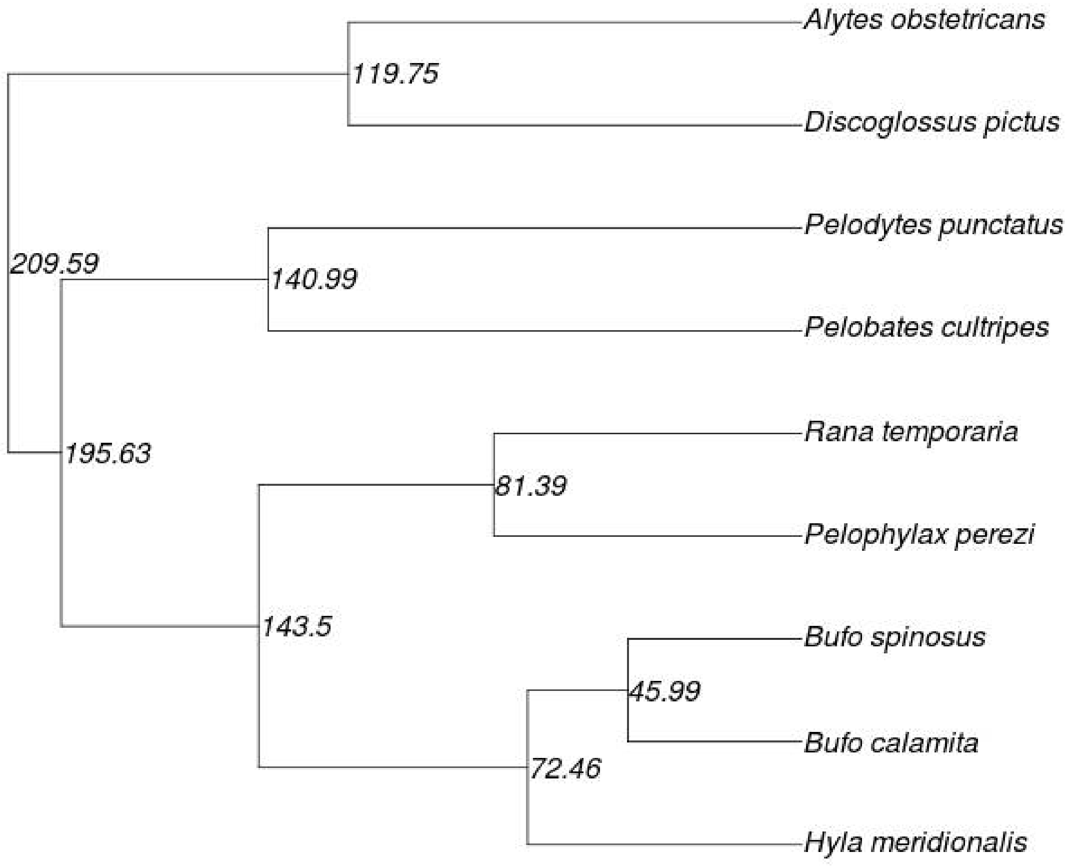


Figure S17: Ultrametric tree used in phylogenetic signal analyses (from Gomez-Mestre et al. [2012]), node labels represent million years since divergence.



PART B

Intraguild competition in tadpoles of *Discoglossus pictus.*

Chapter 4

Differential trophic traits between invasive and native anuran tadpoles.

[published in *Aquatic Invasions*]

How trophic resources are managed is a key factor in our understanding of the success of invasive species, or any species by extension. Concerning tadpoles, it is known that abundant and high-quality food can increase their final size and reduce their larval period. Therefore, in time-constrained tadpoles that usually occupy ephemeral ponds, the capacity to acquire and select trophic resources might be especially important. In addition, as a pond dries, both the larval density increases and food resources become more even limited.

The aim of this work was to assess the trophic traits related to food acquisition and choice of tadpoles of the invasive (originally North African) anuran *Discoglossus pictus* compared to those of native European *Epidalea calamita* tadpoles. Under controlled laboratory conditions, food of two different levels of quality was supplied, and the feeding activity and food preferences of both species were analysed alone and in co-occurrence.

The invasive *Discoglossus pictus* was perfectly capable of modifying its behaviour and food preferences. On the other hand, the native *Epidalea calamita* displayed much milder differences between treatments, thus having more limited abilities in this sense. Both alone and in co-occurrence with the native species, the invasive tadpoles obtained higher feeding activity values and showed a stronger preference for high-quality food than the natives. Additionally, when high densities of the two species were forced to share food resources, the feeding activity results indicated a potential displacement of the native tadpoles to low-quality resources. *Discoglossus pictus* thus presents trophic traits that are favourable for invasion and could limit the fitness of *Epidalea calamita* when resources are limited or there is a risk of pond desiccation.

PART B

Competència intra-gremi en capgrossos de la granota pintada, *Discoglossus pictus.*

Capítol 4

Trets tròfics diferencials entre capgrossos invasors i autòctons.

[publicat a la revista *Aquatic Invasions*]

Entendre com es gestionen els recursos tròfics és un factor clau per entendre l'èxit de les espècies invasores. En amfibis que acostumen a criar en basses que s'assequen en un període curt de temps, les capacitats d'adquirir recursos i de seleccionar-los favorablement són especialment importants, ja que un cop el nivell i la superfície de l'aigua comencen a disminuir també ho fan els recursos alimentaris disponibles, alhora que forçosament augmenta la densitat de competidors. Menjar abundantment i amb alta qualitat proteica s'ha demostrat que efectivament aconsegueix augmentar la mida final dels capgrossos i reduir-ne la durada del desenvolupament fins a la metamorfosi. L'objectiu d'aquest treball va ser avaluar les característiques tròfiques dels capgrossos de l'espècie invasora d'estudi, la granota pintada, en comparació amb les dels capgrossos del gripau corredor (*Epidalea calamita*) una espècie autòctona. En condicions de laboratori, vam subministrar als capgrossos alimentació de dos nivells diferents de qualitat proteica, per seguidament analitzar-ne l'activitat d'ingestió i la preferència per un o altre tipus d'aliment, tant cada espècie per separat, com junts en un mateix tanc d'aigua. Els capgrossos de granota pintada són perfectament capaços de modificar el seu comportament i les seves preferències alimentàries segons demani la situació per tal de millorar el seu creixement; mentre que en el cas del gripau corredor aquestes capacitat, si bé presents en algun cas, són molt més tímides globalment. Tant sols, com també en presència dels capgrossos de gripau corredor, els capgrossos de la granota invasora van aconseguir obtenir valors d'activitat d'ingesta més alts i van mostrar unes capacitats de tria d'aliments d'alta qualitat més clara que els de l'espècie autòctona. A més a més, les dues espècies es veuen forçades a compartir recursos alimentaris en altes densitats, els resultats indiquen que és possible que l'activitat d'ingesta dels capgrossos del gripau autòcton quedi forçadament relegada a aliments de més baixa qualitat proteica. La granota pintada presenta trets tròfics favorables per a la seva capacitat d'invasió, i podrien limitar les capacitats de supervivència del gripau corredor en situacions de recursos limitats o en risc de dessecació de les basses.

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Research Article

Differential trophic traits between invasive and native anuran tadpoles

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Abstract

How trophic resources are managed is a key factor in our understanding of the success of invasive species. In amphibians that usually occupy ephemeral ponds, the capacity to acquire resources and food selection are especially important because as a pond dries, the larval density increases and food resources are limited. Abundant and high-quality food can increase the final size and reduce the duration of development of amphibians. The aim of this work was to assess the trophic traits of tadpoles of the invasive (originally North African) anuran *Discoglossus pictus* compared to those of native European *Epidalea calamita* tadpoles under laboratory conditions. Food of two different levels of quality was supplied, and the feeding activity and food preference of the two species were analysed alone and in co-occurrence. *D. pictus* was capable of modifying its behaviour and food preferences; while *E. calamita* displayed much milder differences between treatments. Both alone and in co-occurrence with the native species, the invasive tadpoles obtained higher feeding activity values and showed a stronger preference for high-quality food. Additionally, when high densities of the two species shared food resources, the feeding activity results indicated potential displacement of the native tadpoles to low-quality resources. *D. pictus* thus presents trophic traits that are favourable for invasion and could limit the fitness of *E. calamita* when resources are limited or there is a risk of pond desiccation.

Key words: diet quality, feeding activity, food preference, *Discoglossus pictus,* invasiveness, anuran tadpoles

Introduction

Invasion by alien species is one of the most important threats to the balance of ecosystems and community structure, and the second most significant cause of biodiversity loss on the global scale (Levine et al. 2003). Approximately ten new species of living organisms become established in Europe each year (Hulme et al. 2009); however, only a small fraction of introductions (20%–30% worldwide) result in invasion (Pimentel et al. 2001). The success of a species in a novel environment is likely to depend on several factors, including the abiotic physical environment, biological interactions and the traits of the introduced species (Shea and Chesson 2002; Duncan et al. 2003; Blackburn et al. 2009). The availability of resources and the capacity of an invasive species to exploit them are critical factors for its survival and reproduction (Thébaud et al. 1996; Tilman 2004).

When an alien amphibian species is introduced, its efficiency at exploiting the available resources during larval stages can be a key factor for the successful establishment of the species and may therefore define its invasiveness, because of the vulnerability during this phase and the crucial effect of food on the development of tadpoles and the subsequent development of toadlets (Werner 1986; Scott 1994; Tejedo et al. 2000). The larval period is considered the most vulnerable phase for amphibians since it is when the highest mortality rate is registered, especially for species that occupy temporary ponds (Calef 1973; Wilbur 1980; Newman 1987; Denver 1998). On the other hand, the effects of diet on the breeding success and metamorphic fitness of amphibians have been repeatedly documented (Kupferberg 1997; Babbitt et al 2000; Álvarez and Nicieza 2002; Jefferson 2014). Both food quantity and food quality are crucial for their development: tadpoles cannot fully develop under desiccated conditions when food is scarce (Enriquez-Urzelai et al. 2013), or low in protein content (Nathan and James 1972; Steinwascher and Travis 1983; McCallum and Trauth 2002; Richter-Boix et al. 2007). Moreover, when food is abundant or highly proteic, tadpoles are capable of accelerating their growth and development (Gotthard and Nylin 1995; Morey and Reznick 2000; Lind and Johansson 2007). Therefore, optimal exploitation of available resources by tadpoles ensures fast growth and high performance, thus favouring the establishment and spread of introduced amphibian species.

Discoglossus pictus auritus (Otth, 1837) is an anuran species introduced from Algeria to Banylus-sur-Mer (France) around 100 years ago (Wintrebert 1908). Its population is currently increasing and its range expanding annually to areas of north-eastern Spain and south-eastern France (Martens and Veith 1987; Montori et al. 2007; Franch et al. 2007). It mostly occupies ephemeral wetlands that are characterized by their low species richness and the low occurrence of potential predators. At the local level, the use of such ponds can be an advantage for the success of this species in the invaded area because in this way it avoids competition and predation risks. However, at the community level, ephemeral pond use leads to spatial limitations on larval development derived from inevitable pond drying (Babbitt et al. 2000; Newman 1987). Traits that favour short developmental periods and high growth rates improve success in colonising this type of ponds.

Often *D. pictus* shares breeding ponds with the native species *Epidalea calamita* Laurenti, 1768. Ephemeral freshwater environments are closed systems with limited resources that intensify interactions between organisms (Wilbur 1980). Competitive superiority in direct interactions or better exploitation of available resources can increase the survival or fitness of one species over that of another when the two share such environments. The greater capacity of the introduced species to take up and use nutrient resources in ephemeral ponds, is a positive biological trait for its establishment and spread, despite the shared habitat. Both *D. pictus* and *E. calamita* exhibit explosive breeding characterized by a high number of eggs per clutch, which leads to high densities of tadpoles in ponds and this intensifies their interactions. Also, *D. pictus* and *E. calamita* present high similarities in the exploitation of their trophic niche, both in larval morphology and from the analysis of digesta (Díaz-Paniagua 1985; Escoriza and Boix 2012; Richter-Boix et al. 2012; San Sebastián et al. 2015).

The aim of this study is to evaluate the capacity of the invasive species (*D. pictus*) to exploit resources, and compare this with the native species. The capacity to exploit resources is evaluated via two measurements: the percentage of tadpoles feeding (feeding activity) and rate of selection of high-quality food (food preference). In order to obtain these measurements, we designed two experiments. The first evaluated whether the invasive species adopts a better strategy to exploit nutrient resources than the native species when they do not co-occur. The second analysed the capacity of the two species to exploit resources in aquariums shared at low and high densities. We expect greater success in the wild of the amphibian species that better exploits high quality resources and/or has a higher activity rate.

Methods

Animals and rearing conditions

Two experiments were conducted in different years (2011 and 2013) but with the same experimental laboratory conditions of temperature (19ºC–20ºC) and photoperiod (12D:12L). The tadpoles used in each experiment were collected as eggs on the 24th and 25th March 2011 (*D. pictus* and *E. calamita*, respectively) and on the 8th April 2013, from 3–4 clutches for each species and year, near to Girona (northeast Spain). The same procedure was followed in each experiment. Clutches of each species were transported to the laboratory in separate containers of dechlorinated water. Eight days after hatching, the tadpoles were mixed together to avoid genetic biases and were then held in plastic containers. The tadpoles were reared and fed with rabbit food until the start of the experiment. The experiments started on 5th April 2011 and 10th April 2013 (day 0) when the tadpoles reached Gosner stage 25 (Gosner 1960). All the individuals were released to their original location after the experiments finished.

Experimental design

Experimental units consisted of 30 L tanks containing dechlorinated water (1.2 × 0.45 × 0.40 m). The tadpoles were randomly allocated to the experimental treatments. The water was changed every two days to prevent eutrophication and the accumulation of lethal levels of metabolic products. We used two food types in the feeding trials that differed in composition: (LP) a commercial rabbit food, which has a low-protein and high-carbohydrate content (16% protein, 3% lipids, 17% carbohydrates, 10% ash); and (HP) the commercial fish food Sera O-nip, which is rich in protein (46% protein, 22% lipids, 2% carbohydrates, 9% ash). Food was supplied *ad libitum* during the experiments. In all the treatments and experiments, the food was available in two small Petri dishes placed randomly in the corners of the containers to avoid learning effects and ensure selection by tadpoles according to diet quality.

The first experiment on the differential selection of diet quality by *D. pictus* and *E. calamita* consisted of three treatments for each species, each replicated six times. All the containers had the same density of tadpoles (10 larvae per container). To evaluate the dietary selection of each species, there were two treatments with one food type (LP or HP) and one treatment with both food types (HLP) for each species: *D. pictus* with HP food (DHP), LP food (DLP), and HP + LP food (DHLP); and *E. calamita* with HP food (BHP), LP food (BLP), and HP + LP food (BHLP).

The second experiment involved two treatments, both containing HP and LP food, and tadpoles at either low density (LD) or high density (HD). The LD treatment consisted of a total of 10 tadpoles, at the same density as in the first experiment, but with five larvae of *D. pictus* and five larvae of *E. calamita*. The HD treatment was designed to increase potential interactions between the species in a more realistic scenario, with 20 larvae of *D. pictus* and 20 larvae of *E. calamita*.

Response variables

Throughout the experiments, we checked each tank daily and we fed the tadpoles with variable frequency to ensure *ad libitum* feeding conditions. For each tank, we checked tadpole behaviour once 5 minutes after food administration, to avoid potential disturbances after dish manipulation. The average number of registers was 16 per aquarium in the first experiment and 23 in the second experiment (more details in Appendix 1). We stopped collecting activity data when 30% of the larvae in the aquarium underwent metamorphosis, to avoid recording behavioural changes as a consequence of reduced tadpole density.

The resource exploitation capacity of *D. pictus* and *E. calamita* were analysed via two variables: feeding activity and food preference. Feeding activity is considered to be a measure of the capacity a species has for resource exploitation, and thereby of competitive capacity (Smith et al. 2004); while food preference helped us to determine whether the tadpoles of each species followed indiscriminate or random foraging, or had a preference for one of the food types.

Feeding activity was calculated as the number of tadpoles feeding (in dishes with HP food, dishes with LP food or two types of food HLP) divided by the total number of tadpoles in each aquarium and then expressed as a percentage, as observed at first glance (Peacor and Werner 1997; Relyea 2002; Pujol-Buxó et al. 2013). Food preference was estimated as the proportion of tadpoles consuming each kind of food divided by the total number of tadpoles in feeding attitude in treatments with both types of food: HP and LP. While feeding activity was calculated including zero activity records, to estimate food selection, we only used counts with tadpole activity higher than zero.

Statistical analysis

Firstly, we compared feeding activity between the species and treatments, and tested their interaction. Then feeding activity in each treatment and food type was explored between species and within the same species. Secondly, the food preference was analysed for each species only in treatments with two types of food (HLP in the first experiment, and LD and HD in the second experiment). Given the presence of a certain amount of zero values and that most assumptions needed to perform classic parametric or non-parametric analyses of variance were not met (McElduff et al. 2010), differences in feeding activity between treatments, species and food types were explored via non-parametric randomization ANOVA (NP-R-ANOVA). We used percentages as the dependent variable and tested the differences applying the function *aovp* from the package *lmPerm* (Wheeler 2010). Potential random variations between aquariums were considered including aquarium as an error in the model. The function aovp() assumes a model of the form: $Y=Xb+Zg+e$, where X is the incidence matrix for fixed effects,

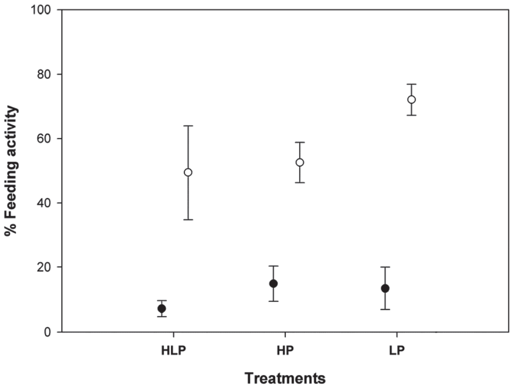

Figure 1. Mean (±SE) feeding activity of *D. pictus* (white circle) and *E. calamita* (black circle) in each treatment.

and Z is the incidence matrix for random effects, with columns representing the different error strata. The algorithm projects Y into strata creating a single error term in each case while X is also projected so that the model in this stratum becomes P(Y)=P(X)bi+ei. This way the model accounts for the lack of independence of the data within each experimental unit.

Our p and F values were calculated after 1,000 permutations, and the level of significance was set at values of p lower than 0.05.

For the food preference analysis, we excluded records with no activity and used only the percentage of feeding larvae in the HP treatment (real distribution). In this case, we generated a new distribution (simulated distribution) that represented our null hypothesis of non-preference for both types of food. This simulated data was generated using a binomial distribution with a "success" probability of 0.5 (that is, an average of 50% tadpoles in each side as null hypothesis: given no preference, half of the active larvae should be eating HP food and the other half LP food) with the standard deviation of the real distribution for each species. This probability was applied on a simulated number of active tadpoles (=events) which followed a Poisson distribution with the same parameters of the real distribution We simulated the same number of null hypothesis data as the number of real data, and then differences between these two distributions were tested using the same procedures as applied to feeding activity. All the analysis were conducted in R (Development R Core Team 2013).

Results

Experiment 1: Differential food selection by D. pictus and E. calamita

Feeding activity: species, treatments and diet quality

We found differences in feeding activity between the species ($F_{1,29}$=337.92 p<0.001), treatments ($F_{2,29}$=7.95 p=0.001) and an interaction between the two ($F_{2,29}$=6.87 p<0.005). *D. pictus* showed higher feeding activity than *E. calamita* in treatments with one ($F_{\mathrm{DHP\text{-}BHP}\ 1,176}$=124.46 p<0.001; $F_{\mathrm{DLP\text{-}BLP}\ 1,203}$=319.03 p<0.001) and two types of food ($F_{\mathrm{DHLP\text{-}BHLP}\ 1,151}$=41.76 p<0.001). The feeding activity of *D. pictus* ranged from 25% to 72%, while that of *E. calamita* did not exceed 20% in any treatment.

Within species, feeding activity levels varied between treatments. *D. pictus* showed significantly more activity in tanks with LP food (DLP) than in DHP ($F_{1,166}$=38.31 p<0.001) or DHLP treatments ($F_{1,161}$=14.43 *p*<0.005), with no significant difference between DHP and DHLP ($F_{1,151}$=0.33 *p*=0.5) (Figure 1). When we compared the feeding activity on each type of food between treatments, we obtained similar results. There were no significant differences in the feeding activity on HP food between the DHP and DHLP treatments ($F_{1,151}$=5.18 *p*<0.05). However, the feeding activity of tadpoles on the LP food was lower in the DHLP treatment than in the DLP treatment ($F_{1,161}$=697.50 p<0.001). Although *D. pictus* showed higher activity in the DLP treatment (72% ± 4.8%), when it had two types of diet available (DHLP) it showed 41% ± 12% feeding activity on HP food and 9% ± 3% on LP ($F_{1,\ 166}$= 38.30 p<0.001).

The feeding activity of *E. calamita* was low in all treatments (Figure 1). Levels of activity were similar in the BHP and BLP treatments ($F_{1,213}$=0.14 *p*=0.7), and in the BHLP and BLP treatments ($F_{1,193}$=5.80 *p*=0.05). However, we observed higher feeding activity in the BHP treatment than in the BHLP treatment ($F_{1,176}$=9.68 *p*=0.01) (Figure 1). When we compared feeding activity on each type of diet, we detected differences between feeding activity on HP food between the BHLP and BHP treatments ($F_{1,176}$= 19.17 p=0.001), and on LP food between the BHLP and BLP treatments ($F_{1,193}$=14.64 p<0.005). The percentage of *E. calamita tadpoles eating* HP food in the BHLP treatment was lower than in BHP, being 5% ± 1% and 15% ± 5% respectively. Also, the BHLP treatment registered lower values of feeding activity on LP food than the BLP treatment. The

BHLP treatment showed 3% ± 2% of tadpoles eating LP food while BLP registered 13% ± 6%. In the BHLP treatment, the native species showed similar feeding activity on the different diets ($F_{1,213}$= 0.14 p= 0.7).

Food preference

D. pictus showed preferential selection in treatments with two types of food ($F_{1,133}$=47.37 p<0.001). On average, 81% of tadpoles were observed feeding on HP food, and only 19% on LP food. However, *E. calamita* displayed no food preference in this treatment ($F_{1,58}$=1.31 *p* = 0.5), with 60% feeding on HP food and 40% on LP (Figure 2).

Experiment 2: Competitive or opportunistic strategy by D. pictus co-occurring with E. calamita (LD and HD)

Feeding activity: species, treatments and diet quality

When the species co-occurred in the same environment, *D. pictus* showed higher feeding activity in both the LD and HD treatments ($F_{LD\ 1,255}$=32.04 p<0.001; $F_{HD\ 1,303}$ = 97.13 p<0.001) (Figure 3). Moreover, in both treatments, the feeding activity of *D. pictus* was higher on the HP food ($F_{LD\ 1,255}$= 33.60 p<0.001; $F_{HD\ 1,303}$= 206.2 p<0.001). Although feeding activity on the LP food was similar for the two species at LD, ($F_{1,255}$=0.75 *p*=0.4), higher *E. calamita* feeding activity was recorded on this food at HD ($F_{1,303}$=5.36, *p*=0.02) (Figure 3).

Density had a significant effect on the feeding activity of the two species (*D. pictus*: $F_{1,274}$=12.16, *p*=0.005; *E. calamita*: $F_{1,274}$=27.76, p<0.001), which was higher under HD conditions than LD (Figure 3). Analysis of the type of food and species revealed that increased density provoked an activity gain of 8% on HP food ($F_{1,274}$=8.04 *p*=0.01) and 3% on LP food ($F_{1,274}$=20.03 *p*=0.001) for *D. pictus*; and a gain of only 1% on HP food and 6% on LP food for *E. calamita*. The proportion of *E. calamita* larvae feeding on the HP food did not vary between density treatments ($F_{1,274}$=0.41 *p*=0.5); whereas for the LP food there was an increase in feeding activity in HD conditions ($F_{1,274}$=72.819 *p*<0.001).

Differences in feeding activity between types of diet were evident for *D. pictus* ($F_{LD\ 1,255}$=67.76 p<0.001, $F_{HD\ 1,303}$=253.34 p<0.001). At all densities the invasive species showed higher feeding activity on HP food than on LP (LD: 18% vs. 2%; HD: 26% vs. 5%). However, the native *E. calamita* only showed higher feeding activity on HP food compared to LP food under LD conditions ($F_{1,255}$=17.14 p<0.001; 6% vs. 1%). In the HD conditions, the feeding activity of *E. calamita* on the two diets was similar ($F_{1,303}$=0.65 p=0.42; 8% LP food vs. 7% HP food).

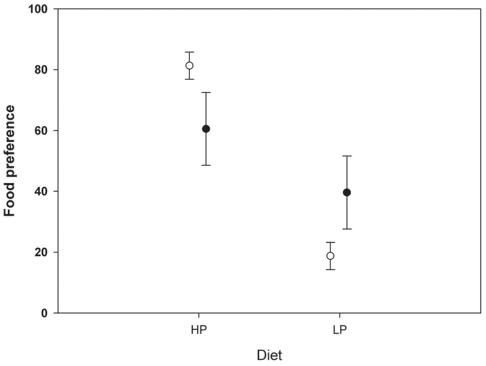

Figure 2. Mean (±SE) food preference for both species in treatments with two types of food. *D. pictus* (white circle) and *E. calamita* (black circle).

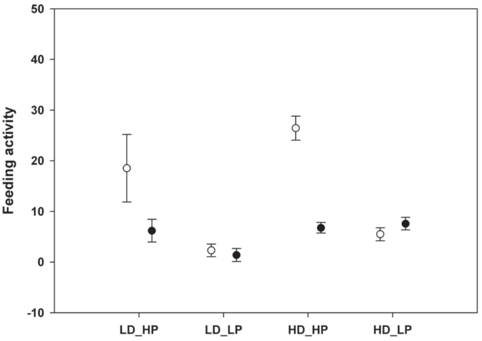

Figure 3. Mean (±SE) feeding activity of *D. pictus* (white circle) and *E. calamita* (black circle) at each density (LD or HD) and quality of diet (LP or HP).

Food preference

In this experiment we detected a preference for type of food by *D. pictus*. More active *D. pictus* chose HP food than LP food in the LD ($F_{1,135}$= 42.95 *p* < 0.001) and HD treatment ($F_{1,277}$= 224.62 *p* > 0.001). A total of 88% and 85% of active larvae of *D. pictus* selected HP food over LP, in the LD and HD treatments respectively. Despite the level of activity recorded for *E. calamita*, it showed no dietary selection in terms of quality of diet. The preference for HP or LP food was similar under LD ($F_{1,67}$ = 1.75 *p* = 0.3) and HD ($F_{1,243}$ = 0.65 *p* = 0.7) conditions. Sixty-one per cent of active *E. calamita* larvae were observed feeding on HP food under LD and 51% under HD conditions.

Discussion

The present study suggests that trophic traits could favour the success of *D. pictus* in the invaded area. *D. pictus* presented higher feeding activity and selection of high-quality food resources, contrasting with the lower feeding activity and apparent non-food selection of the native species either when co-occurring or when alone. As found in other species (Kupferberg 1997; Nicieza and Álvarez 2002) the capacity of amphibian species to exploit resources could predict fast development, high reproductive success and the increased fitness of its metamorphs; especially advantageous for invasive species and with potential negative effects for less competitive species.

Resource exploitation capacity of the invasive and the native species

It is widely documented, in both the field and the laboratory, that food quality is of primary importance in mediating interspecific differences in amphibian reproductive success and performance (for a review see Kupferberg 1997). These effects have been confirmed in *E. calamita* and in *Discoglossus galganoi* (Capula, Nascetti, Lanza, Bullini and Crespo 1985) (in Griffiths et al. 1993; Nicieza and Álvarez 2002; Martins et al. 2013), among other species (Schiesari 2006; Pandian and Marian 1985; Richter-Boix et al. 2007). Those studies show that high-protein food increases the final size and reduces the time required for development in amphibians. These features are directly associated with post-metamorphosis survival rates, a reduction of mortality risk in drying ponds, juvenile performance, enhanced fecundity and presumably juvenile dispersion capacity (Howard 1980; Smith 1987; Babbitt et al. 2000; Tejedo et al. 2000; Martins et al. 2013). Accordingly, high-quality food selection could favour and ensure short-term reproductive success and indirectly enhance the invasive capacity of introduced amphibian species. The results of the present study indicate differential selection of food quality by the invasive species (*D. pictus*) while the native species (*E. calamita*) seems to follow a random feeding pattern, with no clear dietary selection when in isolation.

Although some species seem to show indiscriminate feeding behaviour (Jenssen 1967; Diaz-Paniagua 1985; Diaz-Paniagua 1989; Hoff et al. 1999), tadpole diets can vary under different environmental conditions (Kupferberg 1996; Taylor et al. 1995; Babbitt et al 2000; de Sousa et al. 2014). *Pelodytes punctatus*, for example, shows no preference in the absence of stress factors but becomes selective under stress factors such as competition, predation, and pond desiccation risk (Richter-Boix et al. 2007). This species is another native anuran of invaded areas with high co-occurrence with *D. pictus* and *E. calamita* in ephemeral ponds (Montori et al. 2007; Escoriza and Boix 2012; Richter-Boix et al. 2012). While the native species (*E. calamita* and *P. punctatus*) apparently show no dietary quality selection in conditions without unfavourable factors, either in laboratory experiments or in nature (Diaz-Paniagua 1985; Diaz-Paniagua 1989), invasive *D. pictus* always shows a preference for high-quality food. This preference could be due to the intrinsic behaviour of the species or to particular stimuli or stress linked to its introduction and process of expansion. It is possible that invasive populations have already undergone an adaptive selection favouring some traits (Phillips et al. 2006). In fact, it has been documented that some invasive species present traits linked to their expansion, with higher competitive capacity (Blossey and Notzold 1995; Müller-Schärer et al 2004), higher growth rates (Davis 2005; Mason et al. 2008) and changes in food preference or diet that ensure rapid development and improve reproductive success (Lach et al. 2000; Bøhn et al. 2004).

Additionally, *D. pictus* seems to be capable of modifying its behaviour to achieve more favourable outcomes. It selected HP food when two types of food were available; but when only low-quality (LP) food was available, the species increased its feeding activity with respect to the DHP and DHLP treatments. As the quantity of food has similar effects on larval development as the quality of the food (Morey and Reznick 2000; Lind et al. 2008; Enriquez-Urzelai et al. 2013), the observed increase in activity in the low-quality treatment suggests active compensation by the species for the low quality of the food available via a higher intake. Therefore, it seems that even in an environment free of threats and under *ad libitum* feeding conditions, the invasive tadpoles maximize resources by consuming either higher-quality food or more. Additional comparative studies between the native and invaded ranges could establish whether the invasive species has these specific trophic traits (favouring its success in recipient systems) in their home ranges also, or whether this behaviour is a consequence of its introduction (Moloney et al. 2009).

Resource exploitation capacity of the invasive and the native species under co-occurrence

D. pictus presented similar trophic traits when it was in co-occurrence with the native species and when it was alone. As found in other invasive species (Petren and Case 1996; Holway 1999; Byers 2000), *D. pictus* showed a greater capacity to exploit available resources than did the native residents. *D. pictus* registered higher feeding activity than *E. calamita* and a preference for high-protein food under both LD and HD conditions. Moreover, the increase in the activity of the two species under HD conditions and some changes in feeding activity of the native species suggested an increase in interactions and indeed competitive processes between the two species.

Feeding activity is a trait associated with competitive capacity and capacity for resource exploitation. The introduction of more competitive species often has negative consequences on native species with less competitive capacity. More active individuals are often more competitive and can expropriate resources from less active individuals (Woodward 1982; Werner 1992; Laurila 2000; Dayton and Fitzgerald 2001). *D. pictus* showed higher feeding activity than *E. calamita* in all the treatments. Our results are also consistent with those of Richter-Boix et al. (2012), who reported a higher competitive capacity of *D. pictus* when it co-occurred with *E. calamita*. That study showed that the survival, body mass, and activity of the native species decreased and the time to metamorphosis suffered an increase, in the presence of the invasive species. In summary, it seems that *D. pictus* not only shows good resource exploitation, but it could also have negative consequences on *E. calamita* when the two co-occur.

The results obtained for food preference seem to corroborate the hypothesis that *D. pictus* has a higher competitive capacity. When the invasive and native species co-occurred (under both LD and HD conditions), the invasive species showed a clear preference and selection for high-protein food, whereas the native species showed no preference. In all habitat types, including those with no other kind of threat, competitive interaction is one of the factors that leads to differential dietary selection in amphibians (Griffiths et al. 1993). Although the native species showed no food preference, its feeding activity experienced some changes. When the two species co-occurred at LD, the feeding activity of *E. calamita* was slightly higher on high-protein food than on low–protein food, which could indicate a mild response to a stress factor such as the presence of another species. At HD, the native species significantly increased its total feeding activity only on low-protein food; while its level of feeding activity on high-protein food was maintained. In view of the preference for HP food shown by *D. pictus*, the increase in feeding activity on LP food by *E. calamita* could suggest a reclusion of the native species to the lower-quality food. *E. calamita* could be exploiting those resources, since they are less used by the alien species. Recently, these laboratory results have been corroborated in nature (San Sebastián et al. 2015). Patterns of displacement and trophic niche partitioning have also been detected in ephemeral ponds occupied by *D. pictus* and *E. calamita*. Despite the similarity in diet of the two species (Díaz-Paniagua 1985; Escoriza and Boix 2012), the hierarchy of the communities seems to be repeated in both experimental and field experiments.

As with other invasive species (Fausch and White 1981; Holway 1999), *D. pictus* seems to present a higher competitive capacity than the native species with which it frequently shares its habitat, thereby favouring invasion in the recipient area.

Conclusion

Our conclusions are consistent with other studies conducted on this invasive species: *D. pictus* presents favourable traits that provide it with a high invasive capacity (see Enriquez-Urzelai et al. 2013; Pujol-Buxó et al. 2013; San Sebastián et al 2015). In this work, this invasive species was found to show a preference for high-quality food and to modify its levels of activity according to the quality of diet available, which could ensure rapid larval development and good performance of metamorphs. It also displayed good resource exploitation capacity when sharing nutrients with its most common competitor in nature, *E. calamita*, suggesting high success rates in ephemeral ponds. This invasive species even seems to exhibit a higher competitive capacity than the native one, which could trigger a reduction in the metamorphic fitness and even in the recruitment success of the native species due to the presence of *D. pictus* under stress factors such as pond desiccation. Studies that explain the coexistence of the two species from different points of view such as scale (population and individual behaviour) or skills (physiological capacity, types of food) should be conducted.

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Appendix 1. Number of registers for each aquarium and treatment.

Experiment	Treatment	Species	Registers	Average*	Minimum	Maximum
I	CHLP	*E. calamita*	83	17	15	19
	CHP	*E. calamita*	104	17	15	21
	CLP	*E. calamita*	121	20	14	23
	CHLP	*D. pictus*	79	13	8	15
	CHP	*D. pictus*	84	14	14	14
	CLP	*D. pictus*	94	16	15	17
II	LD	*E. calamita - D. pictus*	131	22	20	24
	HD	*E. calamita - D. pictus*	155	26	25	26

*Average per aquarium

PART B

Intraguild competition in tadpoles of *Discoglossus pictus.*

Chapter 5

Fast evolutionary responses in a native–invasive competitive system after several generations of coexistence

[under review in *Journal of Animal Ecology*]

Invasive species create a series of previously non-existent competitive interactions. If native competitors are not totally displaced, these interactions can force behavioral, ecological and morphological changes both in the native and the invasive species, making biological invasions interesting natural experiments where to study the role of evolutionary change in the competitive abilities of species. Anuran tadpoles are very plastic in terms of behavior and morphology, reacting to a wide array of stress factors, including pond desiccation risk and competitive pressure. These reactions have short and long term effects on individual fitness, thus becoming important targets for natural selection and yielding fast evolutionary responses.

Here, we examine the behavioral and morphological changes mutually induced by tadpoles of an invasive (*Discoglossus pictus*) and a native anuran (*Epidalea calamita*) linked to different combinations of pond desiccation risk and interspecific competitive pressure. To test possible evolutionary changes in this competitive interaction, we repeated the same experiment using tadpoles from two locations with different evolutionary histories: one near the first introduction of the invasive frog (*i.e. circa* 110 years of coexistence of invasive and native competitors), and another from the expansion front of the species (less than 3 years of coexistence). As the native competitor is present all along the invasion range of the invasive frog, we hypothesized that outcomes should be more favorable to the invasive species in short-coexisting populations from the expansion front, where the native competitor is naive and the invasive species is not. Interestingly, most of our results support this idea: in the origin area, invasive tadpoles exposed to native competitors grew less, developed slower and displayed a morphology linked to competitive stress and loss of canalization, while native tadpoles increased their developmental stability and canalization, and mildly improved their rates of growth and development. While results from the expansion front can be described as an asymmetric competition where the invasive species is dominant, competitive outcomes in the origin are arguably much more symmetric, making this an interesting example of fast local adaptation following an invasion.

PART B

Competència intra-gremi en capgrossos de la granota pintada, *Discoglossus pictus.*

Capítol 5

Reaccions evolutives ràpides en un sistema competitiu autòcton-invasor després de diverses generacions de coexistència

[enviat a la revista *Journal of Animal Ecology*]

Les espècies invasores creen una sèrie d'interaccions competitives prèviament inexistents. Si els competidors autòctons no són desplaçats completament degut a aquesta competència, aquestes interaccions poden forçar-ne canvis de comportament, ecològics i morfològics, tant en les espècies autòctones com en les invasores. Això fa que les invasions biològiques siguin interessants experiments naturals on estudiar el paper del canvi evolutiu en la capacitat competitiva de les espècies. Els capgrossos són molt plàstics – és a dir, flexibles – en termes de comportament i de morfologia, modificant aquests dos aspectes en reacció a una àmplia gamma de factors d'estrès, incloent-hi el risc de dessecació de la bassa i la pressió competitiva. Aquestes reaccions tenen efectes a curt i llarg termini sobre les aptituds individuals per la supervivència, convertint-se així en importants dianes per la selecció natural i essent marcs d'acció típics per respostes evolutives ràpides.

En aquest estudi, examinem els canvis morfològics i de comportament induïts mútuament per capgrossos d'una espècie invasora (la granota pintada, Discoglossus pictus) i una d'autòctona (el gripau corredor, Epidalea calamita) sota diferents combinacions de risc de dessecació i de pressió competitiva interespecífica. Per detectar possibles canvis evolutius en aquesta interacció competitiva, es va repetir el mateix experiment fent servir capgrossos de dues ubicacions – La Jonquera i Sant Celoni – amb diferents històries evolutives: una, La Jonquera, ben a prop de la primera introducció de la granota invasora – és a dir, amb al voltant de 110 anys de coexistència entre els dos competidors –, i una altra, Sant Celoni, del front d'expansió de l'espècie – implicant menys de 3 anys de coexistència. Com que el competidor autòcton està present al llarg de tota la distribució invasora de la granota pintada, la nostra hipòtesi era que els resultats haurien de ser més favorables a l'espècie invasora en les poblacions de curta coexistència, és a dir, les del front d'expansió. Això seria degut a que allà el competidor autòcton manca de coneixement evolutiu previ de l'espècie invasora, mentre que en el sentit contrari aquest desconeixement evolutiu no hi seria present, afavorint l'invasor. De manera molt interessant, la majoria dels resultats donen suport a la nostra hipòtesi: en l'àrea d'origen, els capgrossos invasors exposats a competidors autòctons creixen menys, es desenvolupen més lentament i mostren una morfologia relacionada amb l'estrès competitiu i a la pèrdua de canalització morfològica. Per altra banda, els capgrossos autòctons de la zona d'origen tenen més alta la seva estabilitat de desenvolupament i la seva canalització morfològica quan competeixen amb l'espècie invasora, i milloren lleugerament les seves taxes de creixement i desenvolupament.

Per tant, en resum, tot i que els resultats del front d'expansió es poden descriure com una competència asimètrica on l'espècie invasora és la dominant, els resultats en l'origen són sens dubte molt més simètrics, fent d'aquest un exemple interessant de ràpida adaptació local d'una espècie autòctona després d'una invasió.

Fast evolutionary responses in a native-invasive competitive system after several generations of coexistence

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Abstract

1. Invasive species create a series of previously non-existent competitive interactions. If native competitors are not totally displaced, these interactions can force behavioral, ecological and morphological changes both in the native and the invasive species, making biological invasions interesting natural experiments where to study the role of evolutionary change in the competitive abilities of species. Anuran tadpoles are very plastic in terms of behavior and morphology, reacting to a wide array of stress factors, including pond desiccation risk and competitive pressure. These reactions have short and long term effects on individual fitness, thus becoming important targets for natural selection and yielding fast evolutionary responses.

2. We examine the behavioral and morphological changes mutually induced by tadpoles of an invasive (*Discoglossus pictus*) and a native anuran (*Epidalea calamita*) linked to different combinations of pond desiccation risk and interspecific competitive pressure. To test possible evolutionary changes in this competitive interaction, we repeated the same experiment using tadpoles from two locations with different evolutionary histories: one near the first introduction of the invasive frog (*i.e. circa* 110 years of coexistence of invasive and native competitors), and another from the expansion front of the species (less than 3 years of coexistence).

3. As the native competitor is present all along the invasion range of the invasive frog, we hypothesized that outcomes should be more favorable to the invasive species in short-coexisting populations from the expansion front, where the native competitor is naive and the invasive species is not.

4. Interestingly, most results support our hypothesis: in the origin area, invasive tadpoles exposed to native competitors grew less, developed slower and displayed a morphology linked to competitive stress and loss of canalization, while native tadpoles increased their developmental stability and canalization, and mildly improved their rates of growth and development.

5. While results from the expansion front can be described as an asymmetric competition where the invasive species is dominant, competitive outcomes in the origin are arguably much more symmetric, making this an interesting example of fast local adaptation following an invasion.

Introduction

Strong competitive ability is considered a common attribute of invasive species and is listed as a key feature for their success in a new environment (Vila & Weiner 2004; Levine, Adler & Yelenik 2004). The establishment and expansion of an invasive species creates a series of previously non-existent competitive interactions which, by definition, reduce the fitness of at least one of the competing parts (Gurnell *et al.* 2004). Selective benefits for individuals which better withstand these new interactions are expected, posing introduced competitors as strong agents of selection on native species, and vice versa. If both competitors sustain viable populations with enough genetic diversity, competition can act as an evolutionary enhancer and selection can lead to mutual coevolution (Strauss, Lau & Carroll 2006; Vellend *et al.* 2007). Therefore, mid- and long-term impacts of an invasion will depend on the evolvability of both natives and invasives, and could result in detectable structured geographical patterns of competitive abilities (Vellend *et al.* 2007). In this context, evolutionary studies are essential for understanding the diverse effects of invasive species on the native community and making realistic predictions of their capacity to survive and continue expanding. Although evolutionary shifts caused by introduced species have been already explored from the perspective of predator-prey interactions (Kiesecker & Blaustein 1997; Nunes *et al.* 2014; Anderson & Lawler 2016), literature on evolutionary shifts linked to competition among animals is rather scarce, receiving this area of study more attention from a plant biology perspective (Callaway *et al.* 2005; Strauss, Lau & Carroll 2006; Vellend *et al.* 2007; Zuppinger-Dingley *et al.* 2014). Documented examples comprising animals include morphological modifications linked to a shift in habitat use (Crowder 1986; Bourke, Magnan & Rodríguez 1999; Stuart *et al.* 2014) and changes in body size in response to interspecific aggression (Sidorovich, Kruuk & Macdonald 1999). Evolutionary change in response to invasive predators can occur very rapidly, being detectable in only a few generations and in a time frame of only decades or less (Kiesecker & Blaustein 1997; Nunes *et al.* 2014, Anderson & Lawler 2016), so it is reasonable to expect similar evolutionary rates with a selective pressure like competition.

Anuran tadpoles have already been used as models for studying competition among native and invasive species (Kupferberg 1997; Williamson 1999; Smith 2005; Cabrera-Guzmán, Crossland & Shine 2013a,b). In studies on tadpole competition, growth and developmental rates are usually taken as the main proxies of individual fitness: among other effects, larger tadpoles are more successful competitors (Peacor & Pfister 2006), survive better to predation risk (Johnson, Burt & DeWitt 2008; Pujol-Buxó, García-Guerrero & Llorente 2016a) and increase their size and survival as metamorphs (Tarvin *et al.* 2015), while higher developmental rates allow tadpoles to leave the pond earlier, avoiding mortality due to pond drying (Richter-Boix, Llorente & Montori 2006a; Gomez-Mestre, Kulkarni & Buchholz 2013). Interestingly, however, both growth and development demand energy expenditure, which creates a trade-off between both processes (Werner 1988; Richter-Boix, Orizaola & Laurila 2014). When exposed to particular stress factors, tadpoles are able to display shifts along this trade-off, not as a simple consequence of the imposed limitations, but rather as integrated plastic responses to them (Richter-Boix, Llorente & Montori 2006a; Gomez-Mestre, Kulkarni & Buchholz 2013). For instance, if enough food is available (Enriquez-Urzelai *et al.* 2013) some species are able to allocate most of the obtained energy in development and reduce their larval period when confronting rapidly dwindling water levels, but they do so at the cost of a smaller size at metamorphosis (Richter-Boix, Llorente & Montori 2006b; Gomez-Mestre, Kulkarni & Buchholz 2013). On the other hand, models on metamorphosis (Wilbur & Collins 1973; Day & Rowe 2002) predict that under food shortage and no desiccation risk, a tadpole will metamorphose nearly after attaining its threshold of minimum size for metamorphosis, and therefore we will observe similar sizes at metamorphosis with large variations in developmental time (Morey & Reznik 2004). Finally, when there are large amounts of available energy, tadpoles can at the same time metamorphose earlier and at larger sizes (Peacor and Pfister 2006; Lind, Persbo & Johansson 2008). Hence, the inherent difficulty to separate shifts in growth from changes in development in systems where several stresses interact makes necessary a global study of the growth-development axis to correctly assess possible effects of competition on tadpole fitness.

Interestingly, tadpoles exhibit several other types of plastic changes that enhance their chances of surviving stressful situations, thus being responsive beyond size and development – for example in behavior and body shape – under a wide array of stimuli and stress factors, including competitive pressure (Relyea 2002; Van Buskirk 2009). For example, tadpoles under high competitive stress are commonly reported to increase their levels of activity to enhance food intake rates (Relyea 2002; Richter-Boix, Llorente, Montori 2004; Relyea 2004). In addition, tadpoles growing in a regime of increased competition usually avoid investing in tail structures, and rather allocate most energy in head structures where feeding and digesting structures lie (Relyea 2002; Relyea & Hoverman 2003; Relyea 2004; Van Buskirk 2009). These behavioral and morphological reactions in response to competitive pressure also influence – and are part of – the energetic management of tadpoles, having immediate and delayed effects on individual fitness (Tejedo, Semlitsch & Hotz 2000; Relyea & Hoverman 2003). Thus, in general terms, if tadpoles are under a disadvantageous situation, we should expect not only shifts in growth and development, but also changes in their behavior and morphology, as part of a global and probably integrated response in energy and risk management. These plastic responses can enhance effectiveness in coping with several and varied situations if they are correctly assessed by the tadpole, and therefore become an easy target for natural selection and fast evolutionary reactions (Nunes *et al.* 2014). Moreover, these reactions – or parts of them – can be easily coopted or mildly modified from preexistent plastic reactions to be reused to confront novel threats (Gomez-Mestre & Díaz-Paniagua 2011; Pujol-Buxó *et al.* 2013; Pujol-Buxó *et al.* 2016a; Grason 2017), making them a key attribute for both invasiveness and the resilience of natives during the first stages of biological invasions (Richards *et al.* 2006; Peacor *et al.* 2006). In the long term, although phenotypic plasticity in the past had been dismissed as unimportant in adaptive evolution – even considered to constrain it by shielding the genotype from selection – it has more recently been considered as a positive enhancer of evolutionary novelty, among other processes, through genetic accommodation

(West-Eberhard 2003; Ghalambor *et al.* 2007; Lande 2009; Moczek *et al.* 2011).

Nevertheless, during the first stages of an invasion not all observed changes are necessarily adaptive (Ghalambor *et al.* 2007). For instance, when confronted with a novel type of stress, phenotypic plasticity might yield non-adaptive – or even maladaptive – outcomes if the organism makes a wrong assessment of the risk (Carthey & Banks 2014). On the other hand, stress factors are known to also induce morphological responses not related to phenotypic plasticity or adaptation when they interfere with the developmental mechanisms that control the structure and occurrence of phenotypic variation, causing decreased levels of developmental stability and canalization (Badyaev & Foresman 2000; Lazić *et al.* 2014). Thus, developmental stability and canalization have repeatedly posed as reliable indicators that are negatively correlated with the degree of stress in which an animal develops (Badyaev, Foresman & Fernandes 2000; Badyaev 2005; Lazić *et al.* 2014). In tadpoles for instance, increased allometric differences or altered patterns of modularity could be signaling losses in developmental canalization when confronting certain perturbations (similarly to Badyaev, Foresman & Fernandes 2000). On the other hand, increased variance in rates of growth and development of tadpoles under high competitive pressure have been reported (Peacor & Pfister 2006), such that increased intra-group disparity in tadpole shape – as a proxy of developmental stability – could be additional evidence of high levels of stress in this situation. All these factors probably influence the outcomes of the competitive interactions during the incipient establishment of an invasive species, posing an additional layer of complexity to the interpretation of results but giving, in turn, additional insights on the strength of impacts of stress factors and how tadpoles deal with them.

Here we conduct an integrative examination of the developmental, morphological and activity responses mutually induced by tadpoles of two anuran competitors that co-occur in the Iberian Peninsula – the invasive Mediterranean Painted Frog *Discoglossus pictus* and the native Natterjack Toad *Epidalea calamita* – undergoing several experimentally controlled stress conditions, and coming from two populations with different evolutionary histories. Controlled stress treatments were designed to create different levels of pond desiccation risk and tadpole density, which are the two most common natural stress factors for the study species. To examine the dependence of plastic responses on recent evolutionary history, the sampled populations were (1) one very near to the first introduction of the invasive frog - translating to at least 110 years of coexistence with the native competitor - and (2) another from the expansion front and current southern distributional limit of the invasive frog – translating to less than 3 years of coexistence with the native species. From an evolutionary point of view, and assuming a generation time of approximately 3 years for both species (Oromi, Sanuy & Sinsch 2012; Oromi *et al.* 2016), we can argue that the native toad must be nearly or totally “naive” in the distributional limit of the invasive species. By contrast, given that the native toad is present all along the invasion range, the invasive frog has accumulated approximately 35 generations of competitive experience with the native species as it was expanding its range southwards. Contrarily, in the area near the original introduction both competitors have had the same time and a similar number of generations to mutually adapt to the presence of each other in the breeding ponds. This design provides a unique opportunity for studying how species competition triggers phenotypic change in the context of species invasions, and how the interplay between phenotypic plasticity and local adaptation proceed across short evolutionary intervals. Specifically, we hypothesize that, if the native toad is indeed progressively adapting to the invasive competitor, competitive hierarchy should be relatively more favorable to the native tadpoles in the origin area. This should translate in signs of competitive stress and deleterious effects accumulating – always in relative terms – in the invasive species. Contrarily, competitive outcomes should be more favorable to the invasive species in short-coexisting populations from the expansion front, where the native competitor is naive and the invasive species is not. In this case, we should detect stronger signs of competitive stress and deleterious effects in the native species.

Methods

Study system

The Mediterranean painted frog *Discoglossus pictus* is native to Northern Africa and Sicily (Zangari, Cimmaruta & Nascetti 2006). It was introduced to mainland Europe from Algeria in around 1906 in Banyuls de la Marenda, France (Zangari, Cimmaruta & Nascetti 2006; Wintrebert 1908). Its invasive range is continuously increasing (Llorente, Montori & Pujol-Buxó 2015), now extending on a wide coastal stripe of approximately 250km from Montpellier (SE France) to Sant Celoni (Barcelona, NE Spain). Although the species shows great versatility in its choice of reproduction points, temporary and ephemeral ponds are usually selected (Richter-Boix *et al.* 2013; Llorente, Montori & Pujol-Buxó 2015). Larvae are assumed to be largely bottom-dwelling (Escoriza & Boix 2012) and depend on very high rates of food consumption in order to grow (Pujol-Buxó *et al.* 2016b). As a known usual native competitor (Richter-Boix *et al.* 2013; San Sebastian *et al.* 2015a,b) we chose the Natterjack Toad (*Epidalea calamita),* which commonly also selects ephemeral and temporary ponds (Richter-Boix *et al.* 2013) and whose larvae are bottom-dwelling as well (Escoriza & Boix 2012), but in this case exhibiting relatively lower food consumption rates (Pujol-Buxó *et al.* 2016b). Competition of both species has repeatedly been studied from different points of view, usually posing the native competitor as the weaker part of a seemingly asymmetric competition relationship (San Sebastian *et al.* 2015a,b).

Experimental settings

We conducted two identical laboratory experiments in consecutive years, each time using individuals only from either the invasive range origin or the expansion front. Both experiments were carried out in the same laboratory (University of Barcelona, Faculty of Biology, Evolutionary Biology, Ecology and Environmental Sciences Department), under the same temperature (21-23ºC) and photoperiod conditions

(12L:12D). On April 5, 2014, we collected seven *D. pictus* spawns and thirteen *E. calamita* spawns in the invasion origin area, in a set of small ponds and wet meadows located near la Jonquera (UTM 31T 492171 4694521). This area is situated about 18 kilometers from the introduction point of *D. pictus* in mainland Europe, comprising abundant populations of both study species. On March 17, 2015, we collected five clutches of each species in a set of small ponds near Sant Celoni (UTM 31T 466745 4620477). This point lies at about 105 km from the introduction point, and only 2km from the southwestern expansion front of *D. pictus*. In both cases, spawns were synchronously collected after rainy nights and brought to the laboratory facilities of the University of Barcelona where they were individually kept in plastic recipients (18*18*30cm) with approximately 5L of water and fed *ad libitum* with commercial rabbit pellets (SANKY PIBAR. S.A., Barcelona, Spain) until they reached Gosner stage 25 (Gosner 1960).

After this point, tadpoles were transferred to the experimental units, which consisted of plastic tanks (21*40*60 cm) filled with 30L of dechlorinated tap water. To avoid clutch effects, we always selected tadpoles in the same proportion from the different available spawns. The water in tanks was progressively dried out according to the curve $D_j = (1–P*(j\ t^{-1})^a)+P-1$ (broadly based on Wilbur [1987], Fig. S1), where j is the time in days since the beginning of the experiment, D_j is the desired depth on day j, t is the target date for the depth to reach zero, P is the maximum depth (13 cm) at the beginning of the experiment, and a is a shape parameter (a=1.8). To standardize across years, we considered as starting day the first hatches. Therefore, when tadpoles were added to the experimental tanks the water column had already been partially reduced. To experimentally manipulate tadpole density and tank desiccation rate, we considered 20 tadpoles per tank as the low density treatment (LD), and 60 tadpoles per tank as the high density treatment (HD). Based on previous experiments, we considered a t of 60 days as slow desiccation rate, and a t of 35 days as fast desiccation rate (Fig. S1). The experimental design consisted of six different treatments, each replicated in four tanks: 1) *E. calamita* control, consisting only of *E. calamita* tadpoles in low density and under slow desiccation rates; 2) *D. pictus* control, consisting only of *D. pictus* tadpoles in low density and under slow desiccation rates; 3) **LDslow**, including ten tadpoles of each species under slow desiccation rates; 4) **HDslow**, including 30 tadpoles of each species under slow desiccation rates; 5) **LDfast**, with ten tadpoles of each species under fast desiccation rates; and 6) **HDfast**, with 30 tadpoles of each species under fast desiccation rates.

Throughout the experimental period, the water was renewed 3 times a week in order to avoid the proliferation of harmful levels of microorganisms, and after each water change the tadpoles were fed *ad libitum* with commercial rabbit pellets (SANKY PIBAR. S.A., Barcelona, Spain). Tadpole behavior was monitored twice a day, once in the morning and once in the afternoon, always at the same time, to record the proportion of active tadpoles at the moment of the first viewing (Skelly 1995). After 27 – in 2014 - and 24 – in 2015 - days after the start we ended the experiments. These had a slightly different duration in the two cases in order to avoid the possible spread and future effects of a bacterial infection in some tanks in 2015. Thus, once the infection was detected we ended the experiment to avoid further infections. Since intrinsic differences between populations prevent inter-population comparisons in our study (see Supplementary Statistics), we analyzed data always in comparison – or statistically standardized – to control treatments, and this slight difference in duration does not affect the interpretation of the results. At the end of the experiment we quantified total survivorship in each tank – overlooking the effects of the recently detected bacterial infection. In addition, we photographed the lateral view of the body of a sample of tadpoles from each tank (10 individuals per species for LD, and 15 for HD treatments and controls), in a standardized position and using a reference grid to record scale. These photographs were then used to record tadpole size, developmental stage and morphology.

Statistical analyses: tadpole survival and activity

We tested for differences in survival among experimental treatments by fitting a generalized linear model (GLM) with a binomial response and using species, sampling locality and treatment, and all interaction terms, as explanatory variables. We then performed a holistic model selection using the function *dredge* implemented in the package *MuMIn* (Bartoń 2016), and tested each term using single-term deletion with likelihood ratio tests (LRTs) against the simpler nested model.

We tested for differences in the activity level of tadpoles across experimental treatments by fitting a generalized linear mixed model (GLMM) with a binomial response (active vs. inactive) and using species, sampling locality, treatment and tadpole age– and allowing for first order interactions – as potential fixed effects, also including the possibility of a three-way interaction among locality, treatment and species to test for the possible independence of the activity of all treatment groups. We always included tank as a random effect to account for the lack of independence among data from the same group of tadpoles. We performed a holistic model selection using the function *dredge* implemented in the package *MuMIn* (Bartoń 2016), and selected the best models using the Akaike Information Criterion (AIC), the Bayesian Information Criterion (BIC) and LRTs among the best models (Burnham & Anderson 2003). We then used Wald´s z test (Bolker *et al.* 2009) to test for differences among the levels of the factors included in the best model. To examine possible differences in tadpole activity levels among treatments, relative to their respective control, we first calculated the percentage of active tadpoles in each experimental tank and then we standardized the observed activity level by subtracting the mean activity percentage across tanks of the corresponding control. Once standardized, we tested for differences across treatments – excluding the control – using randomization ANOVAs as implemented in the function *lmp* of the *lmPerm* R-package. In this case, the model included species, location, treatment and all possible interaction terms as predictors.

Statistical analyses: tadpole growth and development

To describe tadpole development, and since developmental stage and body growth are tightly related features (Wilbur & Collins 1973; Werner 1988; see Introduction), we analyzed these aspects together. We used the lateral photographs taken at the end of the experiment to determine the final developmental stage of each tadpole recording the Gosner stage (Gosner, 1960). In order to quantify tadpole size (and shape, see further on) we used geometric morphometric techniques (Rohlf & Marcus 1993; Adams, Rohlf & Slice 2013). For this purpose, we digitized 4 landmarks and 17 semilandmarks in the same photographs (Fig. S4) using tpsDig2 (Rohlf 2010). Because tail fin shape is a very plastic feature not necessarily related to body mass or size, we excluded tail fin landmarks (Fig. S4) before performing a Procrustes superimposition (Rohlf & Slice 1990) from which we obtained the centroid size (hereafter, Csize) of each tadpole as a surrogate of its body size. During superimposition, the position of semilandmarks was optimized through a sliding procedure to minimize bending energy among specimens (Bookstein 1997).

To examine variation in development and growth depending on experimental treatment, species, and sampling locality, we first centered the data of each group relative to the species/locality control treatment. We then explored possible differences among species, sampling localities and treatments – and all possible interactions – in Csize and Gosner Stage using MANOVA. Because all factors and interaction terms exhibited significant effects (see Results) we performed non-parametric randomization pairwise comparisons among all treatments using self-written R routines (Script S1, based on the procedures used in Turner, Collyer & Krabbenhoft 2010). To test the influence of stress on the developmental stability of each group in relation to developmental and growth rates, we also tested for possible differences in intra-group disparity in developmental stage and Csize attained by the end of the experiments among treatments, locations and species. To that end, we used a self-written pairwise permutation test on bivariate variance in R (Script S2).

Statistical analyses: tadpole shape

Before the analysis of tadpole shape, and because in this case we were not interested in directly comparing shape patterns across species, we performed a Procrustes superimposition for each species separately, excluding extreme outliers in each case, and using the full set of landmarks and semilandmarks (Fig. 2, Fig. S4). We performed all geometric morphometric analyses using the functions provided in *geomorph* (Adams & Otarola-Castillo 2013; Adams *et al.* 2016) and self-written R routines when specific functions are not cited. To account for effects of positioning during the photographic procedure due to tail fin position, we first standardized the position of the tail of the tadpole using the function *fixed.angle*. In addition, to account for possible effects of development on body shape, the shape of each tadpole was standardized by its Gosner stage, through a multivariate regression of shape on Gosner stage.

Regarding tadpole shape analyses, we first performed a randomization ANOVA - using the function *procD.lm* - to test for mean shape differences among treatments irrespective of size variation, including population, treatment and their interaction as dependent variables. Given that results were significant in all cases (see Results) we performed pairwise comparisons – using the function *advanced.procD.lm* - on the same model. Furthermore, we also tested for differences in the amount of intra-group morphological disparity among treatments – using the function *morphol.disparity* of *geomorph* R-package, including size and Gosner stage as covariates to account for differences in expected disparity in different moments of tadpole development and growth. After that, we proceeded to test allometric differences performing randomization ANOVAs, including sampling locality, treatment and size, and all possible interaction terms as explanatory variables, also using the function *procD.lm*. Given that the three-way interactions were significant for both species (see Results), we performed pairwise tests to examine which treatment-by-locality groups exhibited differences in their allometric trajectories. Moreover, we were interested in knowing if tadpole allometries in front of the different competitive stresses were under different levels of canalization, and therefore if they responded with more variable allometries in one population than the other. To that end, we tested if the several allometries were equally variable in direction and length between populations, using self-written permutation tests in R (Script S3). To test if the detected changes in morphology – be them in mean morphology disregarding size effects or differences in the static allometries – consisted in similar phenotypic reactions, we used trajectory analysis (Collyer & Adams 2013) implemented in the *trajectory.analysis* function of *geomorph*, which allows, in this case, to test for differences in direction and amount of morphological change separately. We always included tadpole size as a covariate in trajectory analyses.

To further investigate whether and how stress due to competitive pressure or desiccation risk influences phenotypic variance, we also analyzed patterns of modularity in tadpole body shape, using the CR coefficient for assessing and comparing the degree of modularity across groups (Adams 2016). To that end, we divided the body of tadpoles into three hypothesized modules based on their function and their energetic and biological value (see Fig. S4 for more details): a “head” module – serving digestive, visceral and sensorial functions with high energetic and biological value –, a tail musculature module – with locomotive function –, and a tail fin module – with hydrodynamic and defensive function (Johnson, Burt & DeWitt 2008). These modules were tested for each species separately. Once confirmed the existence of these modules in both full datasets (see Results), we proceeded to test if the degree of modularity varied among different stress-inducing treatments and locations using self-written randomization tests (Script S4).

All statistical analyses were carried out using R (R Core Team 2015). To illustrate the possible differences in different measures, we usually calculated bootstrapped 95% CIs if they were not yet available in *geomorph* (Script S5 for a generalized example script). Due to clear differences among controls (see Supplementary Statistics) we avoid direct comparisons between similar treatments across populations. Moreover, for clarity, although we performed all multiple pairwise comparisons among all treatments when conducting pairwise analyses, we usually overlook results not involving the control treatment from the corresponding species and location.

Results

Tadpole survival and activity

Survival did not exhibit any clear pattern of variation across treatments, locations or species (Tables S1 and S2), being very high in all cases (>80% in all tank by species interaction, >90% in mean for any species, treatment or location). Although the interaction between location and species was significant when tested against a complex model (Table S2), it lost its importance when tested alone against the best -null - model ($Chisq_3$=-6.14, P=0.105).

Tadpole activity depended on species, location, treatment and age as single effects (Fig. S2), but not all interactions received full support according to AIC and BIC. Namely, neither the three-way interaction of location×age×species (LRT against best model: $Chisq_4$=1.531, P=0.8211) nor location×age or treatment×species terms were usually selected in the best set of models, while the rest of interactions were (Table S3). While the location×age interaction received some support as a possible explanatory variable according to LRTs ($Chisq_1$=4.67, P=0.0306), treatment×species was not supported ($Chisq_4$=6.76, P=0.1492). Thus, both species reacted equally to all treatments in both locations, and the age of tadpoles had similar effects on both locations. Both possible best models (models 496 and 512, see Table S3) could be positively validated through residual analysis. Deep exploration of the simplest of the two - the best model according to BIC - revealed that invasive tadpoles were more active than natives (Table S4); that tadpoles from the expansion front were generally more active (Table S4); and that this increase in activity was more exaggerated in invasive tadpoles (Table S4). However, while both species reacted similarly to stress factors, this reaction depended on sampling locality (Table S4). Thus, while in the expansion front both species had lower activity in most treatments relative to the corresponding control, in the origin area this activity was more or less constant across treatments. The most exaggerated differences in activity levels among locations were found in the LDslow treatment (Table S4, Fig. S2), which was the treatment with lowest activity rates in the expansion front but with highest activity rates in the origin area. An increasing age of tadpoles was always correlated with lower levels of activity, being this relationship less exaggerated in the native *E. calamita* and in treatments with high densities (Table S4). On the other hand, the activity of tadpoles relative to the control (Table S5, Fig. S3) depended only on species – having *D. pictus* higher relative activity levels –, on location – having the expansion front lower relative activity levels –, and on the interaction between location and treatment, due to opposite tendencies in LDslow treatments of the two locations (Fig. S2 and S3). Despite having *D. pictus* a tendency to display higher relative activities in the expansion front and in relation to *E. calamita*, results of the interaction species x location in this case were not significant (P=0.079, Table S5).

Tadpole growth and development

Mean tadpole growth and development was affected by species, location, treatment and all interactions among them (all P<0.001, Table S6). Pairwise comparisons and graphical exploration of the data showed that while *E. calamita* exhibited a strong relationship between developmental stage and body size, *D. pictus* had greater flexibility and independence between both features (Fig. 1). Tadpoles of both species under HDfast conditions were always highly impaired in terms of growth and development (Tables S7 and S8), except for the case of *D. pictus* in the expansion front, which attained sizes as large as the control treatment (Fig. S7). Similarly, the HDslow treatment was always the second most impairing, but HDslow tadpoles for *D. pictus* were even larger than the corresponding control, displaying only a mild reduction of their developmental rates (Fig. 1, Table S7). Moreover, in *D. pictus*, the relative rates of development were always faster in the expansion tadpoles than in the origin area, excepting LDslow, where differences were not significant (Table S7). This also meant that *D. pictus* from the expansion front in low densities were able to significantly accelerate their development – compared to control rates of development – in response to fast desiccation (P=0.015, Table S7), while the observed acceleration was not statistically significant in the origin area (P=0.096, Table S7).

On the other hand, when reared under desiccation risk and low densities of tadpoles of both species (LDfast), *E. calamita* did not accelerate development and had the same results as control tadpoles in both locations (Fig. 1, Table S7). Furthermore, we detected costs in *Epidalea calamita* LDslow tadpoles in the expansion area (P<0.001) but not in the origin area (P=0.093). Costs were detected in all *E. calamita* tadpoles undergoing high densities (all P<0.001, Table S7), equally deleterious in the case of fast desiccation (P=0.948), but mildly less deleterious in the expansion area for low desiccation risks (P=0.033). Concerning the disparity of size and development (Table S9, Fig. S5), *E. calamita* did not exhibit any differences among treatments. However, *D. pictus* showed a clear tendency to increase the intra-group disparity of size and development in all HD treatments. Specifically, the increase of disparity in the HDslow treatment of both locations was nearly significant (0.05 < P < 0.07 in both cases, Table S9), and was significant for the HDfast treatment (P=0.011, Table S9) from the expansion front.

Tadpole shape

Differences in tadpole body shape across different treatments and populations were found in both species. The native *E. calamita* exhibited significant differences – disregarding size effects – among locations ($F_{1,473}$=27.90, P<0.001), treatments ($F_{4,473}$=4.03, P<0.001) and in the interaction among location and treatment ($F_{4,473}$=4.85, P<0.001). Posterior pairwise tests showed that differences against control morphologies were located only in slow desiccation treatments of the origin area (Table S10; Fig. 2). Posterior trajectory analysis of these two morphological reactions showed that they were mildly but significantly different in direction (44.8 degrees, P=0.047), but similar in magnitude (P=0.077). When testing possible effects of size, all terms were significant, including the three-way interaction size×location×treatment (Table S11), pointing at allometric differences among treatments depending on location. Posterior pairwise tests showed that differences against control allometric trajectories were located in slow desiccation treatments from the expansion area (Tables S13, Fig. S6). In both cases, the angle of the static allometry – and not the amount of shape change observed across the allometric trajectory – was significantly different from control (HDslow: P=0.049; LDslow: P=0.026). Trajectory analysis on these two morphological reactions in the expansion area showed that they were clearly different in direction (91.9 degrees, P=0.002) but not in magnitude (P=0.408). Globally, allometries from the expansion front as a whole were more variable than those from the origin area in the native species (P=0.034). Finally, intra-group body shape disparity of the HDfast treatment from the expansion front was significantly higher than that observed in the control treatment (Table S15, Fig. S7).

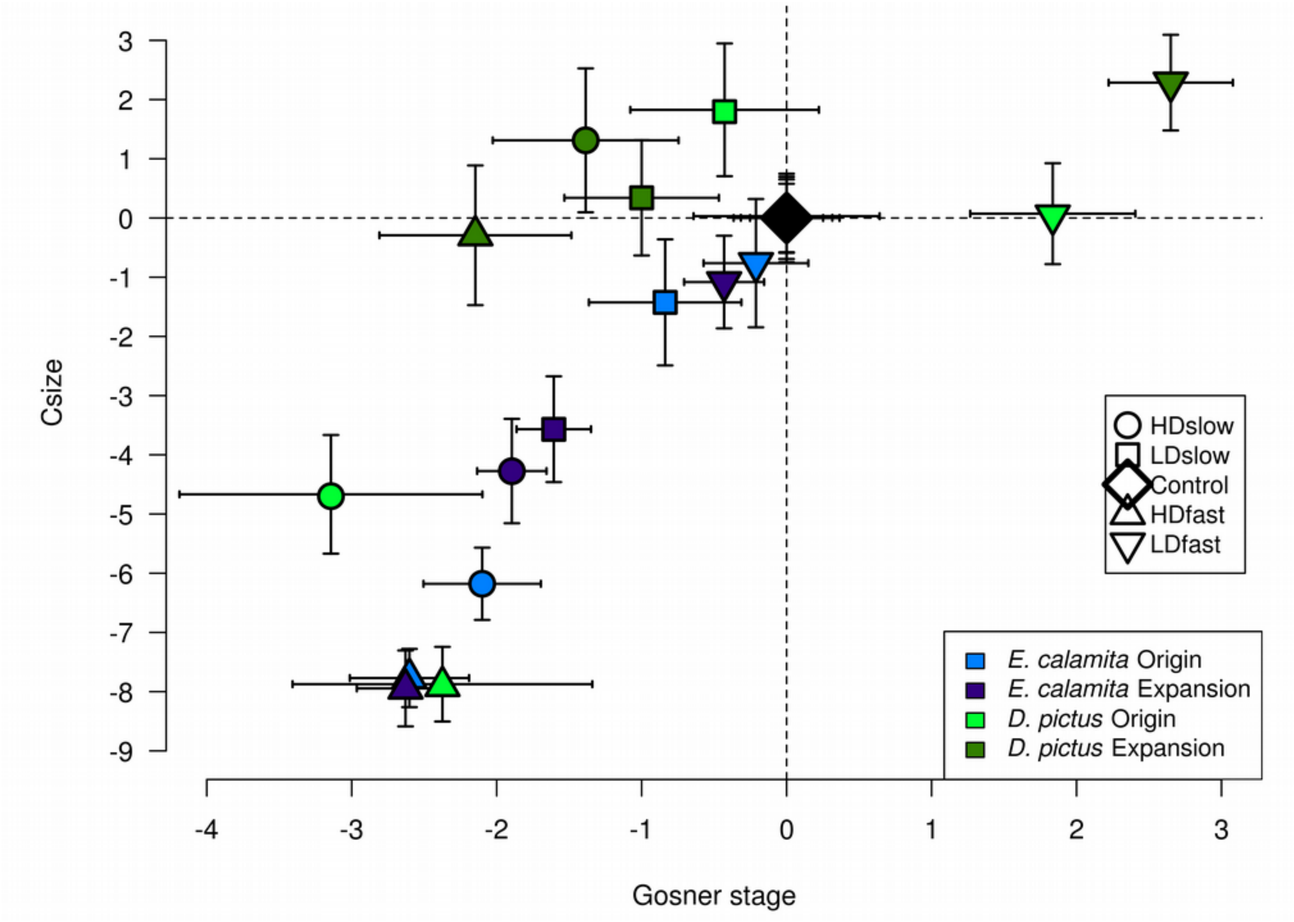

Figure 1. Mean +/- 95% CI of tadpole centroid size – Csize - and development – Gosner stage - at the end of the experiment, with all treatments standardized relative to the corresponding control. Thus, all four control treatments (two species in two locations) are placed in zero – zero coordinates, and the position of each treatment expresses relative changes compared to its corresponding control.

Concerning the invasive *D. pictus*, we found differences in tadpole shape – disregarding size effects – among locations ($F_{1,452}$=55.56, P<0.001), treatments ($F_{4,452}$=4.58, P<0.001) and in the interaction among location and treatment ($F_{4,452}$=4.70, P<0.001). Posterior pairwise tests indicated that differences against control mean morphologies were only detectable in both HD treatments of the origin area (Fig. 2, Table S10). Trajectory analysis of these morphological reactions showed that they were similar in direction (angle= 29 degrees, P=0.157), but the changes observed in the HDfast treatment were significantly more exaggerated in magnitude (P<0.001). When testing possible effects of size and its interactions, all terms were significant, including the three-way interaction size×location×treatment (Table S12), pointing at allometric differences among treatments depending on location. Moderate differences against control allometric trajectories were only found in the angle among static allometries - not the amount of shape change - in the HDslow treatment from the origin area (Fig. 3, Table S14). Contrary to what was observed in the native *E. calamita*, allometries from the origin area as a whole were globally more variable than those from the expansion front area (P<0.001). We did not find differences in body shape disparity between experimental treatments and the corresponding control in any case (Table S15, Fig. S8). Finally, both species globally showed a marked degree of modularity (*D. pictus*: CR=0.682, P=0.006; *E. calamita*: CR=0.729, P=0.007). We found significant losses in the strength of modularity - compared to the corresponding control - in the LDslow and HDfast treatment of *E. calamita* tadpoles from the expansion front (Table S16, Fig. S9), but we did not find any differences in neither treatment or location for *D. pictus* (Table S16, Fig. S10).

Discussion

It has commonly been accepted that a fitness decrease of a species in the presence of an alleged competitor is because of the negative effects of competition. Using similar assumptions in an invasion context, if the fitness of an invasive competitor progressively decreases through the history of its interaction with a native species, the shared, mutual evolutionary interaction between the two species can be interpreted to lead to the competitive inferiority of the invasive species. Here we reveal several changes in the competitive relationship between the native and invasive counterparts of our study according to time since invasion (Table 1) and, more interestingly, we report better outcomes for the native species at the more ancient region of the invasion: in the origin area (*circa* 110 years of coexistence), invasive tadpoles exposed to native competitors grew less, developed slower and displayed a morphology linked to competitive stress and loss of canalization, while native tadpoles increased their developmental stability and canalization, and mildly improved their rates of growth and development.

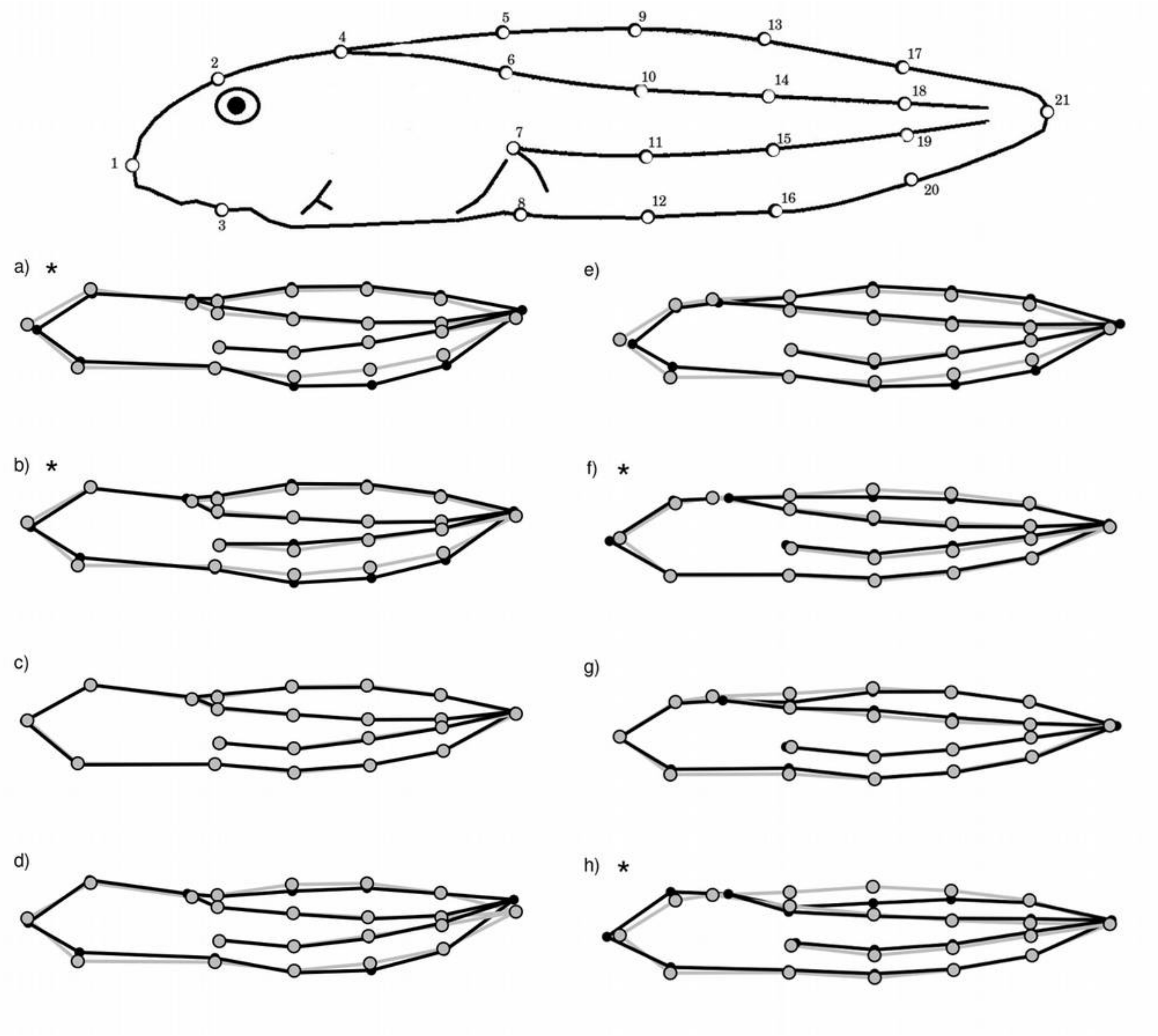

Figure 2. Landmarks and semilandmarks used in the study (top) and mean morphologies of each treatment (bottom) for tadpoles of native *E. calamita* – from a) to d) - and invasive *D. pictus* – from e) to f) - from the origin area. Differences among treatment tadpoles - black lines and points – and control tadpoles - grey lines and points – are twice magnified in order to ease visualization. Letters: a) and e): LDslow; b) and f): HDslow; c) and g): LDfast; d) and h): HDfast. Asterisks mark significant differences from control shape with alpha=0.05.

The main reliable indicator of fitness loss in a group of tadpoles undergoing high levels of competitive stress could have been a decrease in survival. In this case, however, mean survival of all treatments and species was always higher than 97% and, according to model selection, the few small changes in survival followed random patterns. Hence, we can consider that survival was not useful to detect possible deleterious effects of this competitive relationship. Despite this apparently negative result, a high survival in all treatments means that both species are plastically dealing with the different degrees of competitive stress, which allows us to reliably study them without being limited to examine only a subset of survivors. After survival, the set comprising mean size and developmental stages attained at the end of the experiment is arguably our most important result to assess possible changes in competitive hierarchy (Fig. 1). Indeed, a larger tadpole size enhances the probability to survive as tadpole and as metamorph (Peacor & Pfister 2006; Johnson, Burt & DeWitt 2008; Tarvin *et al.* 2015; Pujol-Buxó, García-Guerrero, Llorente 2016a), while fast developmental rates allow them to leave the pond earlier and to avoid mortality due to pond drying (Richter-Boix, Llorente & Montori 2006a; Gomez-Mestre, Kulkarni & Buchholz 2013). In accordance to our predictions and to previous observations (Enriquez-Urzelai *et al.* 2013), when experimentally exposed to combined levels competitive stress and desiccation risk, these cornerstone processes were usually impaired. This was clearly the case for the native *Epidalea calamita*, which exhibited the same pattern of decrease in growth and development across treatments, following a gradient of Control < LDfast < LDslow < HDslow < HDfast in both locations, with scarce differences among populations in the actual amount of change (Fig. 1, Table S7). This order clearly suggests an interaction between both stressors, which has similar – though not exactly the same - effects in both locations. From the differences observed between locations, it is important to highlight that native tadpoles simply exposed to invasive competitors – kept in low tadpole densities and low desiccation risk – displayed significant deleterious effects in the expansion front but not in the origin area (Table S7). Thus, the simple presence of the invasive competitor – without further stresses – can no longer induce significant fitness losses in the origin area, possibly providing evidence of habituation to the presence of the invasive competitor. On the other hand, however, another difference between populations is found in the HDslow treatment, where origin tadpoles mildly developed and grew relatively slower than expansion tadpoles, being one of the few instances of results contradicting our hypotheses. It is important to say that for *E. calamita*, both significant changes in mean size and development are rather small in magnitude and might not represent biologically significant fitness changes, particularly given that these differences are not accompanied by other behavioral or morphological differences between populations in the same direction.

On the invasive side of the competitive relationship, however, growth and development responses to experimental treatments exhibited much clearer differences between locations. In the expansion front of the invasive *Discoglossus pictus*, none of the induced stress types led to a decrease in the growth rate of tadpoles and only high density treatments could slow down tadpole development (Fig. 1, Table S7). Tadpoles from the invasive expansion front significantly accelerated their development in response to fast desiccation and low tadpole densities, surprisingly doing so – further discussed below – without losses in their mean size in turn. All these results oppose the outcomes in the origin area, where tadpoles of the invasive species reared in high densities of competitors clearly displayed deleterious effects in size and development - similar in magnitude to those displayed by the native species - and where tadpoles under a combination of desiccation risk and mild competition were not able to significantly accelerate their development, despite displaying a clear tendency to do so (Fig. 1, Table S7). Summing up, growth and development of the invasive species are comparatively faster at the expansion front, particularly in strong competition treatments, meaning mainly that *D. pictus* tadpoles from the expansion front never displayed size costs under any situation. Taking the results from both species together, these clearly suggest more unfavorable competitive outcomes for the invasive species in the origin area in terms of growth and development rates, with few changes – or mild improvements – for the native species. What are the proximate causes of these changes? Can we uncover other consequences of this competition? Activity and morphology may help to expose possible mechanisms and other alterations undetected by simple measurements of growth and development.

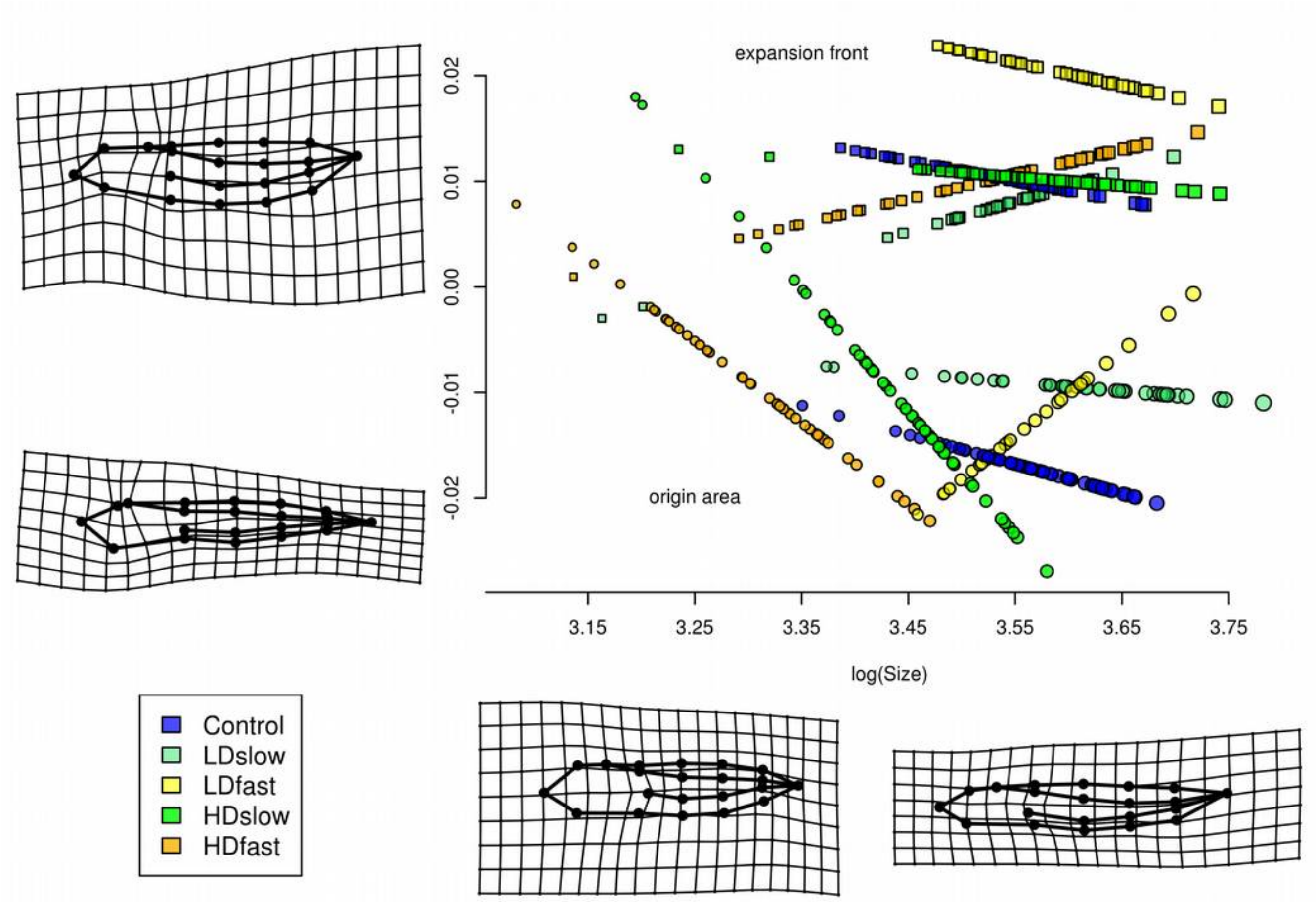

Figure 3. Predicted allometric trajectories for tadpoles of the several treatments of invasive *Discoglossus pictus*, plus reference extreme specimens along each axis to ease visualization of shape changes. log(Size): natural logarithm of tadpole centroid size.

Interestingly, even though our results on tadpole activity suggest a coupled pattern of activity of both species across experimental conditions and populations (Figs S2 and S3), in this pattern (1) the invasive species is globally more active than the native, and (2) these differences are visibly reduced in the origin area (Tables S3 to S5). In the first case, it is important to highlight that invasive *D. pictus* tadpoles are active, very consumptive and base their growth mainly on a high food intake, while native *E. calamita* rely more on assimilation and efficiency to grow (Pujol-Buxó *et al.* 2016b). This means that globally higher activity levels in the invasive species were indeed expected beforehand (as observed in Richter-Boix *et al.* 2013; San Sebastián *et al.* 2015b), and could be simply interpreted as part of each species' idiosyncrasy, at least in the areas studied here. However, in the second case, the significant interaction between location and species means that these differences in activity are exaggerated in the expansion front. Activity of tadpoles has been repeatedly related to their foraging capacities and therefore linked to their competitive ability (Werner 1992; Dayton & Fitzgerald 2001; San Sebastián *et al.* 2015b). Hence, for an intensive consumer species like *D. pictus*, a relative decrease – compared with its direct competitor - in energy intake and accumulation could constitute a proximate cause of the decrease in growth and development rates documented at the origin area. Here it is important to also note that although all tadpoles were fed *ad libitum,* the food supplied was not homogeneous. *Discoglossus pictus* tadpoles are known to be able to discriminate food sources and focus on the most nutritious parts, while *E. calamita* largely lack this capacity, or are clearly less inclined to do so (San Sebastián *et al.* 2015b). Therefore, greater activity than its competitor can enhance the ability of the invasive species to consume the best parts of the food supplied and, even if *ad libitum*, the synergy between an increase of activity and food discrimination may result in significant increases in energy intake, making possible to enhance both growth and development. This synergy could explain, for instance, how *D. pictus* tadpoles from the expansion front under mild levels of competition – discussed before - were able to accelerate their development without incurring in growth losses in turn.

Beyond activity and rates of growth and development, tadpole body shape has also been repeatedly used as a reliable source of information about the environment in which a tadpole develops, being also an important component influencing energy management when confronting a particular stress (Tejedo, Semlitsch & Hotz 2000; Relyea & Hoverman 2003). In this case, interestingly, we only found the expected morphological reactions in front of a known competitor (as in Relyea 2002; Relyea & Hoverman 2003; Relyea 2004) in invasive *D. pictus* tadpoles from the origin area that were withstanding high densities of competitors. Specifically, these tadpoles exhibited a relative increase of their head size – where all feeding and digesting structures lie – and a relative decrease in their investment on tail structures (Fig. 2), being probably the second a necessary consequence of the first. This reaction was found in both treatments of the origin area involving high levels of larval competition, it occurred in the same morphological direction – thus arguably meaning that it was the same plastic reaction –, and it was only exaggerated in magnitude when high desiccation risk was also present. The fact that this stress-linked reaction was not found in the expansion front is in complete agreement with the lack of decreases in growth and development for the invasive tadpoles in this location, lending further support to the hypothesis that native *E. calamita* is not able to inflict a competitive stress on the invasive species during the first stages of the invasion.

Species	*E. calamita*, native	*D. pictus*, invasive
Survival	No significant changes	No significant changes
Activity	Invasive species generally more active in both locations. Clear reductions of mean activity in the OA compared to the EF, but more marked in the case of invasive *D. pictus*	
Mean growth and development rates	Mild improvements in LDslow treatment and mild decreases in HDslow treatment in the OA compared to the EF	Clear decreases in strong competition treatments, decreases in developmental acceleration in response to desiccation risk under mild competitive pressure in the OA
Disparity in growth and development	No significant changes	Increased in all HD treatments, significantly so in HDfast from the EF
Mean tadpole shape	Unexpected shape changes in the LDslow and HDslow treatments, only in the OA	Shape changes in both HD treatments as expected under competitive pressure, only in the OA
Disparity in shape	Increased in the HDfast treatment of the EF	No significant changes
Allometry canalization	Losses of canalization in the EF compared to the OA	Losses of canalization in the OA compared to the EF
Changes in modularity	Losses in modularity in LDslow and HDfast treatments of the EF	No significant changes

Table 1. Summary of results obtained in the study. OA: Origin Area, EF: Expansion front.

However, possible stress-linked losses of developmental stability provide a somewhat different perspective. Generally, a clear tendency to increase disparity in growth and development rates is present in all high density treatments of invasive *D. pictus*, regardless of the location (Fig. S5). In fact, although the four experimental treatments were not statistically different among them in the amount of disparity in growth and development (Table S9), a significant increase compared to the control was achieved only in the HDfast treatment of the expansion front. This might indicate that, at least mildly, the native species is able to inflict competitive pressure on the invasive competitor during the first stages of invasion. In general terms, however, stress signs in the invasive species are largely more present in the origin area, and patterns of canalization in tadpole shape also reflect this difference. Indeed, we found a clear increase of variability in the static allometries of the invasive tadpoles in the origin area when exposed to the studied combination of stresses (Fig. 3). This points to a loss of developmental canalization confronting them, and parallels reported changes in studies dealing with habitat perturbation (Badyaev, Foresman & Fernandes 2000; Lazić *et al.* 2014). Thus, from a general perspective, the clear decreases in growth and development rates of the invasive species that were only found in the origin area – where the native species has had the opportunity to evolve resistance to the invader – are also matched by other morphological signs of competitive distress, like stress-linked modifications in body shape, plus decreases of developmental stability or canalization.

On the native side of the competitive interaction, the study of tadpole shape also provided very interesting insights. Concerning canalization, native *E. calamita* tadpoles displayed the opposite pattern to the invasive competitor, thus signaling also an arguably stress-linked loss of canalization, in this case in the expansion front (Fig. S6). In addition, the groups of tadpoles from the expansion front that underwent both strong competition and desiccation stress significantly increased their shape disparity – but did not in the origin area –, suggesting also a loss of developmental stability during the first stages of invasion (Fig. S7). Hence, the expansion front – the location in which the native species is more vulnerable according to our hypotheses – is the location which accumulates stress-linked and deleterious outcomes for the native *E. calamita*. Two treatments of these native tadpoles from the expansion front were the only instances in which we found significantly altered patterns of modularity – concretely, a significant loss of modularity in both cases. This could signal that the general relationship among different parts of the tadpole morphology can be modified as a consequence of an environmental stress – in this case, competitive stress (Badyaev & Foresman 2000) – a direction that should be more deeply explored in further studies. Summing up, in the native side of the competitive interaction, decreases in canalization and developmental stability, plus possibly altered patterns of modularity, are linked to the area where the native species is in evolutionary disadvantage, that is, where native *E. calamita* is naive and the invasive competitor is not. In this occasion, it is interesting to note that results of size and development alone did not reflect clear losses for the native species in the expansion front, meaning that the different levels of competitive stress could have been largely undetected if tadpole shape had not been studied.

The study of tadpole shape uncovered an unexpected – and potentially interesting – outcome for the native species: we observed significant shifts in mean shape in tadpoles from the origin area that are not directly explainable as a result of competitive stress. These shifts were found in both slow desiccation treatments and were visually very similar (Fig. 2), although differences in their direction of shape change rise doubts on whether they can be considered different expressions of the same response. In both cases, shape changes consisted of a mild reduction of the relative size of head structures, and an increase of the ventral surface of the tail fin (Fig. 2), thus largely opposing the shifts expected under competitive stress. As a first explanation, this pattern could simply reinforce the idea that native tadpoles from the origin area are less stressed than their conspecifics from the expansion front – which would completely agree with our hypotheses and the rest of results of the study – but such an explanation would overlook that these tadpoles from the origin area indeed invest more energy in tail structures than their corresponding controls. Another possible solution could be that the native tadpoles are wrongly assessing cues from their environment, and thus erroneously reacting to another stress factor, like predation risk, which usually yields results similar to those observed (Gomez-Mestre & Díaz-Paniagua 2011; Pujol-Buxó *et al.* 2013; Nunes *et al.* 2014). However, this should have been more expected in the expansion front – where the native species is really naive – and should consist of an increase in the whole tail surface linked to other shifts in activity, development and growth (Pujol-Buxó *et al.* 2013; Nunes *et al.* 2014) that were not observed here. As a reasonable adaptive interpretation of these morphological changes which should be further explored, observed morphological changes might be related to habitat use (Escoriza & Boix 2012). Thus, this morphological change might be linked to adaptive shifts in the use of the water column and microhabitats present in natural ponds (Crowder 1986; Bourke, Magnan & Rodríguez 1999; Escoriza & Boix 2012; Stuart *et al.* 2014) that simple activity measurements in a lab environment – where all food remains in the bottom of the experimental tanks – might have prevented us from detecting. More importantly, if tadpole morphology is indeed modified in response to shifts in habitat use, the lab venue might have prevented us from observing the benefits of this change. Recent studies using stable isotopes in natural ponds near the expansion front have reported mild differences in the diet of *E. calamita* tadpoles in competition with the invasive *D. pictus* tadpoles (San Sebastian *et al.* 2015a), so it is possible that after several more generations of selection, the native species is able to undergo adaptive shifts in habitat use to better cope with particular regimes of competition or desiccation risk. This suggests very interesting directions for future studies, for example focusing on potential evolutionary changes in the trophic ecology of both competitors, or directly assessing the habitat use and fitness of both species in natural ponds along the invasion range.

Is fast evolution in the competitive abilities of the native competitor the main driver behind the observed changes in the competitive hierarchy? Curiously, we found the most marked differences between locations in invasive tadpoles, so at first sight it could seem that losses in the competitive ability of the invasive species might be the underlying process. However, as previously commented, the simplified lab environment, despite being essential to discard other environmental effects, might mask possible benefits for the native species. In addition, a loss in the competitive abilities of invasive frogs from the origin area would clearly oppose previous theory and literature about selective patterns during a range expansion (Burton *et al.* 2010). Since differences in evolutionary history of both locations mainly affect the native toad – which is naive in the expansion front, but not in the origin area – we believe the most plausible explanation is indeed an evolutionary change in the native competitor. Moreover, if we (and according to all previous studies: Richter-Boix *et al.* 2013; San Sebastián *et al.* 2015 a,b) consider native toads the weaker part of an initially asymmetric competition, we can state that competitive selection – and therefore evolutionary change – has probably acted more strongly on the competitive subordinate than on the dominant competitor. Thus, once improvements in native's competitive ability take over, the alleviation of competitive pressure translates to decreases in deleterious effects of stress for the native species, while the increase in competitive pressure for the invasive species – losing its clear role of dominant competitor – translates to clear declines on growth rates (Fig. 1) and other stress signals or deleterious effects on tadpole morphology (Figs 2 and 3). Finally, it is interesting to remark that most deleterious effects of competition on both species – and consequently, the most interesting evolutionary changes – were detected under high densities of tadpoles, even when the desiccation stress was lacking. This identifies high tadpole densities as the most stressful factor for both species in this competitive system – at least considering the stress levels chosen in our study – and poses pond typologies where the two species do not coincide, or large ponds which impede resource depletion or tadpole crowding, as a possible stronghold for the native competitor during the first stages of invasion. In evolutionary terms, this could also have led to competitor avoidance when the adults choose the sites for reproduction, which is yet another interesting possibility that should be explored in the future.

Authors' contributions

EPB, GUG, BA and GLL conceived the ideas and designed methodology; EPB, GUG and BA conducted the fieldwork, the experimentation and collected the data; EPB, GUG, BA, AK analyzed the data; all authors contributed to the discussion of the results and focus of the manuscript; EPB led the writing of the manuscript; all authors contributed critically to the drafts and gave final approval for publication.

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Supplementary Figures for Pujol-Buxó *et al.* (2017):

Fast evolutionary responses in a native-invasive competitive system after several generations of coexistence

Number of supplementary Figures: 10

Figure S1. Curves of desiccation (water depth at each experimental day) of both types of treatments

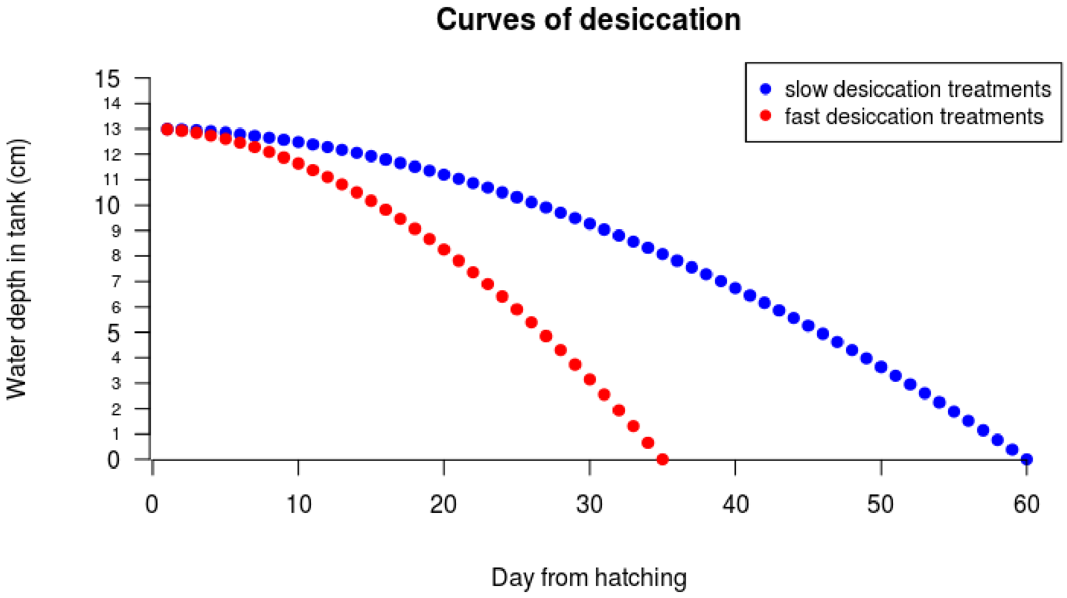


Figure S2. Mean +/- 95% bootstraped confidence intervals of activity of tadpoles, for all treatments, species and locations:

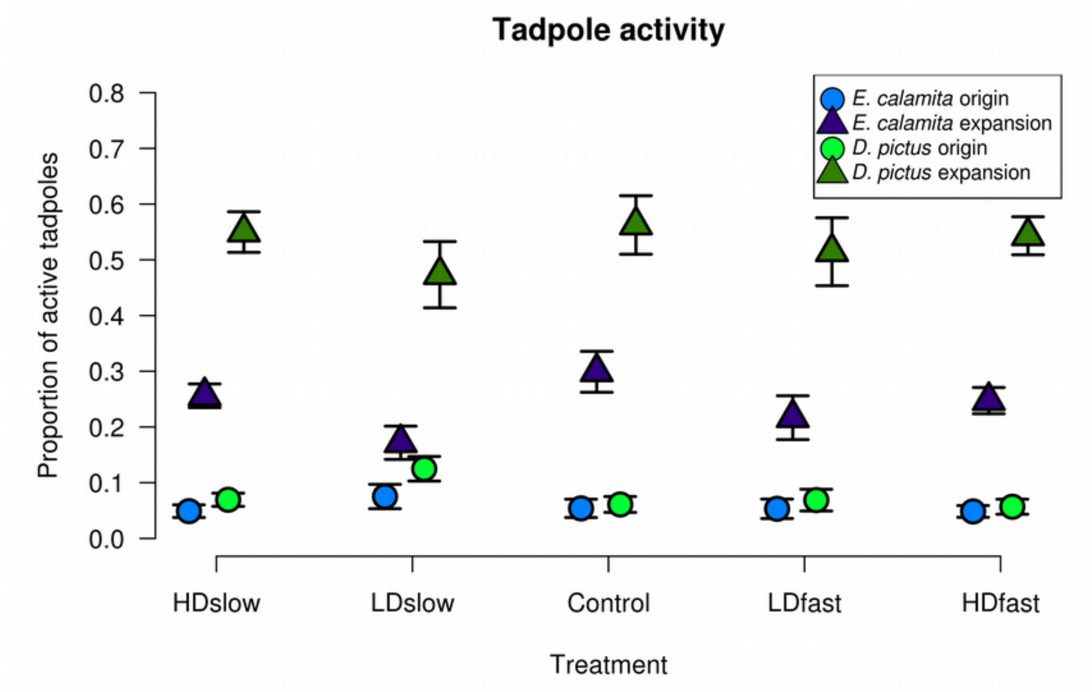


Figure S3. Mean +/- 95% CIs activity of tadpoles, all treatments standardized relative to its control. Thus, all four control treatments (two species in two locations) are placed in a zero mean, and the position of each treatment is expressing relative changes compared to its corresponding control.

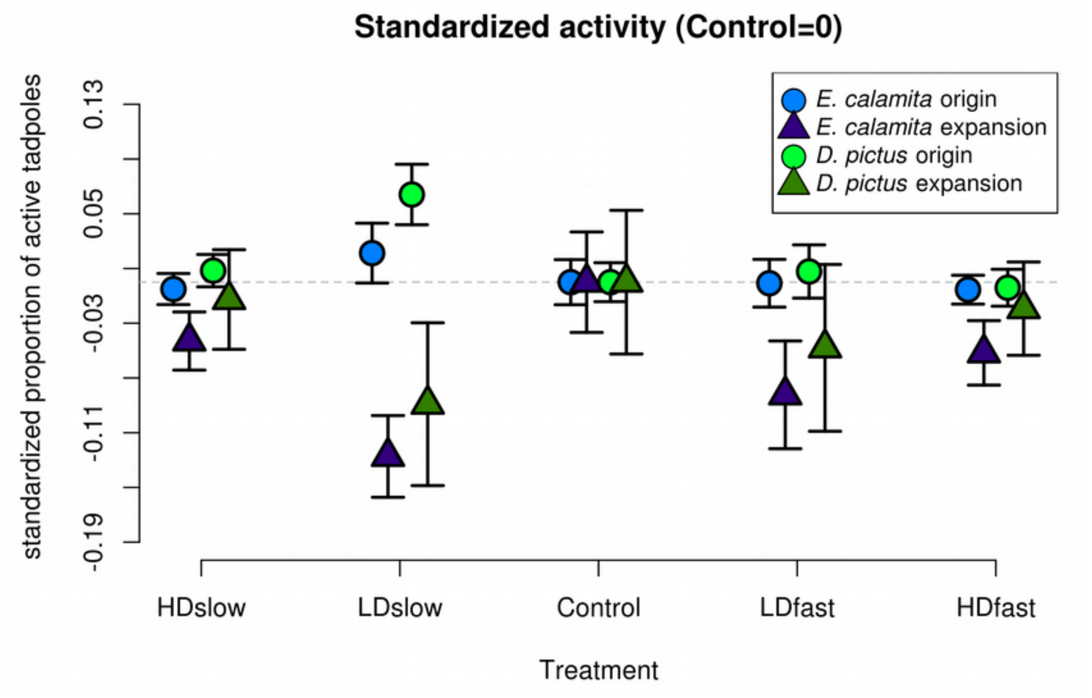
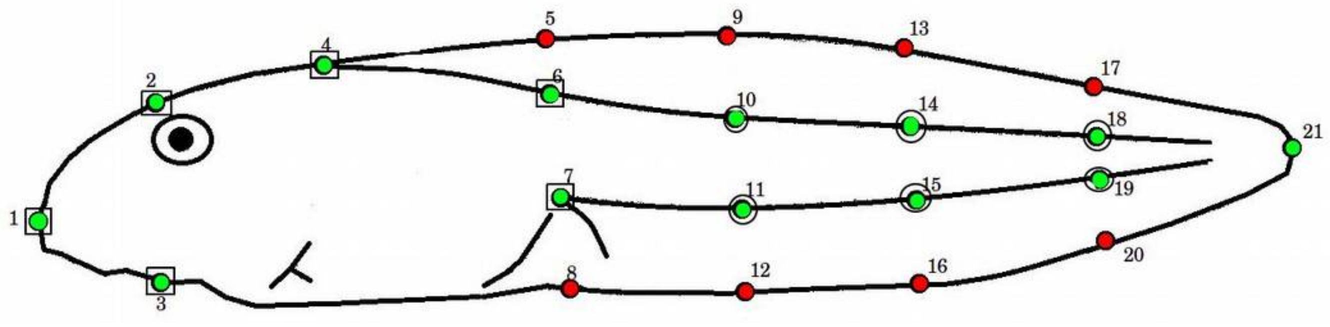


Figure S4. Landmarks (numbers 1, 4, 7 and 21) and semilandmarks (the rest) used in the study. Semilandmarks in red were discarded before superimposing individuals to obtain centroid size. All landmarks and semilandmarks were used when superimposing individuals to study morphological changes. Squared landmarks and semilandmarks correspond to module 1 (head), circled landmarks and semilandmarks correspond to module 2 (tail musculature), and the rest correspond to module 3 (tail fin). Definitions of landmarks and information on modules below.



Landmark definitions:

- Landmark 1 - Most anterior point of the headbody, tip of the snout.
- Semilandmarks 2 & 3- Respectively, dorsal and ventral points of the headbody at one quarter of the distance between landmarks 1 and 7
- Landmark 4 - Intersection between the headbody and the dorsal edge of the tail fin.
- Landmark 7 - Intersection between the headbody and the lower edge of the tail muscle
- Semilandmarks 5, 6 & 8 - Respectively, dorsal side of the tail fin, dorsal side of the the tail musculature and ventral side of the tail fin at the same vertical line as of landmark 7, being “horizontal” relative to landmark 21.
- Semilandmarks 8 to 12 - Respectively, dorsal side of the tail fin, dorsal side of the the tail musculature, ventral side of the tail musculature and ventral side of the tail fin at half the distance between landmarks 7 and 21.
- Semilandmarks 13 to 16 - Respectively, dorsal side of the tail fin, dorsal side of the the tail musculature, ventral side of the tail musculature and ventral side of the tail fin at half the distance between landmarks 7 and 21.
- Semilandmarks 17 to 20 - Respectively, dorsal side of the tail fin, dorsal side of the the tail musculature, ventral side of the tail musculature and ventral side of the tail fin at three quarters of the distance between landmarks 7 and 21.
- Landmark 21 - Tip of the tail fin.

Module descriptions:

→ Module 1: “head” module with visceral, sensorial functions with high energetic and biological value.

→ Module 2: tail musculature module with locomotive function, with middle energetic value but low biological value.

→ Module 3: tail fin module with hydrodynamic and defensive function, and very low energetic and biological value.

Figure S5. Disparity – as mean distance to centroid - of centroid size and development reached at the end of the experiment for each treatment, location and species, +/- its 95% bootstrapped confidence intervals.

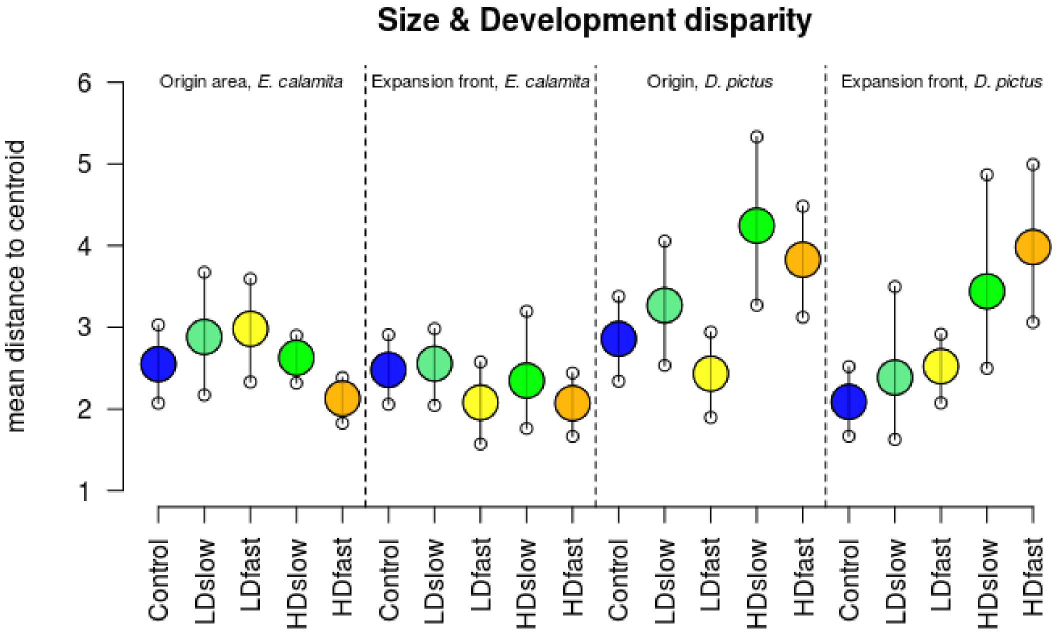


Figure S6. Allometric trajectories from the several treatments of *Epidalea calamita*. Line represents the predicted values for each treatment. Squares: expansion front; Circles: origin area; Deep blue: Control treatment; Light blue-green: LDslow; Green: HDslow; Yellow: LDfast; Orange: HDfast.

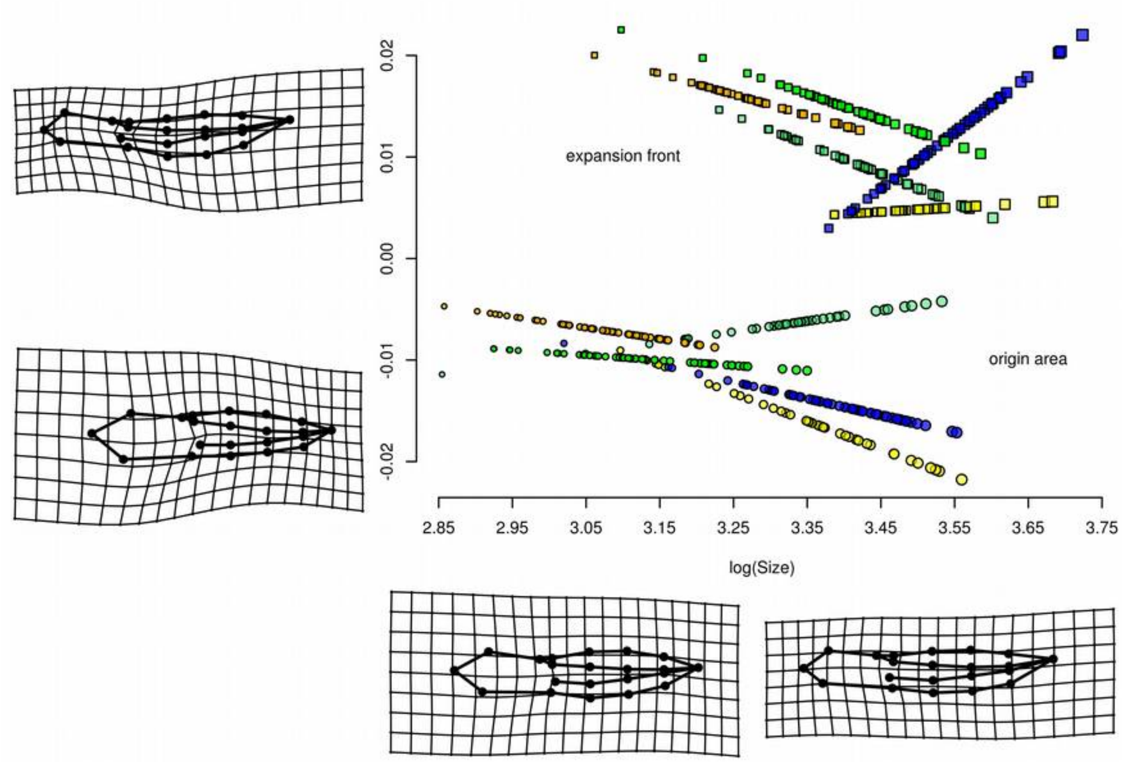


Figure S7. Disparity in tadpole body shape – expressed as 10^3 times the size-corrected Procrustes variance, to ease visualization – for all treatments of *Epidalea calamita.*

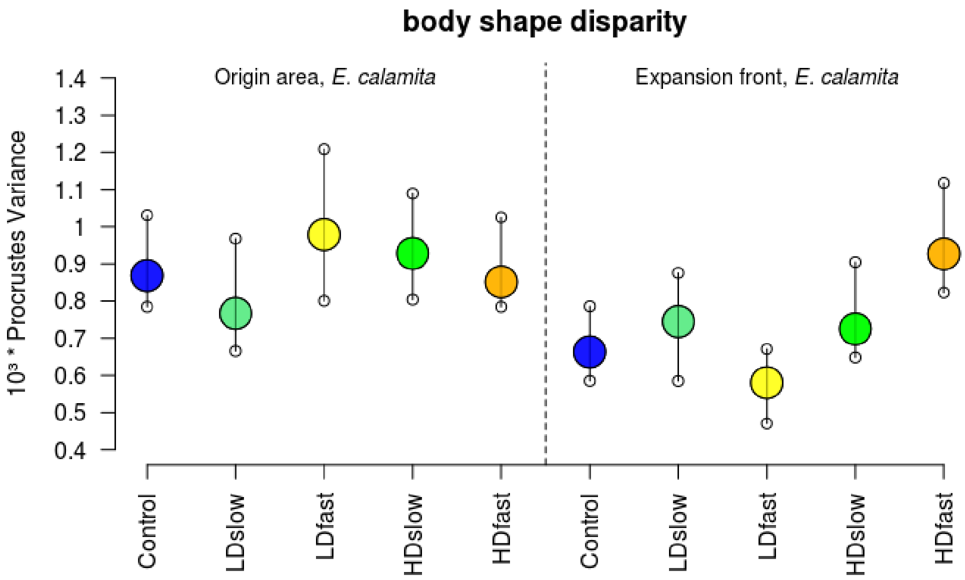


Figure S8. Disparity in tadpole body shape – expressed as 10^3 times the size-corrected Procrustes variance, to ease visualization – for all treatments of *Discoglossus pictus.*

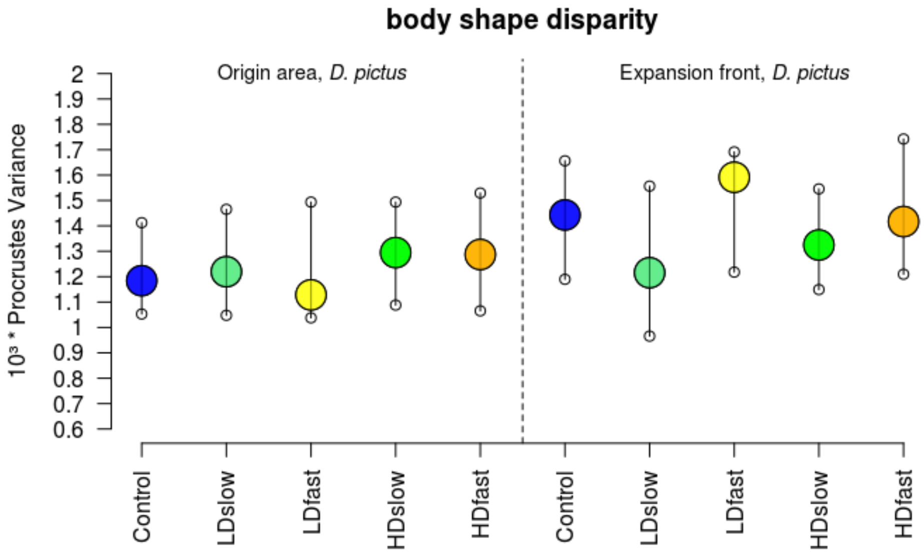


Figure S9. Degree of modularity in *Epidalea calamita* – as the value of the covariance ratio, CR - according to treatment and location, +/- bootstrapped 95% confidence intervals.

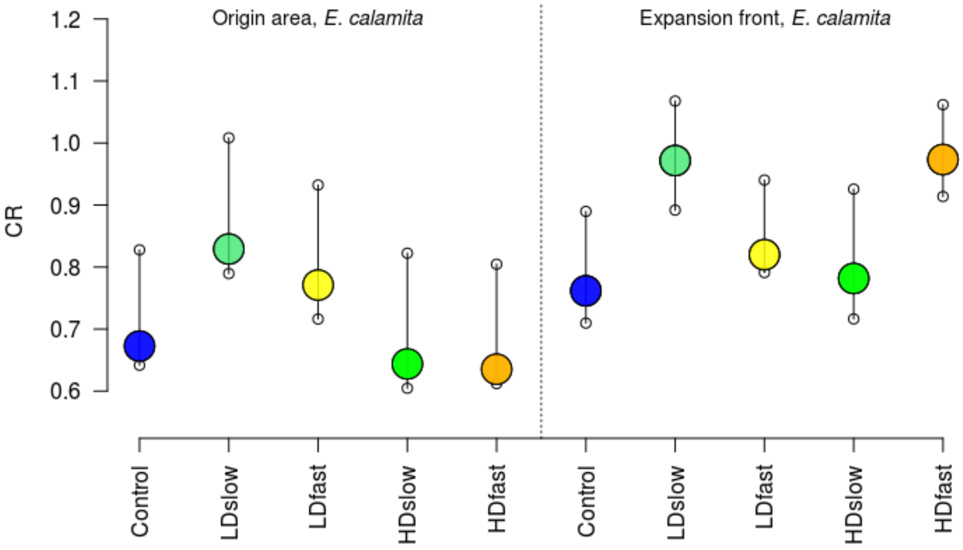
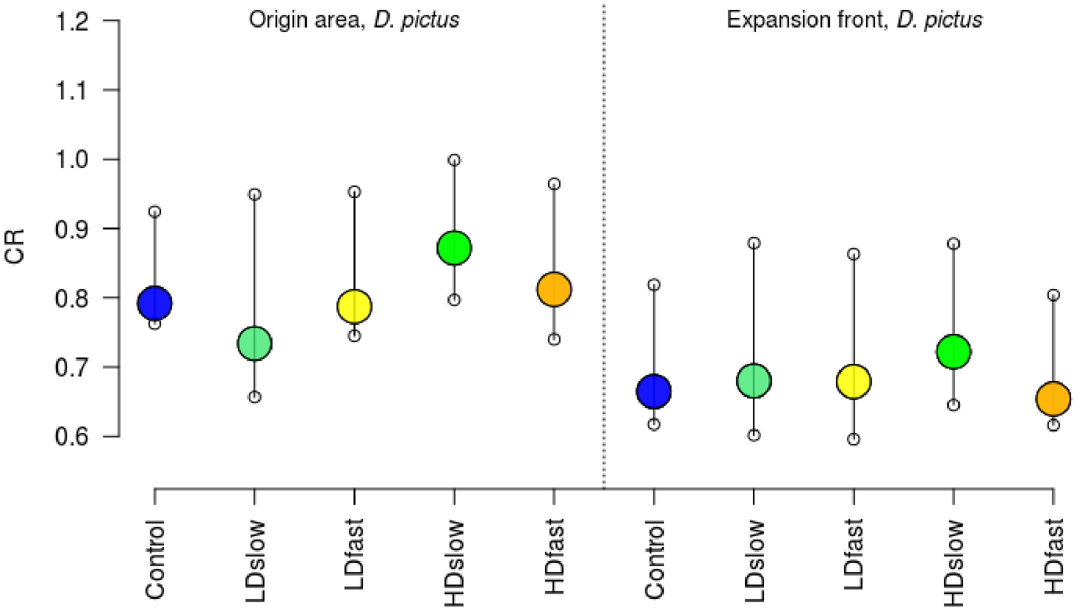


Figure S10. Degree of modularity in *Discoglossus pictus* – as the value of the covariance ratio, CR - according to treatment and location, +/- bootstrapped 95% confidence intervals.



Supplementary Information for Pujol-Buxó *et al.* 2017: Fast evolutionary responses in a native-invasive competitive system after several generations of coexistence

Supplementary Statistical Analyses and R Scripts.

Preliminary analysis: comparison among population controls.

Statistical analyses: morphological comparison among population controls

After procrustes superimposition, standardization of the tail position of the tadpole, and shape standardization for Gosner stage (see article), we first performed a preliminary analysis only among control treatments of the two sampling localities. This was done to investigate to what extent results from these populations are directly comparable, or whether there are some morphological differences between them due to external factors not related to the experimental procedures (e.g. local adaptation), that prevent direct comparison among the results of the several treatments. These consisted, for each species, in (1) a t-test for size differences, (2) a t-test for gosner stage differences, (3) randomization ANOVAs to test differences in mean tadpole body shape, and (4) randomization ANOVAs to test differences in body shape allometry. Thus, all four tests were run for the pair of control treatments of both species.

Results and Discussion: morphological comparison among population controls

Control treatments of both species displayed several interpopulation differences, be them in mean size (*D. pictus*: $F_{1,112}$=9.99, p=0.002; *E. calamita*: $F_{1,116}$=91.6, p<0.001), mean gosner stage (*D. pictus*: $F_{1,112}$=2.08, p=0.152; *E. calamita*: $F_{1,116}$=54.3, p<0.001), mean shape (*D. pictus*: $F_{1,110}$=19.6, p<0.001; *E. calamita*: $F_{1,114}$=18.1, p<0.001), or allometry (*D. pictus*: $F_{1,110}$=1.88, p=0.083; *E. calamita*: $F_{1,114}$=2.42, p=0.012). Thus, since we found that, for both species, the origin area and expansion front populations had significant morphological differences, we had to discard most inter-population comparisons and we always took in consideration each population's control to interpret treatment results.

Script S1: Permutation pairwise test (loosely based on Turner *et al.* 2010) for differences in mean size and development of each treatment:

```
PHpermMultiV <- function(Y, xfactor, nperms=999) {
  # Y is a matrix of cases x continuous variables
  # xfactor is a factor for this cases
  # nperms is the number of permutation desired.

  meansfactor <- tapply(X = Y, INDEX = xfactor, FUN = mean)
  realdists <- dist(meansfactor, method="euclidean", diag=T)
  ### real combination of values and distances.

  ### and now:
  count.p <- ceiling(realdists/1000)
  # create matrix of counters of significant cases. Function will add 1
  # to each counter each time condition for non-significance in that case is met.

  ### loop start
  for (i in 1:nperms) {
    shuffled <- Y[sample(1:nrow(Y)),]
    meansshuf <- aggregate(shuffled ~ xfactor, FUN = mean)
    shufdists <- dist(meansshuf[,-1], method="euclidean", diag=T)
    count.p <- count.p + ceiling((shufdists-realdists)/1000)
  }
  ### loop end
  ### we have shuffled all rows of data nperms times.
  ### each time and for each pairwise case, if real distance is
  ### smaller than distance by chance (random) we add
  ### a "non-signficance 1" to counter.

  ### convert counter into pvalues
  pvals <- count.p/(nperms+1)

  # clean up:
  pvalsfull <- as.data.frame(as.matrix(pvals))
  diag(pvalsfull) <- 1
  rownames(pvalsfull) <- levels(xfactor)
  colnames(pvalsfull) <- levels(xfactor)

  # output is a pairwise table of pvalues:
  pvalsfull}
```

Script S2: Permutation pairwise test for bivariate disparity – as distance to group centroid - in size and development of each treatment:

```
PHmultivar <- function(Y, xfactor, nperms=999) {
  # Y is a matrix of bivariate continuous data
  # xfactor is a factor
  # nperms is the number of permutations for the loop
  # keep bootstrap95 to F to increase speed.

  dadCC <- Y[complete.cases(Y),]
  tlsCC <- tls[complete.cases(Y)] # cleaning from incomplete cases
  meandists <- numeric(nlevels(tlsCC))
  names(meandists) <- levels(tlsCC)

  for (i in levels(tlsCC)) {
    datagroups <- dadCC[tlsCC==i,]
    lsm <- apply(2, X=datagroups, FUN=mean)
    distances <- apply(MARGIN=1, X=datagroups,
                   FUN= function(x) {sqrt((x[1]-lsm[1])^2 + (x[2]-lsm[2])^2)}) #calculate linear distance
    meandists[i] <- mean(distances)}
    # create numeric vector of the real mean distances to centroid.

  realdists <- dist(meandists, method="euclidean", diag=T)
  # create matrix of counters of significant cases. Function will add 1
  # to each counter each time condition for non-significance in that case is met.
  count.p <- ceiling(realdists/1000) # start counter from 1.

  # start loop:
  for (p in 1:nperms) {
    dadCCshuf <- dadCC[sample(1:nrow(dadCC)),]
    meandistsSH <- numeric(nlevels(xfactor))
    names(meandistsSH) <- levels(tlsCC)
    # shuffle data
    for (i in levels(tlsCC)) {
      datagroups <- dadCCshuf[tlsCC==i,]
      lsmSH <- apply(2, X=datagroups, FUN=mean)
      distancesSH <- apply(MARGIN=1, X=datagroups,
                     FUN= function(x) {sqrt((x[1]-lsm[1])^2 + (x[2]-lsm[2])^2)})
      meandistsSH[i] <- mean(distancesSH)}
    # calculate distances for each group, from randomized data
    shufdists <- dist(meandistsSH, method="euclidean", diag=T)
    count.p <- count.p + ceiling((shufdists-realdists)/1000)
    # if real distance is lesser than distance expected by chance
    # we add a non-significance 1 to corresponding counter
  } # loop end

  pvals <- count.p/(nperms+1) # convert counter matrix into p-values matrix
  pvalsfull <- as.matrix(pvals)
  diag(pvalsfull) <- 1
  #
  output <- setNames(list(pvalsfull,meandists),nm=c("pvalues","realdisparity"))}
  output} # output: real disparities and pairwise pvalues.
# end
```

Script S3: Permutation test for differences between populations in the variability of allometries:

```
library(geomorph) # linear models functions taken from geomorph.

# we previously need:
COORDS # three-dimensional array of superimposed landmark coordinates
Csizes # vector of sizes of the specimens
loc    # factor: location of specimens
treat  # factor: treatment of speciments

# to ease posterior analyses, save two formulas:
fullform <- formula(COORDS ~ log(Csizes) * loc * treat)     # full model
alternate <- update(fullform, . ~ . -treat:loc:log(Csizes)) # /wo three-way interaction

# run analysis simply to get description of each allometry
TESTcs1 <- advanced.procD.lm(f1 = fullform, seed="random",
                             f2 = alternate,
                             slope = ~ log(Csizes),
                             angle.type = "deg",
                             groups =  ~ loc*treat,
                             iter=1)
allodata <- cbind(TESTcs1$slopes,TESTcs1$slope.lengths) # create matrix: all slopes + slope lengths

# test statistic: difference between populations of mean distances among allometries:
mean(expansdistallo) - mean(origindistsallo)

### since there are a lot of possible allometries, to represent the null hypothesis
### we create a great number of random allometries by randomizing all
### individuals (morphologies and sizes together) to the several treatments and locations

nperms <- 999 # number of permutations for test. Careful: slow function!
#
alldiffs <- numeric(nperms+1) # create vector to keep results
diffreal <- mean(expansdistallo) - mean(origindistsallo)
alldiffs[1] <- diffreal
# real result is case [1]

pcount <- 1
# create counter of signif. cases. Function will add 1
# to counter each time condition for non-significance is met.

# keep formulas to ease analysis later:
Rfullform <- formula(Rcoords ~ log(Rcsize) * loc * treat)    # full model
Ralternate <- update(fullform, . ~ . -treat:loc:log(Rcsize)) # /wo three-way interaction

# start loop:
for (i in 1:nperms) {
  neworder <- sample(1:dim(COORDS)[3])                  # start to randomize individuals
  Rcoords <- COORDS[,,neworder]                         # randomize morphol info of indiv
  dimnames(Rcoords) <- dimnames(COORDS)                 # put same names
  Rcsize <- Csizes[neworder]                            # randomize (same order) sizes.
  names(Rcsize) <- names(Csizes)                        # end to randomize individuals
  # this way, each size and morphology are still linked, the linkage is only lost with
  # the treatment at which the individual belonged
```

```
  # run analysis simply to get description of each allometry
  Rtest <- advanced.procD.lm(f1 = fullform, f2 = alternate, seed="random",
                             slope = ~ log(Rcsize), print.progress = F,
                             angle.type = "deg",
                             groups =  ~ loc*treat,
                             iter=1)
  Rallodata <- cbind(Rtest$slopes,Rtest$slope.lengths) # obtatin random allometries
  # create matrix: all random slopes + random slope lengths
  #
    Rorigindistsallo <- dist(Rallodata$x[1:5,],  diag=F, upper=F) # "random" dists among origin area
allometries
   Rexpansdistallo  <- dist(Rallodata$x[6:10,], diag=F, upper=F) # "random" dists among expansion front
allometries
  diffR <- mean(Rexpansdistallo) - mean(Rorigindistsallo)   # "random" result of statistic

  alldiffs[i+1] <- diffR                                # keep result for posterior visulization
  if(diffR>diffreal) {pcount <- pcount+1}     # if  expected by chance distance is greater than the real,
                                            # add a non-significance 1 to counter.
}

# end loop

# convert counter to pvalue by divide by total iterations available
pvalue <- pcount/(nperms+1)

# visualize pvalue graphically.
hist(freq=F, alldiffs, breaks=30, col="grey", main="")
abline(v=alldiffs[1], lty=2, col="red")
text(x=alldiffs[1],y=60,pos = 4,paste("p = ",pvalue), col="red")

## end
```

Script S4: Permutation pairwise test for differences in the degree of modularity among treatments:

```
library(geomorph)
# function to test modularity is from geomorph
# plus other useful functions too.
?modularity.test

CRcomparison <- function(A, group, modules, nperms) {

  initialtime <- Sys.time() # the function is slow, optionally
  # do the test with few permutations and see how much time it takes
  # using a timer at the begining and the end.

  partition <- coords.subset(A=A, group=group) # subsetting data by group

  modtest <- lapply(partition,
                    function(x) {modularity.test(x, modules, iter=1,
                                  seed="random", print.progress=F)})
  # run modularity test with one iteration, just to obtain CR values

  CRreal <- numeric(length(modtest))
  names(CRreal) <- levels(group)
  for (i in 1:length(modtest)) {CRreal[i] <- modtest[[i]]$CR}
  # keep real CR values

  distreal <- dist(CRreal) # calculate real distances among CR values

  pcount <- distreal
  pcount[1:length(distreal)] <- 1
  # create a "pvalue counter" that will increase in number
  # each time the condition is met

  print("real distances calculated")
  now <- Sys.time()
  timeelapsed <- now - initialtime
  print(timeelapsed)
  # this last four lines just to let know the user that function is working

  # start loop
  for (j in 1:nperms) {
    #
    nspecs <- dim(A)[3]
    Ar <- A[,,sample(1:nspecs)]
    Rpartition <- coords.subset(A=Ar, group=group)
    # randomize individuals
    #
    modtestr <- lapply(Rpartition,
                       function(x) {modularity.test(x,modules,iter=1,
                                    seed="random",print.progress=F)})
    # again, run modularity test with only one iteration, just to obtain CR values
    CRrand <- numeric(length(modtestr))
    names(CRrand) <- levels(group)
    for (k in 1:length(modtestr)) {CRrand[k] <- modtestr[[k]]$CR}
    # keep results from randomization
```

```
    #
    distrand <- dist(CRrand) # calculate distances from random results
    #
    for (h in 1:length(pcount)) {pcount[h] <- ifelse(distreal[h]>distrand[h],
                                    pcount[h] <- pcount[h]+1,
                                    pcount[h])}
    # add 1 to "pvalue" counter if condition is met.
    # in this case, if real distances between pair of treatments is greater
    # than expected by chance (random distances) p-value increases.
    #
    now <- Sys.time()
    timeelapsed <- now - initialtime
    print(paste(j,"/",nperms,"permutations done"))
    print(timeelapsed)
    # this last four lines just to let know the user that function is working
  }
  #
  #end of loop

  ptable <- as.matrix(pcount)
  colnames(ptable) <- rownames(ptable) <- levels(group)
  # organize table of counter values
  #
  ptable <- ptable/(nperms+1)
  # convert to p-values by dividing for the total number of results
  diag(ptable) <- 1
  ptable <- round(ptable,4) # clean up
  ptable} # output: table of pvalues of differences in the degree of modularity
        # among all pairs of treatments

# end
```

Script S5: Bootstrap to obtain +/- 95% Confidence Intervals on a measure.

```
bootstrappingCIs <- function(X, INDEX, FUN, niter=1000) {

  data <- data.frame(X,INDEX)
  data <- data[order(INDEX),]
  # create ordered data matrix

  Mdata <- data$X
  index <- data$INDEX
  # extract ordered data and index

  bootmat <- matrix(ncol= length(unique(index)), nrow= niter)
  #create matrix for all results of each iteration

  measures <- tapply(X = Mdata, INDEX = index, FUN = FUN)
  bootmat[1,] <- measures #assign real values to first row
  colnames(bootmat) <- names(measures)

  # loop start
  for (i in 2:niter) {
    # randomization with replacement, intra-group:
    Onums <- tapply(X=     1:length(Mdata),
                    INDEX= index,
                    FUN =  function(x) sample(x,replace=T))
    Nums  <- unlist(Onums, use.names=F)
    randdata <- Mdata[Nums]
    # reordering array according to random order
    #
    # repeat obtention of measure, with "randomized" data:
    Rmeasures <- tapply(X=randdata, INDEX=index, FUN = FUN)
    bootmat[i,] <- Rmeasures
    #assign to corresponding row or matrix of results
  } # loop end

  # now we have a matrix of ntreatments x niterations
  # that contains all results
  ordered <- apply(bootmat,2,sort) # ordering each column
  # "ordered" now is the same matrix but with results
  # from small to large values

  CIs <- matrix(ncol = ncol(bootmat), nrow=3)
  colnames(CIs) <- colnames(bootmat)
  rownames(CIs) <- c("CIinf","value","CIsup")
  # create matrix to include results for plotting

  # fill the matrix:
  CIs[1,] <- ordenat[0.025*niter,] # first row, inferior limit of 95% CI
  CIs[2,] <- bootmat[1,]           # second row, real value of measure
  CIs[3,] <- ordenat[0.975*niter,] # third row, superior limit of 95% CI

  # output: bootstrap matrix and results
  setNames(list(bootmat,CIs),c("boots","CIs95"))}
#end
```

Supplementary tables for Pujol-Buxó *et al.* (2017):

Fast evolutionary responses in a native-invasive competitive system after several generations of coexistence

Number of supplementary tables: 16

Survival

Table S1. Model selection table for survival, using AICc for ranking. The presence of "+" means the factor is present in the model, the absence of "+" means the factor is absent from the model. Abbreviations: loc: location; sp: species; trt: treatment; df: degrees of freedom of the model; logLik: log likelihood of the model; delta: difference in the value of the ranking method among model and best model.

loc	sp	loc:sp	trt	loc:trt	sp:trt	df	logLik	AICc	delta
						1	-54.412	110.9	0
+	+	+				4	-51.338	111.2	0.33
			+			5	-50.488	111.8	0.91
+						2	-53.9	112	1.08
	+					2	-54.228	112.6	1.74
+	+	+	+			8	-47.402	112.8	1.96
+			+			6	-49.975	113.1	2.23
+	+					3	-53.717	113.7	2.88
	+		+			6	-50.304	113.8	2.88
+	+		+			7	-49.791	115.1	4.26
+			+	+		10	-47.199	117.6	6.71
+	+	+	+	+		12	-44.618	117.9	7.02
+	+	+	+		+	12	-45.537	119.7	8.86
+	+		+	+		11	-47.014	119.9	9.04
	+		+		+	10	-48.443	120.1	9.2
+	+		+		+	11	-47.929	121.7	10.87

Table S2. Tests on single term deletions for three levels of complexity of survival models, showing the corresponding Akaike Information Criterion (AIC) and likelihood ratio tests (LRT, p). Abbreviations: loc: location; sp: species; treat: treatment; Df: degrees of freedom of the model.

```
Model: survival ~ sp + loc + treat + sp:loc + sp:treat + loc:treat + sp:loc:treat
term deletion  Df    AIC     LRT      p
<none>               117.62
sp:loc:treat    4   117.79   8.162    0.08582
----
Model: survival ~ sp + loc + treat + sp:loc + sp:treat + loc:treat
term deletion  Df    AIC     LRT      p
<none>               117.79
sp:loc          1   120.29   4.5064   0.03377 *
sp:treat        4   113.23   3.4482   0.48580
loc:treat       4   115.07   5.2864   0.25915
----
Model: survival ~ sp + loc + treat
term deletion  Df    AIC      LRT     p
<none>               113.58
sp              1   111.95   0.3683   0.54393
loc             1    12.61   1.0269   0.31088
treat           4   113.43   7.8527   0.09712
```

Activity

Table S3. Model selection table for the activity of tadpoles. We only present the models that were among the ten best according to both the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC). Values for each model of both ranking values are presented in the table. The presence of “+” means the variable is present in the model, the absence of “+” means the variable is absent from the model. Abbreviations: *Nmod*: number of the model; *loc*: location; *age*: age of tadpoles, in days; *sp*: species; *trt*: treatment; *df*: degrees of freedom of the model; *logLik*: log likelihood of the model; Δ: difference in the value of the ranking method among model and best model.

Nmod	loc	age	sp	trt	loc:age	loc:sp	loc:trt	age:sp	age:trt	sp:trt	df	logLik	AIC	AIC Δ	BIC	BIC Δ
512	+	+	+	+	+	+	+	+	+		20	-2290.7	4621.5	0	4723.9	2.36
1024	+	+	+	+	+	+	+	+	+	+	24	-2287.3	4622.7	1.24	4745.7	24.09
496	+	+	+	+		+	+	+	+		19	-2293.1	4624.2	2.75	4721.6	0
1008	+	+	+	+		+	+	+	+	+	23	-2289.7	4625.4	3.91	4743.2	21.64
384	+	+	+	+	+	+	+		+		19	-2304.9	4647.8	26.36	4745.2	23.6
448	+	+	+	+	+	+		+	+		16	-2308.8	4649.6	28.14	4731.6	10.01
368	+	+	+	+		+	+		+		18	-2307.4	4650.9	29.41	4743.1	21.53
432	+	+	+	+		+		+	+		15	-2311	4652	30.5	4728.8	7.25

Table S4. Results of z wald tests applied on the fitted model 496 (from the superior table):

	Estimate	**Std. Error**	**Z value**	**Pr(>|z|)**
loc(Expansion)	1.8742	0.1244	15.05	< 0.0001
age	-0.4508	0.0529	-8.516	< 0.0001
species(Dpi)	0.3094	0.0764	4.045	< 0.0001
treat(LDslow)	0.6486	0.1483	4.373	< 0.0001
treat(HDslow)	0.0441	0.1339	0.329	0.7422
treat(LDfast)	0.0938	0.1645	0.57	0.5688
treat(HDfast)	-0.0518	0.1356	-0.382	0.7028
loc(Expansion):species(Dpi)	0.9188	0.0887	10.34	< 0.0001
loc(Expansion):treat(LDslow)	-1.0954	0.1844	-5.939	< 0.0001
loc(Expansion):treat(HDslow)	-0.0016	0.1638	-0.01	0.9923
loc(Expansion):treat(LDfast)	-0.349	0.1965	-1.775	0.0758
loc(Expansion):treat(HDfast)	0.0189	0.165	0.115	0.9088
age:species(Dpi)	-0.2229	0.0416	-5.355	< 0.0001
age:treat(LDslow)	0.2161	0.0778	2.774	0.0055
age:treat(HDslow)	0.5385	0.0606	8.872	< 0.0001
age:treat(LDfast)	0.1597	0.0822	1.942	0.0522
age:treat(HDfast)	0.4094	0.0612	6.683	< 0.0001

Table S5. Results of NP-R-ANOVA on activity standardized relative to the control treatment:

	Df	**SS**	**MSS**	**F**	**p-value**	
sp	1	0.157	0.15704	10.37	0.005	**
loc	1	1.2129	1.21285	80.1	<0.0001	***
sp:loc	1	0.0184	0.01839	1.214	0.0789	
treat	3	0.064	0.02135	1.41	0.1549	
sp:treat	3	0.0203	0.00677	0.447	0.8156	
loc:treat	3	0.6023	0.20078	13.26	<0.0001	***
sp:loc:treat	3	0.0114	0.00381	0.252	0.8601	
Residuals	976	14.776	0.01514			

<u>Growth and development</u>

Table S6. Results of the MANOVA on growth and development:

	Df	**Pillai**	**approx F**	**Df**	**Pr(>F)**	
(Intercept)	1	0.36405	265.905	2	0.001	***
sp	1	0.15129	82.8	2	0.001	***
loc	1	0.07811	39.357	2	0.001	***
treat	4	0.47689	72.796	8	0.001	***
sp:loc	1	0.06444	31.996	2	0.001	***
sp:treat	4	0.14355	17.979	8	0.001	***
loc:treat	4	0.1313	16.336	8	0.001	***
sp:loc:treat	4	0.07098	8.555	8	0.001	***
Residuals	930					

Table S7. Pairwise comparisons in mean growth and development among treatments, species and location, for *Discoglossus pictus* (bottom triangle) and for *Epidalea calamita* (Top triangle):

		ORIGIN	ORIGIN	ORIGIN	ORIGIN	ORIGIN	EXPNSN	EXPNSN	EXPNSN	EXPNSN	EXPNSN
		HDslow	LDslow	Control	HDfast	LDfast	HDslow	LDslow	Control	HDfast	LDfast
ORIGIN	HDslow	-	0.001	0.001	0.057	0.001	0.033	0.008	0.001	0.061	0.001
ORIGIN	LDslow	0.001	-	0.093	0.001	0.485	0.002	0.037	0.095	0.001	0.772
ORIGIN	Control	0.001	0.082	-	0.001	0.501	0.001	0.002	1	0.001	0.256
ORIGIN	HDfast	0.002	0.001	0.001	-	0.001	0.001	0.001	0.001	0.948	0.001
ORIGIN	LDfast	0.001	0.017	0.096	0.001	-	0.001	0.001	0.495	0.001	0.866
EXPNSN	HDslow	0.001	0.319	0.041	0.001	0.003	-	0.509	0.001	0.001	0.001
EXPNSN	LDslow	0.001	0.158	0.328	0.001	0.015	0.323	-	0.001	0.001	0.009
EXPNSN	Control	0.001	0.067	1	0.001	0.109	0.038	0.336	-	0.001	0.28
EXPNSN	HDfast	0.001	0.009	0.018	0.001	0.002	0.054	0.21	0.015	-	0.001
EXPNSN	LDfast	0.001	0.007	0.003	0.001	0.043	0.001	0.001	0.003	0.001	-

Table S8. Pairwise comparisons in mean size and developmental stage among treatments, species and location, among *Discoglossus pictus* (D.pi) and *Epidalea calamita* (E.ca):

			E.ca	E.ca	E.ca	E.ca	E.ca	E.ca	E.ca	E.ca	E.ca	E.ca
			ORIGIN	ORIGIN	ORIGIN	ORIGIN	ORIGIN	EXPNSN	EXPNSN	EXPNSN	EXPNSN	EXPNSN
			HDslow	LDslow	Control	HDfast	LDfast	HDslow	LDslow	Control	HDfast	LDfast
D.pi	ORIGIN	HDslow	0.034	0.001	0.001	0.001	0.001	0.158	0.052	0.001	0.001	0.001
D.pi	ORIGIN	LDslow	0.001	0.005	0.07	0.001	0.025	0.001	0.001	0.072	0.001	0.007
D.pi	ORIGIN	Control	0.001	0.097	1	0.001	0.492	0.001	0.001	1	0.001	0.271
D.pi	ORIGIN	HDfast	0.063	0.001	0.001	0.909	0.001	0.001	0.001	0.001	0.915	0.001
D.pi	ORIGIN	LDfast	0.001	0.017	0.094	0.001	0.062	0.001	0.001	0.083	0.001	0.037
D.pi	EXPNSN	HDslow	0.001	0.005	0.034	0.001	0.014	0.001	0.001	0.04	0.001	0.015
D.pi	EXPNSN	LDslow	0.001	0.103	0.319	0.001	0.232	0.001	0.001	0.317	0.001	0.185
D.pi	EXPNSN	Control	0.001	0.117	1	0.001	0.499	0.001	0.001	1	0.001	0.271
D.pi	EXPNSN	HDfast	0.001	0.096	0.015	0.001	0.05	0.001	0.002	0.016	0.001	0.067
D.pi	EXPNSN	LDfast	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001

Table S9. Pairwise comparisons in disparity of size and development *Discoglossus pictus* (bottom triangle) and *Epidalea calamita* (top triangle):

		ORIGIN	ORIGIN	ORIGIN	ORIGIN	ORIGIN	EXP.	EXP.	EXP.	EXP.	EXP.
		HDslow	LDslow	Control	HDfast	LDfast	HDslow	LDslow	Control	HDfast	LDfast
ORIGIN	HDslow	-	0.758	0.914	0.47	0.643	0.704	0.933	0.834	0.439	0.482
ORIGIN	LDslow	0.22	-	0.65	0.349	0.89	0.49	0.695	0.62	0.329	0.338
ORIGIN	Control	0.062	0.6	-	0.553	0.555	0.76	0.995	0.921	0.501	0.541
ORIGIN	HDfast	0.552	0.476	0.147	-	0.3	0.753	0.562	0.581	0.937	0.948
ORIGIN	LDfast	0.044	0.37	0.639	0.104	-	0.428	0.597	0.522	0.278	0.285
EXP.	HDslow	0.28	0.833	0.393	0.58	0.255	-	0.795	0.853	0.712	0.732
EXP.	LDslow	0.021	0.319	0.535	0.06	0.958	0.19	-	0.907	0.534	0.548
EXP.	Control	0.003	0.142	0.265	0.016	0.698	0.069	0.685	-	0.574	0.599
EXP.	HDfast	0.735	0.383	0.098	0.818	0.079	0.463	0.039	0.011	-	0.989
EXP.	LDfast	0.037	0.357	0.676	0.089	0.919	0.245	0.872	0.603	0.063	-

Morphology

Table S10. P-values for pairwise differences among mean morphologies (disregarding size effects) of the several treatments and locations of invasive *Discoglossus pictus* (bottom triangle) and *Epidalea calamita* (top triangle):

		ORIGIN	ORIGIN	ORIGIN	ORIGIN	ORIGIN	EXP.	EXP.	EXP.	EXP.	EXP.
		HDslow	LDslow	Control	HDfast	LDfast	HDslow	LDslow	Control	HDfast	LDfast
ORIGIN	HDslow	-	0.062	0.002	0.003	0.07	0.02	0.757	0.14	0.244	0.411
ORIGIN	LDslow	0.003	-	0.001	0.013	0.018	0.426	0.742	0.734	0.873	0.836
ORIGIN	Control	0.034	0.062	-	0.073	0.983	0.158	0.632	0.068	0.258	0.392
ORIGIN	HDfast	0.337	0.003	0.002	-	0.577	0.302	0.841	0.151	0.251	0.604
ORIGIN	LDfast	0.317	0.673	0.857	0.128	-	0.556	0.912	0.366	0.599	0.741
EXP.	HDslow	0.427	0.854	0.453	0.307	0.188	-	0.013	0.309	0.646	0.35
EXP.	LDslow	0.936	0.597	0.826	0.974	0.64	0.926	-	0.683	0.1	0.947
EXP.	Control	0.4	0.086	0.04	0.547	0.022	0.111	0.962	-	0.855	0.449
EXP.	HDfast	0.77	0.892	0.508	0.458	0.356	0.86	0.989	0.597	-	0.357
EXP.	LDfast	0.237	0.146	0.031	0.117	0.004	0.042	0.123	0.312	0.147	-

Table S11. NP-R-ANOVA on body shape of Epidalea calamita:

	Df	SS	MS	Rsq	F	Z	p-value	
log(CSMORF)	1	0.0113	0.0113	0.02716	16.4991	10.5889	0.001	**
treat	4	0.0282	0.007	0.068	10.3251	7.8854	0.001	**
log(CSMORF):treat	4	0.0082	0.002	0.0197	2.9916	2.5125	0.001	**
loc	1	0.0346	0.0346	0.0832	50.5349	24.7605	0.001	**
log(CSMORF):loc	1	0.0015	0.0015	0.00377	2.2925	2.0277	0.024	*
treat:loc	4	0.0104	0.0026	0.02512	3.8141	3.6043	0.001	**
log(CSMORF):treat:loc	4	0.0044	0.0011	0.01066	1.619	1.6155	0.016	*
Residuals	463	0.3171	0.0006					
Total	482	0.416						

Table S12. NP-R-ANOVA on body shape of Discoglossus pictus:

	Df	SS	MS	Rsq	F	Z	p-value	
log(CSMORF)	1	0.0139	0.0139	0.02222	12.6919	8.3159	0.001	**
treat	4	0.0226	0.0056	0.03603	5.1447	4.0926	0.001	**
log(CSMORF):treat	4	0.0139	0.0034	0.02217	3.1666	2.5461	0.001	**
loc	1	0.0587	0.0587	0.09351	53.4109	24.7223	0.001	**
log(CSMORF):loc	1	0.0075	0.0075	0.01205	6.8838	5.8054	0.001	**
treat:loc	4	0.0164	0.0041	0.02625	3.7485	3.4801	0.001	**
log(CSMORF):treat:loc	4	0.0087	0.0021	0.01389	1.984	1.9312	0.004	**
Residuals	442	0.4859	0.001					
Total	461	0.6279						

Table S13. P-values for differences in the allometric slopes length (bottom triangle) and differences in the allometric slopes angle (top triangle) for *Epidalea calamita*:

		ORIGIN	ORIGIN	ORIGIN	ORIGIN	ORIGIN	EXP.	EXP.	EXP.	EXP.	EXP.
		HDslow	LDslow	Control	HDfast	LDfast	HDslow	LDslow	Control	HDfast	LDfast
ORIGIN	HDslow	-	0.311	0.372	0.476	0.406	0.07	0.324	0.738	0.032	0.073
ORIGIN	LDslow	0.234	-	0.069	0.222	0.071	0.308	0.235	0.839	0.22	0.305
ORIGIN	Control	0.451	0.1	-	0.509	0.809	0.859	0.985	0.17	0.714	0.784
ORIGIN	HDfast	0.188	0.06	0.243	-	0.837	0.612	0.864	0.648	0.05	0.084
ORIGIN	LDfast	0.495	0.108	0.876	0.286	-	0.863	0.944	0.033	0.417	0.056
EXP.	HDslow	0.359	0.08	0.84	0.407	0.741	-	0.931	0.049	0.998	0.529
EXP.	LDslow	0.274	0.091	0.348	0.833	0.396	0.499	-	0.026	0.919	0.303
EXP.	Control	0.395	0.149	0.507	0.766	0.528	0.624	0.881	-	0.07	0.477
EXP.	HDfast	0.81	0.451	0.757	0.124	0.874	0.666	0.206	0.294	-	0.659
EXP.	LDfast	0.472	0.244	0.571	0.955	0.593	0.663	0.914	0.838	0.319	-

Table S14. P-value for differences in the allometric slopes length (bottom triangle) and differences in the allometric slopes angle (top triangle) for *Discoglossus pictus*:

		ORIGIN	ORIGIN	ORIGIN	ORIGIN	ORIGIN	EXP.	EXP.	EXP.	EXP.	EXP.
		HDslow	LDslow	Control	HDfast	LDfast	HDslow	LDslow	Control	HDfast	LDfast
ORIGIN	HDslow	-	0.121	0.05	0.286	0.008	0.04	0.232	0.393	0.031	0.854
ORIGIN	LDslow	0.194	-	0.799	0.55	0.748	0.912	0.81	0.435	0.689	0.482
ORIGIN	Control	0.597	0.214	-	0.67	0.673	0.961	0.715	0.197	0.618	0.654
ORIGIN	HDfast	0.736	0.088	0.703	-	0.128	0.732	0.595	0.581	0.2	0.745
ORIGIN	LDfast	0.335	0.07	0.234	0.294	-	0.903	0.967	0.265	0.973	0.206
EXP.	HDslow	0.61	0.334	0.676	0.534	0.285	-	0.885	0.509	0.777	0.612
EXP.	LDslow	0.235	0.998	0.256	0.13	0.086	0.424	-	0.424	0.993	0.196
EXP.	Control	0.165	0.787	0.172	0.064	0.062	0.272	0.829	-	0.171	0.225
EXP.	HDfast	0.274	0.898	0.288	0.14	0.106	0.369	0.916	0.686	-	0.194
EXP.	LDfast	0.333	0.681	0.75	0.52	0.076	0.978	0.678	0.602	0.771	-

Table S15. P-values for differences in the amount of disparity of tadpole body shape – previously corrected by size - for *Discoglossus pictus* (bottom triangnle) and *E. calamita* (top triangle):

		ORIGIN	ORIGIN	ORIGIN	ORIGIN	ORIGIN	EXP.	EXP.	EXP.	EXP.	EXP.
		HDslow	LDslow	Control	HDfast	LDfast	HDslow	LDslow	Control	HDfast	LDfast
ORIGIN	HDslow	-	0.095	0.488	0.374	0.592	0.027	0.062	0.003	0.988	0.001
ORIGIN	LDslow	0.673	-	0.298	0.404	0.053	0.704	0.861	0.331	0.15	0.089
ORIGIN	Control	0.448	0.847	-	0.844	0.237	0.118	0.208	0.024	0.536	0.005
ORIGIN	HDfast	0.963	0.694	0.529	-	0.209	0.19	0.292	0.033	0.463	0.014
ORIGIN	LDfast	0.376	0.674	0.788	0.413	-	0.02	0.036	0.003	0.606	0.002
EXP.	HDslow	0.849	0.565	0.341	0.798	0.311	-	0.835	0.508	0.043	0.168
EXP.	LDslow	0.674	0.985	0.849	0.683	0.654	0.506	-	0.372	0.095	0.139
EXP.	Control	0.352	0.189	0.078	0.33	0.101	0.445	0.176	-	0.007	0.433
EXP.	HDfast	0.451	0.237	0.137	0.4	0.124	0.575	0.22	0.873	-	0.001
EXP.	LDfast	0.075	0.044	0.022	0.065	0.018	0.121	0.043	0.395	0.304	-

Table S16. P-values for differences in the degree of modularity – using the covariance ratio index, CR – among treatments for *Discoglossus pictus* (bottom triangle) and *Epidalea calamita* (top triangle):

		ORIGIN	ORIGIN	ORIGIN	ORIGIN	ORIGIN	EXP.	EXP.	EXP.	EXP.	EXP.
		HDslow	LDslow	Control	HDfast	LDfast	HDslow	LDslow	Control	HDfast	LDfast
ORIGIN	HDslow	-	0.035	0.697	0.93	0.163	0.112	0.001	0.143	0.001	0.05
ORIGIN	LDslow	0.147	-	0.086	0.034	0.532	0.629	0.104	0.457	0.116	0.91
ORIGIN	Control	0.353	0.54	-	0.667	0.262	0.175	0.001	0.251	0.001	0.071
ORIGIN	HDfast	0.481	0.383	0.797	-	0.149	0.072	0.001	0.128	0.002	0.029
ORIGIN	LDfast	0.409	0.617	0.959	0.819	-	0.9	0.015	0.922	0.031	0.601
EXP.	HDslow	0.096	0.904	0.374	0.301	0.524	-	0.027	0.798	0.03	0.677
EXP.	LDslow	0.036	0.598	0.226	0.154	0.28	0.668	-	0.017	0.987	0.088
EXP.	Control	0.018	0.462	0.131	0.08	0.217	0.492	0.875	-	0.017	0.518
EXP.	HDfast	0.011	0.397	0.108	0.063	0.198	0.43	0.796	0.894	-	0.096
EXP.	LDfast	0.054	0.597	0.239	0.173	0.299	0.669	0.996	0.887	0.803	-

PART B
Intraguild competition in tadpoles of *Discoglossus pictus.*

Chapter 6
Mild ecological segregation in the breeding preferences of an invasive anuran (*Discoglossus pictus)* and its native competitor (*Epidalea calamita*)

[under review in *Amphibia–Reptilia*]

The choice of breeding sites by anuran species has notable consequences for the fitness of larvae. Pond-breeding anurans are known to prefer the use of a certain type of ponds – usually based on their hydroperiod –, but beyond that they are also known to discriminate among very similar ponds according only to certain features, like pond depth, the presence of vegetation or the presence of competitors. When two species with similar larval features reproduce in the same pond typology are expected to compete, and ecological segregation at the pond level – favouring allotopy – might be expected, be this through differential selection of similar ponds or due to direct competitor avoidance. When one of the potential competitors is an alien species, the lack of shared evolutionary history might impede proper ecological differentiation, making fine-grained assessments of the possible axes of segregation between native and invasive competitors very important to correctly assess the long-term evolutionary outcomes of the invasion or to undertake – if needed – reasonable actions to artificially benefit the native.

Here, we have studied several possible sources of ecological segregation between the invasive *Discoglossus pictus* and the native *Epidalea calamita,* focusing on their use of ephemeral and short-temporary ponds as breeding points, where the native toad was only occasionally forced to competition before the arrival of the invasive species. We report mild differences in the phenology and the features that each species uses to choose among similar ponds for reproduction – with divergent selection in some features that could potentially be used to artificially benefit the native –, and a possible tendency to avoid larval syntopy in both species. Even with these differences, ecological segregation between both species ends up being always gentle, and patterns of co-occurrence in natural systems are far from being low. This justifies the repeated consideration of *Epidalea calamita* as usual competitor for the invasive species, and points at other processes as the key to their coexistence hitherto.

PART B

Competència intra-gremi en capgrossos de la granota pintada, *Discoglossus pictus.*

Capítol 6

Segregació ecològica moderada en les preferències reproductores d'un anur invasor (*Discoglossus pictus)* i un anur autòcton (*Epidalea calamita*). [enviat a la revista *Amphibia–Reptilia*]

La tria dels punts de reproducció per part de les espècies d'anurs té conseqüències notables per a les probabilitats de supervivència de les seves larves, és a dir, els capgrossos. A la majoria d'anurs que crien en basses se'ls hi coneix la preferència per l'ús d'un determinat tipus de punt d'aigua – en general en funció del seu hidroperiode –, però més enllà de preferències generals, també se sap que els adults en període de cria són capaços de discriminar entre basses molt semblants només segons certes característiques, com la profunditat, la presència de vegetació circumdant o submergida, o la presència de competidors. Quan dues espècies amb característiques similars en la fase de capgròs es reprodueixen a la mateixa tipologia de bassa, estany o de punt d'aigua, s'assumeix amb raó que són molt probablement competidors. En aquest cas, la segregació ecològica a nivell de punt d'aigua – afavorint l'al·lotopia – n'és un resultat possible, es produeixi mitjançant la selecció diferent de punts d'aigua aparentment similars o mitjançant l'evitament directe del competidor. Quan un dels potencials competidors és una espècie al·lòctona, la manca d'història evolutiva compartida entre les dues espècies pot impedir fàcilment la diferenciació ecològica que seria esperable o adient. En aquest sentit, fer avaluacions fines dels possibles eixos de segregació entre competidors autòctons i al·lòctons és molt important per avaluar correctament possibles resultats evolutius de la invasió a llarg termini o per dur a terme – si cal – mesures raonades per beneficiar artificialment l'espècie autòctona.

Aquí, hem estudiat diverses fonts possibles de segregació ecològica entre la granota pintada i el gripau corredor, centrant-nos en el seu ús de les basses efímeres i temporals com a punts de cria, on el gripau autòcton estava obligat només ocasionalment a competir abans de l'arribada de la granota invasora. Els nostres resultats indiquen diferències lleus en la fenologia i les característiques que cada espècie utilitza per triar entre basses similars per a la reproducció, i una possible tendència a directament evitar la sintopia de les larves. En el primer cas, detectem algunes divergències en la selecció de les basses podrien ser fàcilment utilitzades per beneficiar artificialment el gripau autòcton. Tot i això, fins i tot amb aquestes diferències, la segregació ecològica entre les dues espècies acaba sent sempre lleu, i els graus de sintopia en els sistemes naturals estan lluny de ser baixos. Això també justifica la consideració repetida del gripau corredor com a competidor habitual de la granota invasora, i assenyala altres processos com a clau per a la seva coexistència.

Mild ecological segregation in the breeding preferences of an invasive anuran (*Discoglossus pictus*) and its native competitor (*Epidalea calamita*)

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Abstract

The choice of breeding sites by pond-breeding anurans has notable consequences for the fitness of larvae. Hence, beyond pond typology and phenology, adults also discriminate according to other features like the presence of vegetation or competitors. When similar species of tadpoles co-occur are expected to compete, and ecological segregation at the pond level – favouring allotopy – might be expected, either through differential selection of similar ponds or direct competitor avoidance. However, the lack of shared evolutionary history might impede proper ecological differentiation with alien species. This makes very important a fine-grained assessment of the possible axes of segregation, either to correctly assess the long-term evolutionary outcomes of the invasion, or to undertake – if needed – reasonable actions to artificially benefit the native competitors. Here, we studied several possible sources of ecological segregation between the invasive *Discoglossus pictus* and the native *Epidalea calamita*, focusing on ephemeral and short-temporary ponds, where the native toad was only occasionally forced to compete before the arrival of the invasive species. We find mild phenological differences, a possible tendency to avoid syntopy in both species, and divergence in the features that each species uses to choose among similar reproduction sites, which could potentially be used to artificially benefit the native. Even with these differences, patterns of co-occurrence in natural systems are far from being low, justifying the repeated consideration of *E. calamita* as usual competitor for the invasive species, and pointing at other processes as the key to their actual coexistence.

Keywords. Amphibian, niche overlap, temporary ponds, ephemeral ponds, interspecific competition, tadpoles, competitor avoidance, oviposition site.

Introduction

The choice of breeding sites by adult anurans has notable consequences for the fitness of their offspring. Water bodies can vary widely in their abiotic and biotic features, and these variations determine great advantages or risks for the larvae, for example in food availability, presence of competitors, pond desiccation risk, water temperature, or predation risk (Alford, 1999). The tendencies in these attributes often correlate or trade-off among them (Wellborn, 1996; Alford, 1999), creating a series of typologies of water bodies that most anuran species have adapted to discriminate, using only some of them as breeding habitats where they preferably invest most of their reproductive effort, and to which their larvae are most adapted to survive (Altig and McDiarmid, 1999; Hero et al., 2001). Interestingly however, beyond the main typologies of water bodies, anuran adults are known to be able to distinguish among very similar sites according only to certain habitat features that can enhance particular aspects of their reproduction (Refsnider and Janzen, 2010), detecting and avoiding the presence of predators (Resetarits and Wilbur, 1991; Petranka et al., 1994), pollutants (Takahashi, 2007), parasites (Kiesecker and Skelly, 2000) and intraspecific competitors (Crump, 1991; Matsushima and Kawata, 2005; Cayuela et al., 2016). Breeding males and females also select sites according to the presence of interspecific competitors, taking them as deleterious for their offspring (Heying, 2004) or, contrarily, as a good signal of the lack of predators (Rudolf and Rödel, 2005). Additionally, several factors can interact: for example the presence of larval competitors may be deemed as deleterious if the pond is small, but as a good proxy of the lack of predators if the pond is sufficiently large to avoid excessive larval competition (Matsushima and Kawata, 2005). All this suggests a fine-grained and flexible selection of breeding sites by adult anurans (Cayuela et al. 2016). In the case of species breeding in ephemeral ponds, usually containing few predators (Smith, 1983; Pearman, 1995; Schneider and Frost, 1996; Richter-Boix, Llorente and Montori, 2006; 2007), competition and desiccation risk might be the most important factors taken in consideration (Schneider and Frost, 1996, Richter-Boix, Llorente and Montori, 2006; 2007). Given that both factors can synergistically affect tadpoles (Enriquez-Urzelai et al., 2013), a precise site selection becomes paramount to avoid both excessive competition pressure and desiccation risk while at the same time favouring other features potentially important for the growth and development of larvae – e.g. expected water temperature, pond depth.

Introduced species can act as powerful competitors which were previously absent in a system. This newly arrived competitors can forcibly entail a sustained decrease in energy intake or trophic modifications that could in turn translate into significant losses of fitness for the native species (Kiesecker, Blaustein and Miller, 2001; Cadi and Joly, 2004; Smith, 2005). Local extinctions, as a result of competitive exclusion, are a commonly reported consequence when the invasive competitor can largely surpass the native's competitive abilities (Holway, 1999; Bertolino et al., 2014). Similarly, for inhabitants of small enclosed systems with relatively simple and short food webs – like ephemeral and temporary ponds –, the few possibilities for ecological segregation may leave allotopy – segregation at the pond level – as the key to relax competition and allow sympatry (similarly as in Finstad et al., 2011). Nevertheless, when allochthonous competitors are introduced or invade a new territory, selection of breeding sites according to interspecific competition might become difficult for natives. For example, the signalling of the presence of invasive competitors in a pond might be unrecognisable, or confused with other cues, due to the lack of previous shared evolutionary history. On the other hand, even though recognized, real possibilities of ecological segregation in the system might be excessively scarce. Alternatively, if similar species already existed in the area, native competitors could generalize cues and competitive risks may be correctly assessed. In this case, if allotopy is indeed also possible, selection should exaggerate and secure shifts in the breeding preferences of the native – or both – species. Thus, depending on several aspects, segregation could be simply observed as a disturbance on the use of available breeding sites (Richter-Boix et al., 2013), or either as a series of directional changes – e.g. phenology, pond preferences –, which could even translate to other types of evolutionary modifications (e.g. Bourke 1999; Stuart et al. 2014). Anyhow, a fine-grained study of the breeding preferences of an invasive species and its potential competitors, and their patterns of co-occurrence, is totally justified from a management point of view in case actions to favour the native competitors are needed in the future.

Here, we have studied several possible axes of ecological segregation between an invasive and a native species breeding in similar ponds. We examined the similarity in the patterns of occurrence, the phenology, and the features used for pond choice between the native Natterjack Toad *Epidalea calamita* and the invasive Mediterranean Painted Frog *Discoglossus pictus*, focusing in ephemeral and short-term temporary ponds. These ponds are the main stronghold of the native toad in areas where the invasive competitor is not present, where the toad is only occasionally forced to compete with other species (Montori et al., 2007; Richter-Boix et al., 2013). Since data comes from three different study areas which translate to approximately 40, 20 and 3 years of coexistence with the native competitor, we also take in consideration possible changes according to the invasion history. Given that the native toad is present all along the invasive range of *D. pictus*, we arguably consider that while the invasive frog has accumulated the same number of sympatric generations with the native species in all three studied areas, the native *E. calamita* increases its previous co-evolutionary history with the invasive competitor as the populations are further from the expansion front. Under these premises, several outcomes are plausible. If both species largely overlap in their breeding preferences and the native toad is initially unable to detect the invasive competitor, selection should act to decrease syntopy, that is, ecological segregation should visibly grow together with the antiquity of the invasion. On the other hand, several factors can lead to other outcomes. For example, if the native species is able to recognize the alien competitor from the onset of the invasion and is able to modulate its pond preferences accordingly, we could find similar results in all three populations. Alternatively, regardless of the detection of interspecific competition, if the native species is strongly constrained not to modify its current breeding preferences – being competitive exclusion avoided by other means –, we will also detect similar results across populations.

Materials and Methods

Study species

The Mediterranean painted frog *Discoglossus pictus* (Anura, Discoglossidae), is native to northern Africa, Sicily, Malta and Gozo (Zangari et al., 2006). It was accidentally introduced in Europe around 1906 in Banyuls de la Marenda, southern France (Wintrebert, 1908; Zangari et al., 2006). Since then, it expanded northwards arriving up to Montpellier (SE France) and southwards to Sant Celoni (NE Spain), with similar dispersion rates in both directions (Montori et al., 2007; Genniez and Cheylan, 2012; Llorente et al. 2015). In the Iberian Peninsula – comprising the southern expansion, where studied populations lie –, the species acts as a generalist, but mostly reproduces in ephemeral and temporal water bodies (Richter-Boix et al., 2013; Llorente et al., 2015), where their larvae are highly consumptive competitors (Pujol-Buxó et al., 2016). Following previous studies (Richter-Boix et al., 2013; San Sebastián et al., 2015a,b), we chose the Natterjack Toad (*Epidalea calamita*) as the native competitor. This species uses a similar reproductive strategy, breeding after strong rains in ephemeral or temporal ponds located in open areas, thus often co-occurring with the invasive *D. pictus* (Montori et al. 2007; Richter-Boix et al. 2013, Escoriza and Boix, 2014). In addition, the native toad is already evidenced to be affected by interspecific competition from *D. pictus*, both according to lab and field studies (Richter-Boix et al., 2013; San Sebastián et al., 2015a,b; authors unpublished data).

Data collection

During spring 2016, we examined thrice a month – within each third of each month –, all along the reproductive season of the studied species, the 69 ponds chosen for the study. We started to survey ponds with the first rains after winter (21th - 28th of February), and finished when the summer droughts started (21st - 31st of May). On each visit, we examined all ponds and took data on the presence of spawns or tadpoles of the invasive *D. pictus* and the native *E. calamita,* or any other anuran species. When the pond was dry, it was assigned as missing data and kept that way in all subsequent transformations of the matrix. If eggs of a species had been overlooked – tadpoles were already present in the next survey of the same pond – the matrix was corrected at the moment of the detection of the tadpoles. Ponds were scattered across three different study areas, all located in NE Catalonia (NE Spain, exact locations in Table S1) which differ in time since invasion: in Girona – hereafter, mid expansion area – where both competitors have coexisted for approximately 40 years, we surveyed ten ponds; in Riudarenes – hereafter, far expansion area – where both species have coexisted for 20 years, we surveyed 19 ponds; and in the area between Hostalric and Sant Celoni – hereafter, the expansion front – where the invasive species had arrived only from 0 to 3 years ago, we surveyed 27 ponds. All chosen ponds were already known to be – according to criteria from (Montori et al., 2007; Richter-Boix et al., 2006; 2013) – ephemeral or temporary at most. Therefore, none of them contains water all year long, and most of them usually contains less than two or three consecutive months of water. These ponds are rarely used as reproduction points by species other than the two competitors of study, and in most cases, were already known to have been used by at least one of the species of the study. Unluckily, thirteen ponds did not have reproduction of any species during that spring and they had to be excluded from the study, leaving a total of 56 ponds. We characterised each surveyed pond using a set of five variables (Table S1): (1) the maximum depth of water column – hereafter, pond depth –; (2) the vegetation cover inside the pond, be them algae, mosses or vascular plants – very low: from 0 to 25% of surface with vegetation, low: from 25 to 50% of surface with vegetation, present: from 50 to 75% of surface with vegetation, abundant: from 75 to 100% of surface with vegetation –; (3) the vegetation cover outside the pond – with the same classification as the previous variable, taking in consideration the first nearest meter of dry land outside the pond; (4) the square root of the surface of the pond when full – given that surfaces increase quadratically, we use the square root to avoid outliers –; and (5) the proportion of this surface usually under direct sunlight – to the nearest 0.05 –, hereafter sunshine on pond or sunshine.

Statistical analyses

As a necessary preliminary processing, we transformed the survey matrix (Table S2) into several matrices of absence / presence (0/1) for the different stages – or combinations of stages – of each species. Then, firstly, to test if the global distributions of the positive detections of both species were significantly similar or dissimilar, we proceeded to test their occurrence similarity using mantel tests. To that end, we previously transformed the matrices of presence / absence of eggs or tadpoles of both species into distance matrices among ponds using the Jaccard method implemented in the function *vegdist* of the package *vegan* (Oksanen et al., 2016), which accepts missing values. Then, using the function *mantel* of the same package, we tested (1) the similarity between the reproductive occurrence patterns of both species – as tadpoles or eggs, indifferently –, and (2) the similarity between the patterns of presence of tadpoles of both species. Both tests were run using the area of study as strata for the permutations. After that, we also repeated these tests separately for each study area when the number of data available allowed it.

Secondly, after testing general similarities in the positive detection of both species, we were interested in testing possible competition avoidance. That is, we wanted to test if eggs of a species co-occurred less than expected with tadpoles of the other species in a certain period of time. To that end, we used the *cooccur* function of the package *cooccur* (Griffith, Veech and Marsh, 2016), applying the function for each period of time – each ten days – separately, to compare the patterns of presence (0 / 1) of the several items of interest. Thus, we run tests for the co-occurrence of eggs of *D. pictus* and tadpoles of *E. calamita* for each possible period of time – limited by the presence of both *E. calamita* eggs and *D. pictus* tadpoles in that period of time –, and we also run tests for the co-occurrence of eggs of *E. calamita* and tadpoles of *D. pictus*, with similar limitations. In the first case we could run the test for all three periods of April plus the second period of May. In the second case we could test all three periods of April and the first two of May. Unluckily, separate tests for populations had to be deemed unreliable – in most cases, they were simply unfeasible – due to excessively low numbers of positive data per time period and location.

Then we wanted to know if both species repeatedly rejected the same ponds as reproductive points. To that end, we performed again permutation tests implemented in the function *cooccur* of the package *cooccur* (Griffith, Veech and Marsh, 2016), in this case to test if there was significant similarity between the ponds used by both species. Thus, in this occasion, before testing we first transformed the presence / absence matrix of both species – and other anurans – to matrices of 1 / 0 corresponding to: (1) pond used at some point of the reproductive season by species / (0) pond never used. After this general assessment, we repeated the same test separately for each study areas.

Once detected possible divergent preferences in relation to pond use (see Results), we wanted to know if certain pond features were shaping the pond choice of the species and, since preferences might change due to evolutionary history or local adaptation, we were also interested in possible changes of these preferences across areas. To that end, we used the automated model selection and multimodel inference procedures for Generalized Linear Mixed Models (GLMMs) implemented in the package *glmulti* (Calcagno, 2013). We used the reproductive presence of the species – as eggs or tadpoles indifferently – at some point of the reproductive season as a binomial response variable (1: present at some point / 0: never present), and used as possible explanatory variables (see data collection): (1) the study area, always included to account for possible differences in abundance or percentage of use of ponds among areas, (2) the pond depth, (3) vegetation cover inside the pond, (4) vegetation cover outside the pond, (5) the pond surface, and (6) the sunshine on pond. To test for possible differences in preferences across areas of study, we allowed for first order interactions among the study area and the rest of explanatory variables. Before model selection we run pairwise correlations to see if some pairs of these variables were excessively correlated to be included in the procedures. For each species, we performed a holistic model selection ranking models using the corrected Akaike Information Criterion (AICc, Burnham and Anderson, 2003). Once we obtained the ranked models, we inferred the statistical importance of each term according to its inclusion in the best models and their model-averaged importance – in this case, the inclusion in the 100 models with lower values of AICc. After model validation and once detected the potentially important terms, we explore and discuss the ecological preferences of each species using the fitted values of the best models.

Finally, we were also interested in possible differences in the reproductive phenology of both species. To test possible differences in the mean moment of reproduction, we first assimilated our surveying chronology to a numeration from 1 (21st to 28th of February) to 10 (21st to 31st of May). We then created a dummy variable which was as long as the number of reproductive events detected in the study, the values of the variable coinciding with the previously created chronological numeration. Thus, for each time in which a reproductive event – egg laying – had been detected for a species, a number – according to the chronological numeration – had been added to the dummy variable. Two classifier variables – the species and the study area to which the reproductive event belongs – allowed us to test differences in the mean numeration for each species, location and their interaction. Given that this data did not follow the assumptions of normality and continuity needed for parametric tests, we used the permutation ANOVAs implemented in the function *aovp* from the package *lmPerm* (Wheeler and Torchiano, 2016). Even if the interaction was not significant (see Results), we repeated the test for each study area for clarity. All analyses and figures of the manuscript were done using R (R core team 2015).

Results

All mantel tests showed a tendency to similarity between the presence of both species in the ponds when a survey is conducted, with mild differences in their significance. At a global scale, the positive similarity between the matrices of presence / absence of eggs or tadpoles indifferently was far from significant (r = 0.0482, P = 0.120), while the positive similarity between the matrices of presence / absence of tadpoles alone was nearly or marginally significant (r = 0.0845, $P < 0.05$). At a population level, the mid expansion area showed a nearly significant association (r = 0.330, P = 0.078 for reproductive presence; tadpoles not possible to reliably test due to low number of data), the far expansion area showed a nearly significant or significant association (r = 0.227, P = 0.053 for reproductive presence; r = 0.381, $P < 0.01$ for tadpoles) and the expansion front showed a non-significant positive association (r = 0.0373, P = 0.32 for reproductive presence; r = 0.0103, P = 0.421 for tadpoles).

The co-occurrence of eggs of *D. pictus* and tadpoles of *E. calamita* along the several surveys in which eggs of the invasive species were present was in all five cases mildly lower than expected, but only significantly so in the last period (Table 1). Thus, adults of *D. pictus* had during all April periods and the first of May a non-significant tendency to avoid tadpoles of *E. calamita* when laying eggs, but a significant tendency to do so in the second period of May. Globally, only in three occasions in this study, adults of *D. pictus* laid eggs in a pond when *E. calamita* tadpoles were already present. On the opposite direction, results were very similar. In this case, the co-occurrence of eggs of native *E. calamita* and tadpoles of invasive *D. pictus* show a non-significant tendency to be lower than expected in the first period (Table 1), patterns very similar to random co-occurrence in the rest of April, and a significant tendency to avoidance in the second period of May.

Time Period	***D. pictus* (invasive)**	***E. calamita* (native)**	**Expected co-occurrence**	**Observed co-occurrence**	***P* value**
1st-10th of April	eggs	tadpoles	0.4	0	0.564
11th-20th of April	eggs	tadpoles	0.6	0	0.460
21th-30th of April	eggs	tadpoles	1.5	0	0.167
11th-20th of May	eggs	tadpoles	3.3	3	0.553
11th-20th of May	*eggs*	*tadpoles*	*3.6*	*0*	*0.006*
1st-10th of April	tadpoles	eggs	1.5	0	0.090
11th-20th of April	tadpoles	eggs	1.6	2	0.569
21th-30th of April	tadpoles	eggs	4.0	4	0.958
11th-20th of May	*tadpoles*	*eggs*	*3.0*	*0*	*0.014*

Table 1. Results of the co-occurrence analysis for all possible combinations of tadpoles and eggs of each species. Expected co-occurrence and observed co-occurrence are expressed in number of ponds. In italics, the combinations that showed significantly lower co-occurrence than randomly expected.

The usage of the same ponds was significantly lower than expected by chance among invasive *D. pictus* and native *E. calamita* (expected co-occurrence = 30.7 ponds, observed co-occurrence = 27, $P < 0.01$), higher than expected by chance between invasive *D. pictus* and other species of anurans occasionally breeding in ephemeral habitats (expected co-occurrence = 9.2 ponds, observed co-occurrence = 12, $P < 0.05$), and not different from random between native *E. calamita* and other species of anurans occasionally breeding in ephemeral habitats (expected co-occurrence = 8.6 ponds, observed co-occurrence = 8, $P = 0.467$). Results of usage of ponds within each population (Table S3) repeat these tendencies thrice, thus largely resembling general patterns, but results are not significant when they are tested separately.

The only explanatory variables that were moderately correlated between them were the vegetation cover outside and inside the pond (Pearson correlation: 0.62, Table S4) while the rest of explanatory variables were largely uncorrelated (Table S4, all values below 0.45). Each species had different probabilities to breed in each area, and used different features to choose ponds to breed. Native *Epidalea calamita* chose ponds according mainly to pond depth, pond surface area, and the interactions of both terms with area (Fig S1, Table S5). The amount of sunshine in the pond and the study area – plus their interaction – had clearly less support and all the rest of potential explanatory variables are practically absent from the best set of models. Thus, according to coefficients and predicted probability (Table S6), *Epidalea calamita* has a decreasing tendency to occur in ponds as they become deeper – being this tendency more marked in the mid expansion area (Fig. 1, Fig S2)

–, and different tendencies across areas concerning pond area, with an increasing tendency in the expansion front but decreasing tendencies in the far and mid expansion study areas (Fig. S3). Finally, the native toad is more prone to use ponds as reproductive points as ponds are less shaded (Fig. S4). On the other hand, invasive *Discoglossus pictus*, chose ponds mainly – in decreasing order of importance – according to the presence of vegetation around them, their depth, their surface, the study area, and the proportion of the pond surface under direct sunshine (Fig. S5, Table S7). In this case, any interaction of an explanatory variables with area was poorly supported. Thus, according to coefficients and predicted probabilities (Table S8), *Discoglossus pictus* clearly prefers ponds with at least some vegetation around and it is more prone to reproduce in them as they are deeper (Fig. 1) and – mainly in the expansion front – more shaded (Fig. S6). An increased pond surface is negatively selected by *Discoglossus pictus* (Fig. S7).

Study area	***D. pictus* (invasive)**	***E. calamita* (native)**	**Mean difference (days)**	***P* value**
Globally	April 11th to 14th	April15th to 20th	3.5	0.406
mid expansion	March 30th to April 3rd	April 10th to April 14th	10.9	0.448
far expansion	April 26th to April 29th	April 28th to May 1st	2.1	0.745
expansion front	April 10th to April 15th	April 10th to April 14th	-0.7	0.941

Table 2. Mean point of reproduction in the spring of 2016 for each species, differences between species in the mean point of reproduction in days (in positive: invasive species reproduces first; in negative: the native species reproduces first), and p-value for the differences.

The phenology of both species in the spring of 2016 was not significantly different according to statistical analyses (Table 2). We detected a significant effect of study area ($F_{2,89}$ = 10.01, $P < 0.001$), but although the mean point of egg laying for *D. pictus* was between the first and the second thirds of April, and the mean point of egg laying for *E. calamita* was between the second and third thirds of April, these interspecific differences cannot be considered as significant ($F_{1,89}$ = 0.143, $P = 0.406$). Although phenological differences were most exaggerated in the mid expansion area, diminishing in the far expansion area, finally being totally negligible in the expansion front, this interaction does not receive enough statistical support ($F_{2,89}$ = 0.362, $P = 0.551$, Table 2).

Discussion

Of all possibilities for ecological segregation in breeding preferences studied here, we mainly found interspecific differences in the features that both species use for pond choice. In addition, we detect a possible tendency, by both species at the end of the reproductive period, to directly avoid larval syntopy. Concerning phenology, differences were too mild to provide significant results but were nonetheless exactly as expected based on previous studies (Montori et al., 2007). Although there are some interesting interpopulation tendencies, in any case differences were clearly marked and evolutionary consequences of different co-existence time are largely undetected in this study. Even with the combination of differences in pond choice and phenology, patterns of co-occurrence are far from being low, and therefore both species seem often forced to end up competing. This justifies the repeated consideration of *E. calamita* as a usual competitor of the invasive *D. pictus* (Montori et al., 2007; Richter-Boix et al., 2013; San Sebastian et al., 2015a,b), and points at other processes as the key to relax competition and allow sympatry.

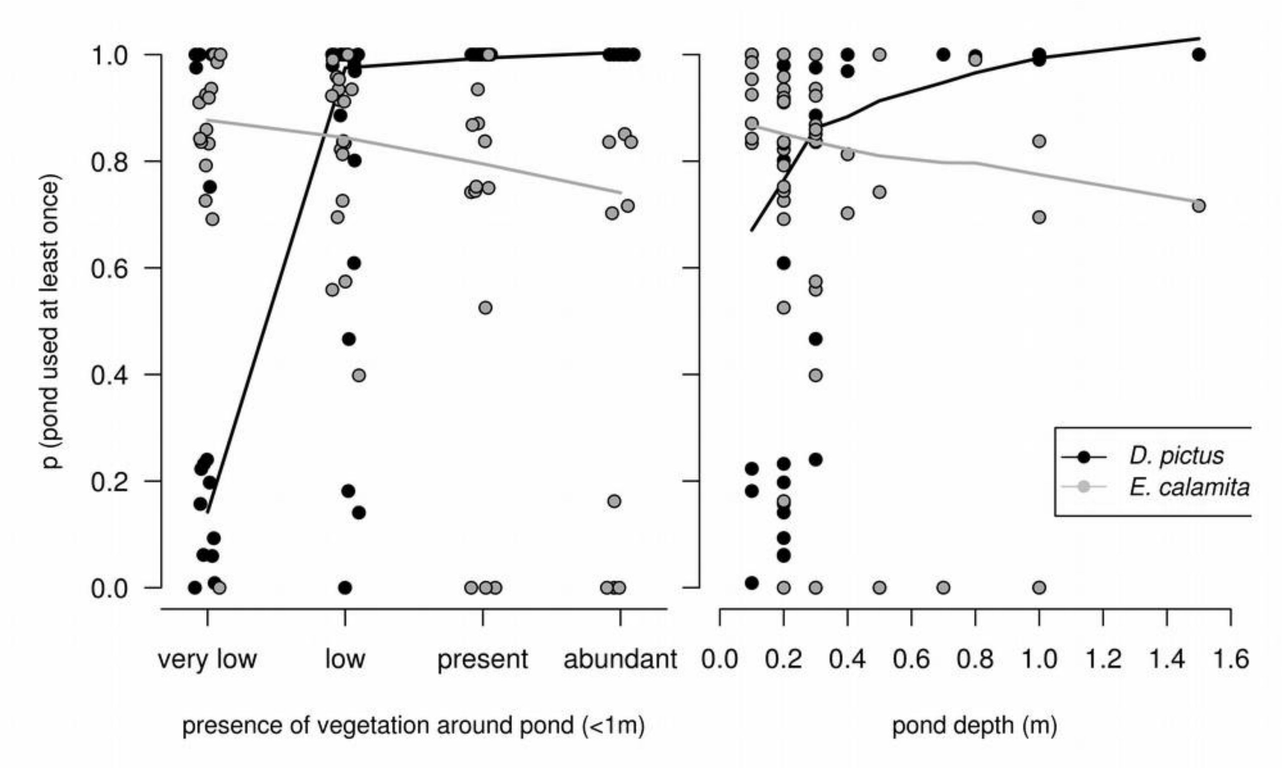

Figure 1. Probability of reproduction of each species in a pond depending on the presence of vegetation around the pond (left) and the pond depth (right).

Interestingly, the different analyses studying co-occurrence in this study show apparently contradictory patterns. Results from mantel tests point at a positive association of the reproductive presence of both species in ponds, suggesting that if we run a random survey in several ephemeral or temporary ponds within the invasive range of *D. pictus*, we will probably detect signs of reproduction of the other species if one of them is already present. This is in accordance with previous studies posing *Epidalea calamita* as a very common native competitor for the invasive

Discoglossus pictus during the larval phase (Montori et al., 2007; Richter-Boix et al., 2013; Escoriza and Boix, 2014). Contrarily, according to analyses of co-occurrence, both species repeatedly show a tendency to avoid laying eggs – significantly in both cases at the end of the reproductive season (Table 1) – where competitor larvae are already present. How are these seemingly contradictory results compatible? The solution lies in the different nature of both analyses and, consequently, the biological interpretation of both results. Mantel tests assess the possible association of the reproductive presence / absence of both species in a survey by pond matrix. Importantly however, data from a particular survey is not independent from the previous one, and not all transitions among different occupancy states of a pond among consecutive surveys are equally possible (and some of them are highly improbable, see flux diagram in Fig. S8). For instance, direct transitions from the presence of a single species to the presence of the other alone are virtually impossible and, even though possible, we did not detect any case in which all tadpoles of a single species secured metamorphosis – releasing the other from competition – before the pond drying out. This makes pond occupancy tend to fall in double absences or double presences (see flux diagram in Fig. S8), even with a random succession of events. The excess of double negatives – e.g. too early in the reproductive season in a particular area, or the pond was dry in the previous survey and it had not yet rained – is in this case easily solved with the usage of Jaccard distances, but the intrinsic tendency of the system to accumulate double presences cannot be controlled with simple methods. Interestingly, similar studies repeatedly discuss that not all temporary and ephemeral ponds are always available (Werner et al., 2007; Cayuela et al., 2012), but the fact that ponds persisting in time will probably accumulate competitors is not so commonly taken in consideration. In contraposition with mantel tests, in the co-occurrence analyses we compared, for each ten days time period, the coincidence of oviposition activity of each species with the presence of tadpoles of the other. Although a complete independence of oviposition patterns among different moments in time is difficult to ensure, tests are run separately, and biases present in the Mantel test are here clearly absent. Thus, here the preferences *among the available ponds* in each moment are tested, and most results tend – significantly so at the end of the reproductive season – to express the opposite outcome, that is, competitor avoidance (Table 1). The tendency to avoid interspecific larval competition is more markedly clear at the end of the oviposition season for both species, probably signalling a reinforced preference to avoid interspecific competition due to the already enhanced constraints for breeding late – shorter expected water persistence in ponds, forcing tadpoles to develop quicker (Enriquez-Urzelai et al., 2013; Richter-Boix et al., 2014). Globally, we could simply consider that the mantel test is methodologically biased for this purpose. Nevertheless, it is interesting to highlight that syntopy studies need to consider that the presence/absence of species tends to be inevitably associated in time-limited systems like temporary ponds (Fig. S8), and is compelling to note that the contradiction of results points an important biological interpretation: although both species try to avoid each other, the natural constraints of the pond typology in which they breed force them to competition.

Are the mild possibilities to avoid competition shaped by particular features of breeding points? The patterns of usage of ponds at least once for reproduction – considering this usage the acceptance of the pond as a valid reproduction point – indicates that, even though both species share the acceptance of a fairly high number of ponds (27 out of 56), these are significantly less that the randomly expected (30.7 out of 56). Although statistically significant, differences can be considered very gentle, reinforcing the idea that both species indeed compete for the same set of ponds. But on the other hand, the result demands further investigation, and the study of environmental variables highlights several interspecific differences in reproductive habitat preferences. Hence, while the native toad *Epidalea calamita* has a preference for shallow ponds with great surfaces, if possible under direct sunlight (Fig. 1 and Figs S2-S4), the invasive frog clearly avoids water bodies devoid of surrounding vegetation, being also more prone to breed in ponds as they are deeper – within the limits of ephemeral or temporary ponds, opposing the results found when all water bodies are surveyed (Escoriza and Boix, 2014) – and smaller in surface, largely irrespective of pond shading (Fig. 1 and Figs S6-S7). Possibly, the native toad likes to breed in shallow ponds because their water temperature is likely to increase markedly during daytime, accelerating embryonic development (Sjögren, Elmberg and Berglind, 1988; Bull and Shepherd 2003). The selection for great pond surfaces might follow in turn this preference for shallow ponds, as a way to avoid excessively small water volumes that might easily dry out. Contrarily, although the invasive *D. pictus* is a usual breeder in ephemeral ponds, it selects among them the ones that feature most elements of temporary ponds, namely the presence of vegetation and deeper water columns. This could be significantly related to its great consumptive needs as a tadpole (Pujol-Buxó et al. 2016), thus selecting ponds with more abundant and diverse feeding resources where tadpoles can use their abilities to select high quality resources (San Sebastián et al. 2015b). This would also fully agree with the positive association found among *D. pictus* and other occasional breeders in ephemeral ponds (*Pelodytes punctatus* and *Hyla meridionalis* [Jakob et al., 2003]), which seem attracted to them only if these feature a certain complexity. Since freezing can be problematic for species that breed early – *D. pictus* is an early breeder, see below – , the choice of deeper ponds could also be explained as an avoidance of water bodies likely to freeze completely in a late cold spell (Petranka and Petranka, 1981). Summing up, even if differences are mild for the most part, we have identified some features that might shape competition avoidance through opposite preferences across species. Importantly, these preferences could be used to artificially favour the native species if needed. In that sense, ponds that are thoroughly sunlit, clearly devoid of surrounding vegetation, and with great water surface but shallow, are the most probably colonized by the native but not the invasive species.

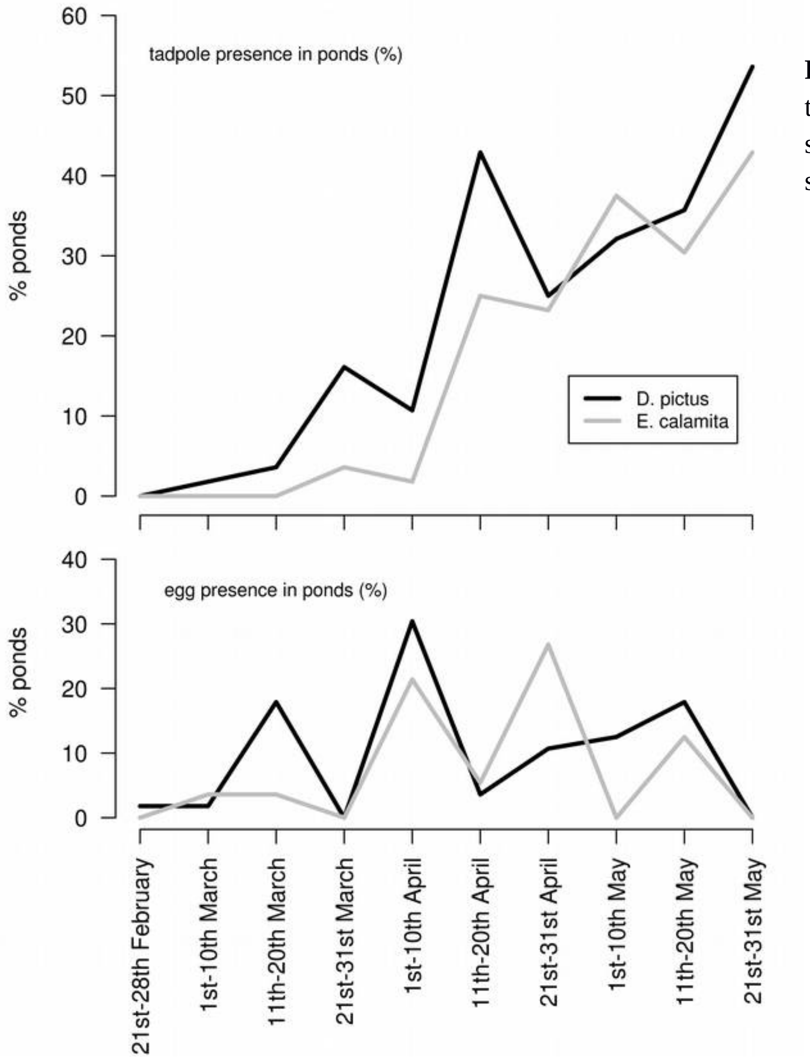

Figure 2. Percentage of occurrence of tadpoles and eggs of each species in the surveyed ponds along all the reproductive season of 2016.

Finally, another possibility for both competitors could be to segregate temporally. Given that ephemeral ponds, by definition, fill up with strong rains and last few weeks until drying out, the prospect to avoid competition segregating temporally is scarce. However, published literature on the biology of both species suggests that *D. pictus* is an earlier breeder than *E. calamita* during spring, being the difference mainly in the starting – but not the end – of the oviposition season (Montori et al., 2007). Our observations totally match this description and uncover clear differences mainly on the amount of oviposition during the first strong rains after winter (10th - 20th of March, Fig. 2), when the reproductive season of the invasive species had clearly started but reproduction by the native toad was largely absent. This implies that during the first batch of reproduction of the invasive frog, some conditions for the reproduction for *E. calamita* were not yet met – e.g. water or air temperature (Sjögren et al., 1988; Bull and Shepherd, 2003), and suggests as well that the native toad is a more explosive breeder. Differences in mean breeding time however, were never significant. This might be a combined effect of studying the phenology of only one year – with a rather small sample size – and the specially irregular rainfall in the studied year.

Do pond preferences or phenology change according to invasion history? Phenological segregation tends to be wider as the invasion is more ancient (Table 2), but results are not statistically significant and more thorough phenological study would be needed to explore this tendency. Pond preferences across areas suggest different preferences in pond depth in *E. calamita*, that avoids deeper ponds – which are positively selected by *D. pictus* – more clearly where the species has coexisted more with the invasive species (Fig. S4). This – like phenological tendencies across populations – follows the expected pattern assuming an active selection to segregate both species. However, not all results are so consistent with expectations. Interpopulation differences in preferred pond surface are difficult to interpret for both species (Fig. S5 and S6), and contrary to expectation, the invasive species is more prone to breed in shaded ponds – where the native species is usually absent – in the expansion front (Fig. S7). Together with the small effects of the potentially evolutionary patterns and their inconsistency, differences across areas and species on observed – and expected – pond occupancy also ask for caution, since unluckily in some locations the available data ended up being small with unequal use of both species (Mid expansion area: n = 10, nine used by *D. pictus* and two by *E. calamita*). Therefore, although tendencies in phenology and pond depth are clearly suggestive, we believe they are not conclusive, at any rate hopefully serving to spur wider-scale studies examining these possibilities.

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Amphibia-Reptilia

Mild ecological segregation in the breeding preferences of an invasive anuran (Discoglossus pictus) and its native competitor (Epidalea calamita)

Eudald Pujol-Buxó [1,2], Gabriel M. Riaño[1], and Gustavo A. Llorente[1,2]

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Supplementary Figures

Number of supplementary figures: 8

Fig S1. Model-averaged importance of terms – proportion of the 100 best models including the term – explaining the use by *Epidalea calamita* at least once of a pond for reproduction:

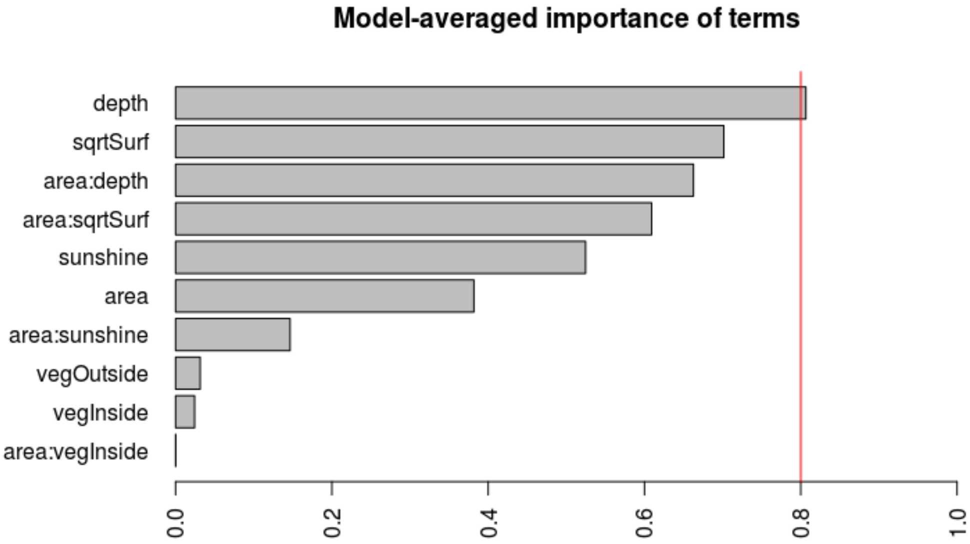


Fig S2. Predicted probabilities of use for reproduction at least once by *Epidalea calamita* according to pond maximum depth.

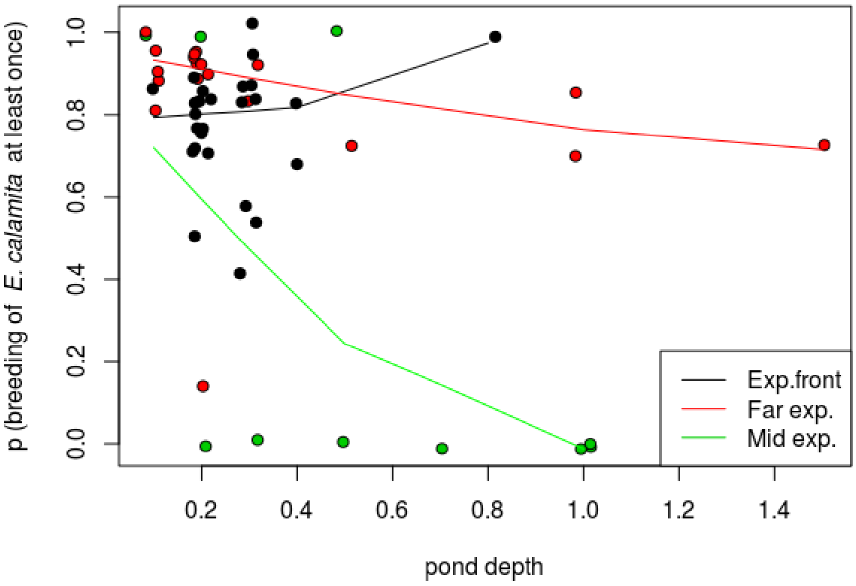


Fig S3. Predicted probabilities of use for reproduction at least once by *Epidalea calamita* according to the square root of the pond area.

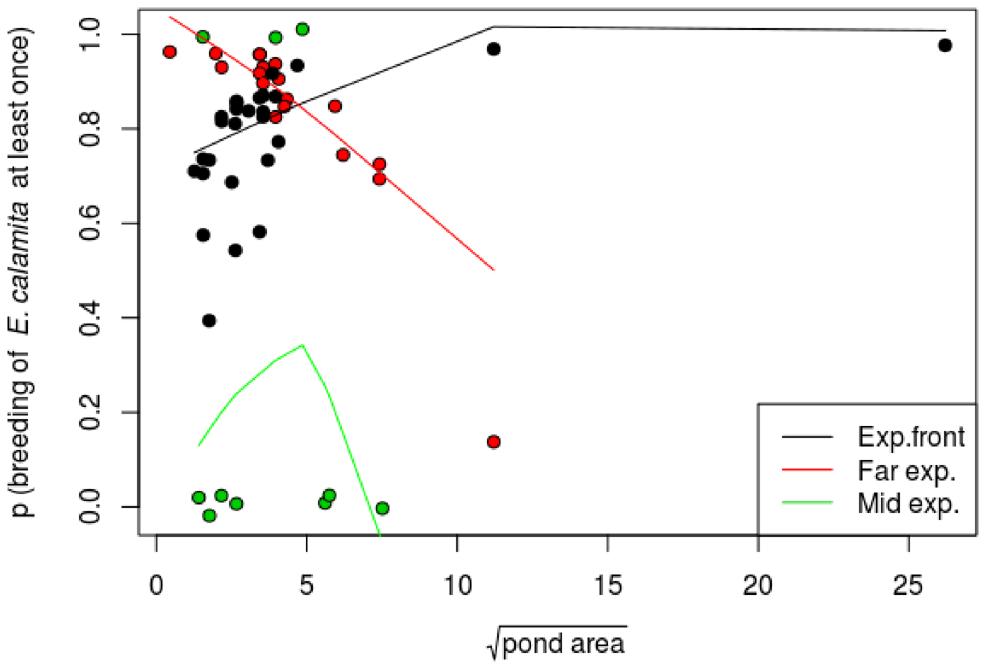


Fig S4. Predicted probabilities of use for reproduction at least once by *Epidalea calamita* according to the proportion of the pond usually under direct sunshine.

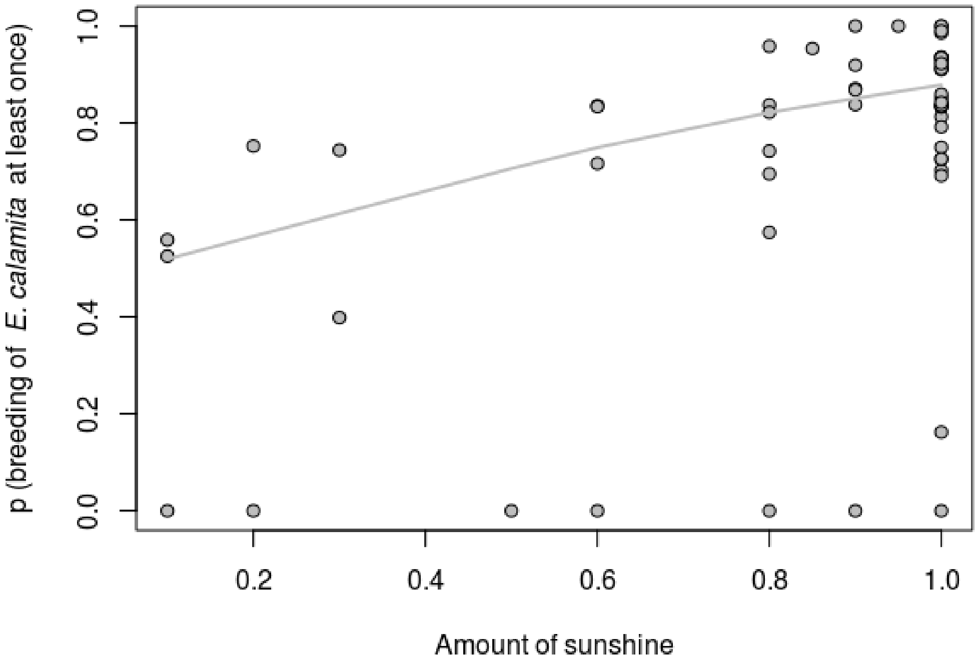


Fig S5. Model-averaged importance of terms – proportion of the 100 best models including the term – explaining the use by *Discoglossus pictus* at least once of a pond for reproduction:

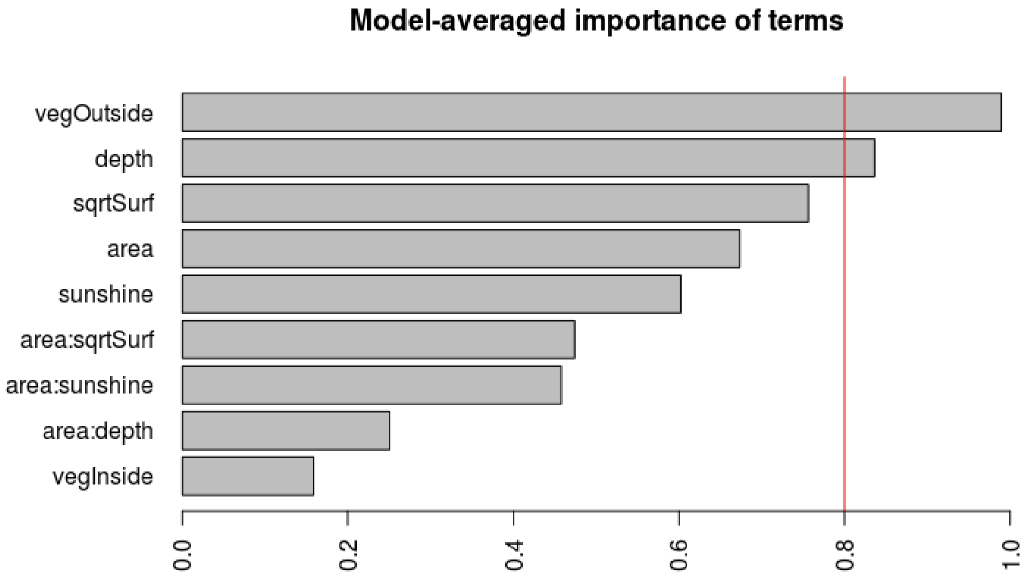


Fig S6. Predicted probabilities of use for reproduction at least once by *Discoglossus pictus* according to the proportion of the pond usually under direct sunshine.

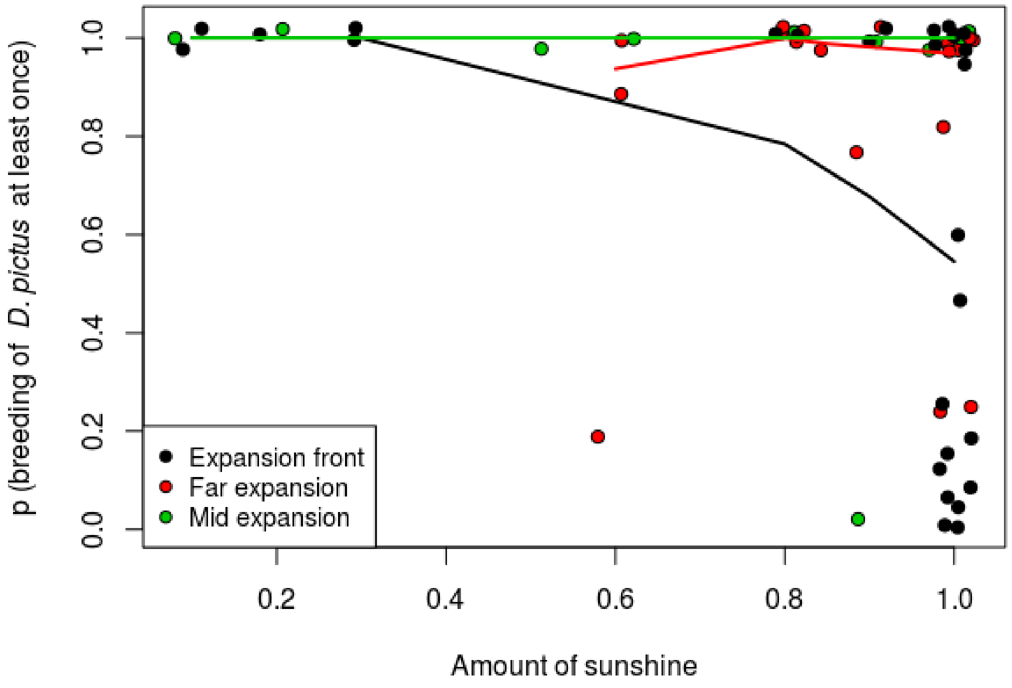


Fig S7. Predicted probabilities of use for reproduction at least once by *Discoglossus pictus* according to the square root of the pond area.

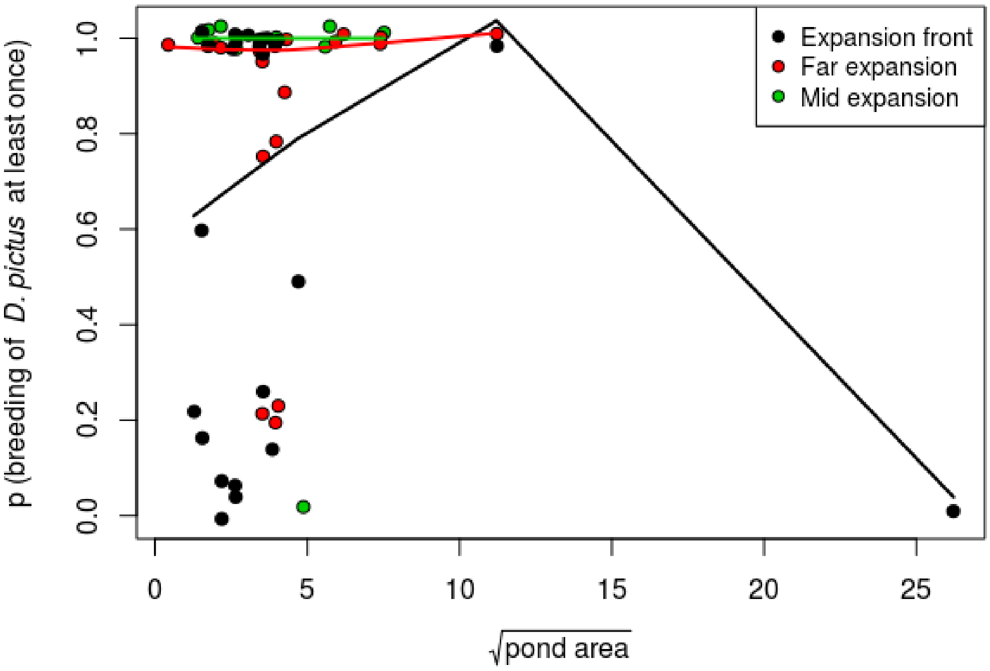
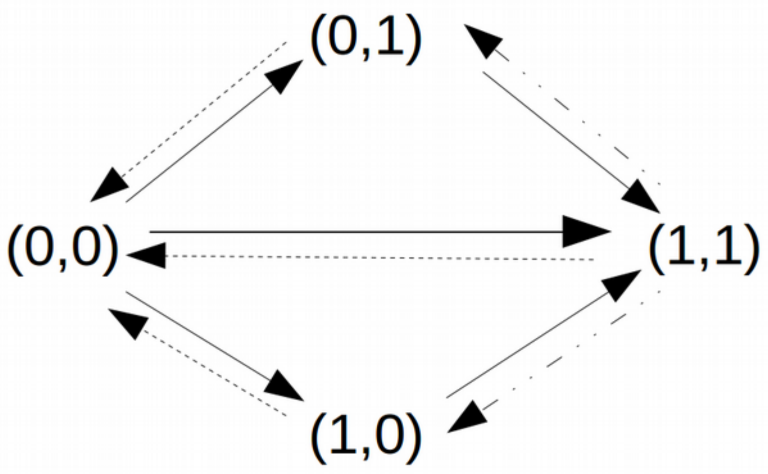


Figure S8. Flux diagram representing pond occupancy states (,) with two competitors (0: absence of a species; 1: presence of a species). Different types of lines correspond to different types of events (Continuous line: breeding events; Fine dashed line: drying of a pond; One dash – two dots line: a whole guild of a species secures metamorphosis). Absence of a line connecting two states indicates that the transition is impossible. *Comment: Given the lack of possible transitions between (0,1) and (1,0), and since the possibility that absolutely all tadpoles of a species secure metamorphosis is fairly uncommon, even more when surveying tadpoles within a single reproductive season, the flux system clearly tends to favour double absences or double presences.*



Amphibia-Reptilia

Mild ecological segregation in the breeding preferences of an invasive anuran (Discoglossus pictus) and its native competitor (Epidalea calamita)

Eudald Pujol-Buxó [1,2], Gabriel M. Riaño[1], and Gustavo A. Llorente[1,2]

[1] Department of Evolutionary Biology, Ecology and Environmental Sciences, University of Barcelona.

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Supplementary Tables

Number of Supporting tables: 8

Table S1. Exact locations and main features of the ponds studied (Depth: maximum depth; Vegetation variables: 1= very low, 2=low, 3=present, 4=abundant; Sunshine: proportion of the pond under direct sunlight).

Code	Latitude	Longitude	Surface (m^2)	Depth (m)	Vegetation inside (1-4)	Vegetation outside (1-4)	Sunshine (0 to 1)
GI.OLI.01	42° 0'15.80"N	2°51'24.36"E	23.6	0.5	1	2	0.9
GI.ABA.02	42° 0'8.78"N	2°50'45.53"E	56.5	1	2	4	0.5
GI.ARG.01	41°59'55.17"N	2°50'38.65"E	2.4	0.1	3	3	0.95
GI.ARG.02	41°59'55.10"N	2°50'38.75"E	4.7	0.5	3	4	0.8
GI.ARG.03	41°59'54.98"N	2°50'39.42"E	2	0.2	3	1	1
GI.ARG.04	41°59'55.63"N	2°50'38.65"E	31.4	1	4	3	0.9
GI.ARG.05	41°59'55.78"N	2°50'38.96"E	15.7	0.2	2	1	1
GI.ARG.06	41°59'55.71"N	2°50'40.02"E	33	1	4	4	0.1
GI.ARG.07	41°59'56.39"N	2°50'39.76"E	3.1	0.7	2	3	0.6
GI.ARG.10	41°59'41.59"N	2°50'44.02"E	7.1	0.3	2	3	0.2
RI.ESP.02	41°49'44.18"N	2°43'5.96"E	55	1	1	2	0.8
RI.MUR.01	41°49'18.12"N	2°41'18.24"E	125.7	0.2	4	4	1
RI.MUR.02	41°49'18.57"N	2°41'16.30"E	11.8	0.2	1	3	1
RI.MUR.03	41°49'18.98"N	2°41'17.29"E	35.3	1	4	3	0.8
RI.MUR.04	41°49'21.87"N	2°41'17.03"E	15.7	0.2	1	2	1
RI.MUR.05	41°49'18.29"N	2°41'17.61"E	16.5	0.2	1	1	1
RI.BAT.01	41°49'25.94"N	2°40'58.33"E	18.8	0.1	3	3	0.9
RI.BAT.02	41°49'28.78"N	2°40'59.91"E	3.9	0.2	4	2	0.8
RI.CAÇ.01	41°49'27.23"N	2°40'51.29"E	55	1.5	4	4	0.6
RI.CAÇ.02	41°49'28.29"N	2°40'45.69"E	38.5	0.5	4	3	0.8
RI.GRE.01	41°47'47.10"N	2°41'45.70"E	12.6	0.1	1	1	1

RI.GRE.02	41°47'46.94"N	2°41'45.71"E	0.2	0.1	2	1	1
RI.GRE.03	41°47'47.34"N	2°41'45.15"E	11.8	0.2	1	2	1
RI.GRE.04	41°47'50.95"N	2°41'42.21"E	12.6	0.3	1	1	1
RI.GRE.05	41°48'18.11"N	2°41'11.26"E	18.1	0.3	2	2	0.6
RI.GRE.06	41°48'12.08"N	2°41'13.59"E	4.7	0.1	1	2	0.85
RI.GRE.07	41°48'11.87"N	2°41'13.80"E	11.8	0.2	2	2	1
RI.GRE.08	41°47'46.66"N	2°41'46.37"E	12.6	0.2	1	1	0.9
RI.PON.01	41°47'54.89"N	2°42'21.67"E	15.7	0.1	3	2	0.6
HO.POL.02	41°43'55.72"N	2°37'7.36"E	2.4	0.2	1	1	1
HO.POL.04	41°44'3.76"N	2°36'38.50"E	687.2	0.3	1	1	1
HO.POL.05	41°43'57.75"N	2°36'32.23"E	9.4	0.2	1	2	0.8
HO.POL.06	41°43'54.72"N	2°36'28.98"E	15.7	0.3	1	3	0.9
HO.POL.07	41°43'54.44"N	2°36'27.90"E	12.6	0.3	1	2	0.9
HO.DUC.01	41°44'7.59"N	2°36'5.05"E	15	0.2	1	2	1
HO.DUC.02	41°44'8.44"N	2°36'4.27"E	12.6	0.4	1	2	1
HO.DUC.04	41°44'7.58"N	2°36'3.41"E	125.7	0.8	4	2	1
HO.DUC.06	41°44'6.89"N	2°36'4.01"E	6.9	0.2	1	1	1
HO.ARI.01	41°43'19.07"N	2°33'52.17"E	3.1	0.2	1	3	1
HO.ARI.02	41°43'19.19"N	2°33'53.15"E	11.8	0.3	4	4	1
HO.ARI.03	41°43'19.03"N	2°33'53.22"E	7.1	0.2	4	4	1
HO.ARI.04	41°43'19.28"N	2°33'53.52"E	7.1	0.2	4	4	1
HO.ARI.05	41°43'19.38"N	2°33'52.14"E	6.3	0.4	4	4	1
HO.MOT.01	41°43'6.53"N	2°33'10.66"E	22	0.3	1	2	1
HO.MOT.02	41°43'6.39"N	2°33'10.33"E	4.7	0.2	1	1	1
HO.MOT.03	41°43'6.46"N	2°33'9.02"E	7.1	0.2	1	1	1
HO.MOT.04	41°43'6.85"N	2°33'2.50"E	1.6	0.2	1	1	1
HO.MOT.05	41°43'6.42"N	2°33'2.98"E	12.6	0.3	1	1	1
HO.MOT.06	41°43'6.63"N	2°33'1.11"E	4.7	0.1	1	1	1
HO.MOT.07	41°43'7.28"N	2°32'58.04"E	2.4	0.2	1	2	1
HO.MOT.08	41°43'7.70"N	2°33'2.28"E	6.9	0.2	1	3	0.1
HO.MOT.09	41°43'8.00"N	2°33'2.05"E	11.8	0.3	1	2	0.1
HO.MOT.10	41°43'8.15"N	2°33'1.89"E	13.7	0.2	1	3	0.3
HO.MOT.11	41°43'8.43"N	2°33'1.37"E	16.5	0.2	1	3	0.2
HO.MEA.01	41°42'49.27"N	2°32'31.71"E	2.4	0.3	2	2	0.8
HO.MEA.02	41°42'49.55"N	2°32'31.17"E	3.1	0.3	1	2	0.3

Table S2. Survey matrix, *i.e.* summary of the presence-absence data for each species, pond and ten days period. Abbreviations: ME= Mid Expansion area, FE= Far expansion area, EF=Expansion Front, **NA**=Pond empty of water, N= No species present, c = *Epidalea calamita* eggs during that period, C = *Epidalea calamita* tadpoles during that period, d = *Discoglossus pictus* eggs during that period, D = *Discoglossus pictus* tadpoles during that period, o = other species (*Pelodytes punctatus* or *Hyla meridionalis*) eggs during that period, O = Other species tadpoles during that period.

Area	**Code**	**20th - 30th February**	**1st - 10st March**	**11th - 20th March**	**21st - 31st March**	**1st - 10st April**	**11th - 20th April**	**21st - 30th April**	**1st - 10th May**	**11th - 20th May**	**21st - 31st May**
ME	GI.OLI.01	N	N	c	C	N	N	N	N	N	N
ME	GI.ABA.02	N	N	d	D	D	D	N	N	N	N
ME	GI.ARG.01	N	N	do	DO	DO	DO	cD	CD	CDo	CDO
ME	GI.ARG.02	N	N	d	D	D	D	D	D	N	N
ME	GI.ARG.03	N	N	N	N	d	D	N	d	D	D
ME	GI.ARG.04	N	N	d	D	D	D	D	Do	DO	DO
ME	GI.ARG.05	N	N	d	D	D	D	cD	D	D	D
ME	GI.ARG.06	N	N	d	D	D	D	D	D	D	D
ME	GI.ARG.07	N	N	N	N	d	D	N	d	Do	DO
ME	GI.ARG.10	**NA**	**NA**	d	D	N	N	N	d	D	N
FE	RI.ESP.02	**NA**	**NA**	**NA**	**NA**	**NA**	do	cDO	DO	DO	DO
FE	RI.MUR.01	N	**NA**	**NA**	**NA**	d	D	d	D	N	N
FE	RI.MUR.02	N	**NA**	**NA**	**NA**	**NA**	**NA**	N	N	N	CD
FE	RI.MUR.03	N	N	N	N	do	DO	N	N	N	N
FE	RI.MUR.04	N	**NA**	**NA**	**NA**	**NA**	**NA**	N	d	cd	CD
FE	RI.MUR.05	N	**NA**	**NA**	**NA**	N	N	c	C	N	N
FE	RI.BAT.01	N	**NA**	**NA**	**NA**	do	DO	N	N	cdo	CDO
FE	RI.BAT.02	N	**NA**	**NA**	**NA**	**NA**	CD	CD	CD	cdo	CDO
FE	RI.CAÇ.01	N	N	N	N	do	DO	DO	O	cO	CO
FE	RI.CAÇ.02	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	N	N	cd	CD
FE	RI.GRE.01	**NA**	**NA**	c	C	C	C	C	C	C	C
FE	RI.GRE.02	**NA**	**NA**	**NA**	**NA**	cd	CD	CD	CD	CD	CD
FE	RI.GRE.03	**NA**	**NA**	**NA**	**NA**	**NA**	d	D	D	D	D
FE	RI.GRE.04	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	cD	D	D	D
FE	RI.GRE.05	**NA**	**NA**	**NA**	**NA**	**NA**	c	C	Cdo	CDO	CDO
FE	RI.GRE.06	**NA**	**NA**	**NA**	**NA**	**NA**	N	c	Cd	CD	CD
FE	RI.GRE.07	**NA**	**NA**	**NA**	**NA**	**NA**	N	c	Cd	CD	CD
FE	RI.GRE.08	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	cd	CD
FE	RI.PON.01	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	c	C	**NA**	**NA**
EF	HO.POL.02	N	N	d	D	N	N	N	N	d	D
EF	HO.POL.04	N	N	N	**NA**	N	**NA**	c	C	C	C
EF	HO.POL.05	N	N	N	**NA**	d	D	**NA**	**NA**	d	D
EF	HO.POL.06	N	N	**NA**	**NA**	d	D	N	**NA**	cd	CD
EF	HO.POL.07	N	N	**NA**	**NA**	d	D	**NA**	**NA**	d	D

EF	HO.DUC.01	N	N	d	D	c	Co	CO	CO	C	C
EF	HO.DUC.02	N	N	N	**NA**	N	N	c	**NA**	do	DO
EF	HO.DUC.04	N	d	D	N	cd	CD	CD	CD	CD	CD
EF	HO.DUC.06	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	c	C	C	C
EF	HO.ARI.01	**NA**	c	d	**NA**	N	N	N	**NA**	**NA**	**NA**
EF	HO.ARI.02	d	cD	D	**NA**	cd	CD	cd	CD	**NA**	**NA**
EF	HO.ARI.03	**NA**	**NA**	**NA**	**NA**	d	cD	N	**NA**	**NA**	**NA**
EF	HO.ARI.04	**NA**	**NA**	**NA**	**NA**	cd	CD	N	**NA**	**NA**	**NA**
EF	HO.ARI.05	**NA**	**NA**	**NA**	**NA**	d	cD	N	**NA**	**NA**	**NA**
EF	HO.MOT.01	**NA**	**NA**	**NA**	**NA**	c	C	C	C	C	C
EF	HO.MOT.02	**NA**	**NA**	**NA**	**NA**	c	C	C	**NA**	**NA**	N
EF	HO.MOT.03	**NA**	**NA**	**NA**	**NA**	c	C	C	C	C	N
EF	HO.MOT.04	**NA**	**NA**	**NA**	**NA**	c	C	C	C	C	C
EF	HO.MOT.05	**NA**	**NA**	**NA**	**NA**	c	C	C	C	C	C
EF	HO.MOT.06	**NA**	**NA**	**NA**	**NA**	c	C	C	**NA**	**NA**	N
EF	HO.MOT.07	**NA**	**NA**	**NA**	**NA**	c	C	C	**NA**	**NA**	N
EF	HO.MOT.08	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	cd	CD	CD	CD
EF	HO.MOT.09	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	cd	CD	CD	CD
EF	HO.MOT.10	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	cd	CD	CD	CD
EF	HO.MOT.11	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	d	D	D	D
EF	HO.MEA.01	N	N	N	N	d	D	D	N	N	N
EF	HO.MEA.02	N	N	**NA**	**NA**	do	DO	DO	DO	DO	DO

Table S3. Tests for co-usage of ponds at some point of the reproductive point. P-values are obtained from permutation tests (see Methods) and indicate probability of significant differences between expected and observed co-occurrence in the direction that these differences are found:

Area	**sp1**	**sp2**	**Observed co-occurrence**	**Expected co-occurrence**	**p**
mid expansion	other anurans	*D. pictus*	3	2.7	0.700
mid expansion	other anurans	*E. calamita*	1	0.9	0.708
mid expansion	*D. pictus*	*E. calamita*	2	2.7	0.300
far expansion	other anurans	*D. pictus*	6	5.1	0.295
far expansion	other anurans	*E. calamita*	5	5.1	0.705
far expansion	*D. pictus*	*E. calamita*	13	13.5	0.578
expansion front	other anurans	*D. pictus*	3	2.0	0.279
expansion front	other anurans	*E. calamita*	2	2.3	0.545
expansion front	*D. pictus*	*E. calamita*	12	14.0	0.063

Table S4. Correlation among the continuous explanatory variables used in the model selection:

	depth	**vegInside**	**vegOutside**	**sunshine**	**sqrtSurf**
depth	1.000				
vegInside	0.411	1.000			
vegOutside	0.416	0.617	1.000		
sunshine	-0.262	-0.008	-0.343	1.000	
sqrtSurf	0.317	0.124	0.047	0.032	1.000

Table S5. Ten best models explaining pond choice for *Epidalea calamita*, sorted downwards starting from the best. Empty spaces indicate that the term is absent from the model, written names indicate that the term is included in the model:

model	**area**	**vegInside**	**vegOutside**	**sqrtSurf**	**depth**	**sunshine**	**area:sqrtSurf**	**area:depth**	**area:sunshine**	**aicc**	**weights**	**Δaicc**
1				sqrtSurf	depth		area:sqrtSurf	area:depth		59.29	0.23	0.00
2				sqrtSurf	depth	sunshine	area:sqrtSurf	area:depth		60.17	0.15	0.88
3	area			sqrtSurf	depth		area:sqrtSurf	area:depth		61.89	0.06	2.60
4					depth			area:depth		62.71	0.04	3.42
5					depth	sunshine		area:depth		62.85	0.04	3.56
6	area					sunshine				62.86	0.04	3.57
7	area			sqrtSurf	depth	sunshine	area:sqrtSurf	area:depth		63.46	0.03	4.17
8	area			sqrtSurf	depth	sunshine	area:sqrtSurf		area:sunshine	63.49	0.03	4.20
9	area									63.86	0.02	4.56
10	area					sunshine			area:sunshine	63.87	0.02	4.58

Table S6. Coefficients for the terms predicting the presence of *E. calamita* in a pond, including as explanatory variables in the model all terms present in more than 40% of the 100 best models.

	coefficient	**Std. Error**
(Intercept)	-0.93	2.26
areaRI	3.65	2.65
areaGI	-28.29	6813.96
sqrtsurf	0.59	0.54
depth	-3.39	7.03
sunshine	1.67	1.40
areaRI:sqrtsurf	-1.14	0.66
areaGI:sqrtsurf	61.05	7910.80
areaRI:depth	4.27	7.28
areaGI:depth	-503.12	59606.46

Table S7. Ten best models explaining pond choice for *Discoglossus pictus*, sorted downwards starting from the best. Empty spaces indicate that the term is absent from the model, written names indicate that the term is included in the model:

model	area	vegInside	vegOutside	sqrtSurf	depth	sunshine	area:sqrtSurf	area:depth	area:sunshine	aicc	weights	Δaicc
1	area		vegOutside	sqrtSurf	depth		area:sqrtSurf			42.88	0.20	0.00
2			vegOutside	sqrtSurf	depth	sunshine	area:sqrtSurf		area:sunshine	43.34	0.16	0.46
3	area		vegOutside	sqrtSurf	depth			area:depth		44.52	0.09	1.64
4	area		vegOutside	sqrtSurf	depth	sunshine			area:sunshine	44.85	0.08	1.97
5	area		vegOutside	sqrtSurf	depth	sunshine	area:sqrtSurf			45.51	0.05	2.63
6	area		vegOutside			sunshine			area:sunshine	45.98	0.04	3.10
7	area		vegOutside		depth	sunshine			area:sunshine	46.03	0.04	3.14
8			vegOutside	sqrtSurf	depth	sunshine		area:depth	area:sunshine	46.86	0.03	3.98
9	area		vegOutside	sqrtSurf	depth	sunshine		area:depth		47.29	0.02	4.41
10	area	vegInside	vegOutside			sunshine			area:sunshine	47.31	0.02	4.42

Table S8. Coefficients for the terms predicting the presence of *D. pictus* in a pond, including as explanatory variables all terms present in more than 40% of the 100 best models.

	coefficient	Std. Error
(Intercept)	170.01	62561.61
areaRI	-158.10	62561.61
areaGI	-137.98	73915.07
vegOutside2	2.12	1.52
vegOutside3	55.37	13714.75
vegOutside4	178.86	34783.02
sqrtSurf	-0.97	0.84
sunshine	-175.12	62561.61
depth	24.66	13.05
areaRI:sqrtSurf	-3.74	3.24
areaGI:sqrtSurf	-36.32	7193.68
areaRI:sunshine	176.21	62561.61
areaGI:sunshine	304.50	73458.00

PART B

Intraguild competition in tadpoles of *Discoglossus pictus.*

Chapter 7

Preliminary exploration of changes in native–invasive trophic competition according to coevolutionary history [in prep.]

Competition is one of the main ways an invasive species might modify the trophic web of a newly invaded ecosystem. Trophic competitive interactions among natives and invasives have been repeatedly studied in animal ecology, highlighting the ability of invasive species to occupy, modify or displace the trophic niche of native competitors. Anuran tadpoles have long been used as models for competition studies – including native-invasive interactions – and recent studies using stable isotopes analysis (SIA) have been successful in exploring ecological patterns of trophic competition.

We here attempt to make a first exploration of fast evolutionary changes in competitive relationships using SIA. We assess possible changes in the effects and direction of interspecific trophic segregation, across a set of locations along an invasion range with naturally created different coevolutionary histories. Both tadpole species – native *Epidalea calamita* and the invasive *Discoglossus pictus* – clearly segregate in their trophic position in ponds, and, interestingly, the nature of this segregation changed across populations and ponds. Concretely, while the magnitude of differences does not seem to vary with time since invasion, there is a tendency to progressively segregate more in the type of consumed organic matter within the same trophic level – carbon signature – rather than in the trophic level itself – nitrogen signature. Globally, this is possibly signalling an ecological segregation created from the very onset of the invasion, but with variations across populations in how is this segregation achieved. However, with the dataset used hitherto, variations across populations can also be alternatively explained from a purely ecological point of view, mainly through interspecific differences in the influence of accompanying vegetal and animal communities. On the other hand, interspecific or differences across populations in niche width were scarce and seemingly unrelated to co-evolutionary patterns. The addition of five more ponds in the study may help to to discern possible evolutionary patterns from alternative ecological explanations.

PART B

Competència intra-gremi en capgrossos de la granota pintada, *Discoglossus pictus*

Capítol 7

Exploració preliminar dels canvis en la competència tròfica interespecífica entre una espècie invasora i una d'autòctona segons la història coevolutiva de les poblacions. [en preparació]

La competència és una de les principals formes que una espècie invasora pot modificar la xarxa tròfica d'un ecosistema recentment envaït. Les interaccions tròfiques competitives entre autòctons i invasors s'han estudiat en nombroses ocasions en l'ecologia animal, sovint posant de relleu la capacitat de les espècies invasores per ocupar, modificar o desplaçar el nínxol tròfic dels competidors nadius. Els capgrossos han estat reeixidament utilitzats com a models per estudis de competència – incloent-hi les interaccions autòcton-invasor – i, més recentment, alguns estudis han tingut cert èxit en l'exploració de patrons ecològics derivats de la competència tròfica en capgrossos fent servir anàlisis d'isòtops estables (Stable Isotopes Analysis, SIA).

En aquest estudi, intentem realitzar una primera exploració de possibles canvis evolutius en relacions de competència tròfica mitjançant l'anàlisi d'isòtops estables, fent servir com a model la competència entre la granota pintada i el gripau corredor. Avaluem possibles canvis en els efectes i la direcció de la segregació tròfica interespecífica, prenent mostres al llarg d'un conjunt de localitzacions al llarg de la distribució invasora de la granota, implicant diferents graus de coevolució creats de forma natural. Com a resultats, cal dir que les dues espècies de capgròs es segreguen clarament en la seva posició tròfica en les basses, i, curiosament, la naturalesa d'aquesta segregació canvia al llarg de diferents poblacions i basses. En concret, mentre que no sembla que la magnitud de la segregació varii segons el temps des de la invasió, hi ha una tendència a separar-se cada vegada més en el tipus de matèria orgànica consumida dins del mateix nivell tròfic – indicat per la signatura de carboni – en lloc de segregació segons el propi nivell tròfic – indicat per la signatura de nitrogen. Globalment, això és possiblement la senyalització d'una segregació ecològica creada des de l'inici de la invasió, però amb variacions interpoblacionals en el com s'aconsegueix. No obstant això, amb el conjunt de dades utilitzat fins ara, les variacions interpoblacionals poden també ser explicades alternativament des d'un punt de vista purament ecològic, principalment a través de diferències interespecífiques en la influència de les comunitats vegetals i animals present a cada bassa. D'altra banda, les diferències interespecífiques o interpoblacionals d'amplitud de nínxol tròfic eren escasses i aparentment sense relacions co-evolutives clares. L'addició de cinc estanys i basses més en l'estudi, que es durà a terme properament, pot ajudar a molt a discernir entre els possibles patrons d'evolució i les explicacions alternatives ecològiques.

Preliminary exploration of changes in native–invasive trophic competition according to coevolutionary history

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Abstract

Trophic competition is one of the main ways an invasive species might modify the trophic web of an ecosystem. Competitive interactions among natives and invasives have been repeatedly studied in animal ecology, highlighting the ability of invasive species to occupy, modify or displace the trophic niche of natives. Anuran tadpoles have long been used as models for competition studies – including native-invasive interactions – and recent studies using stable isotopes analysis (SIA) have been successful in exploring several ecological patterns. Using SIA, we explore fast evolutionary changes in a competitive relationship between a native – *Epidalea calamita* – and an invasive – *Discoglossus pictus* – tadpole species. To that end, we examine the trophic position of both species across a set of locations along the invasion range, which embody a naturally created gradient of different coevolutionary histories. Studied species clearly segregate in their trophic position in ponds, and, interestingly, the nature of this segregation changes across populations and ponds. Concretely, while the magnitude of differences does not seem to vary with time since invasion, there is a tendency to progressively segregate rather in the type of consumed organic matter within the same trophic level – carbon signature – than in the trophic level itself – nitrogen signature. This possibly signals that co-evolutionary history might simply shape the nature of a segregation which is created from the very onset of the invasion. However, with the dataset used hitherto, interpopulation variations can also be alternatively explained under a purely ecological point of view, through interspecific differences in the influence of accompanying vegetal and animal communities. On the other hand, differences in niche width across species and ponds were usually scarce and seemingly unrelated to co-evolutionary patterns. The addition of five more ponds in the study may help to to discern possible evolutionary patterns from alternative ecological explanations.

keywords. Stable isotopes, syntopy, Epidalea calamita, Discoglossus pictus, trophic competition.

Introduction

Trophic competition is – along with other types of competition and predator-prey interactions – one of the main ways an invasive species might modify the trophic web of a newly invaded ecosystem. Competitive interactions among natives and invasives have been repeatedly studied in animal ecology, highlighting the ability of invasive species to overlap, modify or displace the trophic niche of native competitors (Blanchet *et al.* 2007, Pérez-Santigosa *et al.* 2011, Bertolino *et al.* 2014). However, only a few of these works assess possible geographic differences in the impacts of the introduced competitor, or possible evolutionary changes in the native-invasive competition along time, hence unwillingly assuming a similar impact along time and space (but see Stuart *et al.* 2014). Theoretically, once a new competitive relationship is created, selective benefits for individuals which better withstand it should be expected, thus shaping the mid- and long-term impacts of an invasion. Introduced competitors therefore become from the start strong agents of selection on native species – and viceversa –, possibly inducing evolutionary changes for example in the usual trophic position of a species, or in its habitat-linked morphology (Crowder 1986, Bourke *et al.* 1999, Stuart *et al.* 2014). Indeed, since evolutionary changes in response to invasive predators can occur very rapidly (Trussel *et al.* 2002, Nunes *et al.* 2014), it should be possible to expect similar evolutionary rates with a selective pressure like competition. This poses the evolutionary point of view as necessary to correctly assess the potential mutability of the competitive roles of natives and invasives, and in turn make realistic forecasts on long-term impacts of an invasion. Indeed, this final impact will largely depend on the capacity of both parts to adapt to the new interaction (Cox 2004).

Anuran tadpoles have long been used as models for competition studies, also including invasive species (Kupferberg 1997a, Kiesecker *et al.* 2001, Smith 2005, Cabrera-Guzmán *et al.* 2013, San Sebastián *et al.* 2015b). Most tadpole species from temperate areas, concretely, are considered to feed mostly on detritus, algae and phanerogams, occasionally incorporating small animals and carcasses in their diets (Diaz-Paniagua 1985, Campeny 2001, Caut *et al.* 2013, Arribas *et al.* 2014). Most previous studies suggest that the quality of resources (Brown & Rosati 1997, Kupferberg 1997b) and trophic competition *per se* (Richter-Boix *et al.* 2013) clearly influence the growth and development of tadpoles, thus potentially hindering the capacity of tadpoles to complete metamorphosis before pond drying (Enriquez-Urzelai *et al.* 2013), and affecting individual fitness before and after metamorphosis, either through increased predation risk or other processes (Morey & Reznick 2001, Pujol-Buxó *et al.* 2017). Thus, trophic competition has direct effects on individual fitness, but also cascades to other decisive components of it, potentially exaggerating the population-level effects of subtle changes in the trophic position of a species. However, in the study of dietary habits in tadpoles, once known the general preferences of each species, further examinations of gut content can become unprofitable in terms of precision and reliability answering fine ecological questions (Altig *et al.* 2007). Probably driven by this constraints, in the last years, stable isotopes analysis (SIA) has become increasingly used as a handy tool to detect subtle changes in the trophic ecology of tadpole species whose general trophic status are already known (Whiles *et al.* 2006, Schiesari *et al.* 2009, Caut *et al.* 2013, Arribas *et al.* 2015, San Sebastián *et al.* 2015a). In the case of inhabitants of small and enclosed systems comprising relatively simple and short food webs, like ephemeral ponds, limited diversity may force trophic changes to consist in modifications of the frequency rather than the list of items, making SIA specially useful.

Using SIA, San Sebastián *et al.* (2015a) reported the ability of tadpoles of the invasive *Discoglossus pictus* (Anura: Discoglossidae) to affect diet patterns and possibly displace the tadpoles of the native *Epidalea calamita* (Anura: Bufonidae) from their preferred trophic niches in ephemeral and temporary ponds, confirming an expected competitive relationship between these commonly co-occurring species (Richter-Boix *et al.* 2013). Nevertheless, the invasive species is continuously expanding (Geniez & Cheylan 2012, Llorente *et al.* 2015), what creates a coexistence gradient with its competitors along the invasion range. Linked to this coexistence gradient, several factors – e.g. progressive exclusion from certain microhabitats, changes in competitive abilities of one or both competitors – can lead to different and geographically structured competitive outcomes. Hence, the expansion of an invasive species can be considered a “natural experiment” (HilleRisLambers *et al.* 2013) in which we can evaluate mutual adaptation and evolutionary modifications on the trophic ecology of both competitors. To test this possibilities, we here examine their trophic niches along a gradient of four different populations with different co-evolutionary histories. These populations – or study areas – lie along the southern expansion of the invasive species (Fig.1): (1) one near – 20km – to the first introduction of the invasive frog, meaning approximately 110 years of coexistence with the native competitor, and hereafter “origin area”; (2) another 60km from this origin area, meaning from 30 to 40 years of coexistence, and hereafter “mid expansion”; (3) a third one 80km from the origin area, meaning approximately 20 years of coexistence, and hereafter “far expansion”; and finally (4) a population less than 3km from the current southern distributional limit of the frog – 100km from the origin area – with less than 5 years of coexistence, hereafter the “expansion front”. From an evolutionary point of view, we can argue that the native toad is totally “naive” for this competitive interaction in the expansion front. Contrarily, assuming a mean generation rate of 3 years (Oromi *et al.* 2012) – it has had from 30 to 35 generations of competitive experience – and therefore natural selection – with the invasive species in the origin area, representing the mid and far expansion areas midpoints between this two extremes. On its side, the invasive species – with a similar generation rate (Oromi *et al.* 2016) – given that the native toad is present all along the invasive range, is equally experienced in all places, what gives it a clear *evolutionary advantage* in the expansion front.

These co-evolutionary patterns can lead to various possible outcomes, mainly depending on the ability of the native species to modify its trophic niche from the start, for example, by means of phenotypic plasticity. If the native species does not undergo a niche differentiation at the onset of the invasion, but is progressively forced to exploit different dietary habits, we should expect much larger trophic overlaps in the expansion front than in the origin area. According to theory (Van Valen 1965, Araújo *et al.* 2008, Bolnick *et al.* 2010) interspecific competition should constraint niche width, but several studies (Codron *et al.* 2011, Abbey-Lee *et al.* 2013, San Sebastian *et al.* 2015a) have found that dominant competitors can force competitive subordinates to increase their niche widths instead, if both have very similar food preferences. Accordingly, we here propose that, if niche segregation were to increase with invasion age, niche widths – only of the native *E. calamita* if it is indeed the subordinate species (San Sebastián *et al.* 2015a,b), or of both species if there is no clear dominant or subordinate competitor (authors *in prep.*) – should decrease accordingly. Contrarily, if the trophic niche is modified from the start, differences among populations – either in mean or niche width – could become largely undetectable. In this case, changes should be possibly found in the nature and direction, or on other outcomes of this trophic partitioning (authors *in prep.*). Between these two extremes, mixed or accumulative solutions – *i.e.* changes both in the amount and the nature of the trophic partitioning – might also be possible.

Materials and Methods

Study Species

The Mediterranean Painted Frog (*Discoglossus pictus*) was accidentally introduced from Northern Algeria to SE France (Banyuls de la Marenda) approximately 110 years ago (Wintrebert 1908, Zangari *et al.* 2006). The species occupies nowadays a wide strip of the Mediterranean coast from Montpellier (SE France) to Sant Celoni (Catalonia, NE Spain), continuously expanding into new areas, mainly northwards and southwards at an approximate rate of 2 km/year (Geniez & Cheylan 2012, Llorente *et al.* 2015). The chosen competitor, the Natterjack Toad (*Epidalea calamita*), is a native species present in all the invasive range of *Discoglossus pictus*. Both species use a similar reproductive strategy, mainly breeding after strong rains in ephemeral or temporary ponds, and therefore in the invaded areas the native toad is often found in syntopy with the invasive

D. pictus (Richter-Boix 2013, authors *in prep.*). Both studied tadpoles have a benthic morphology and habits (Escoriza & Boix 2012) and the invasive tadpoles have the greatest consumption rates of all the local community, with a clearly consumption-based growth physiology (Pujol-Buxó *et al.* 2016). Consequently, the native species is reported to suffer from the invasive competition according to both laboratory and field studies, usually acting as a competitive subordinate (Richter-Boix *et al.* 2013, San Sebastian *et al.* 2015a,b, authors *in prep.*).

Field sampling and lab procedures

During springs of 2016 we monitored several ephemeral and small temporary ponds that gathered the features allowing a possible coincidence in space and time of the two study species (authors *in prep.*). All ponds were all located along the southern expansion of *D. pictus,* clearly grouped in the already described four study areas (Table S1). Once the reproductive season was on course, we sampled ponds from the former selection under the following criteria: (1) four or five ponds per study area, (2) absence of any other tadpole species, (3) both study species had to be at least at Gosner stage 25 (Gosner 1960) and have attained middle or large sizes. Unluckily, even though all field work was planned for the spring of 2016, the year was extremely arid in the study area and prevented the completion of the study, forcing to delay until spring 2017 the sampling whole origin area – where an extremely dry winter and spring possibly impeded absolutely the reproduction of both species – and an additional pond to compete the mid expansion. Here the results for the ponds studied hitherto (twelve in total, Table 1) are presented.

As a surrogate of the trophic niche of each species, we obtained the isotopic niche using SIA. We used the isotopic signatures of nitrogen (δ15N) and carbon (δ13C), which are the most commonly used in animal trophic ecology and similar studies (Whiles *et al.* 2006, Schiesari *et al.* 2009, Caut *et al.* 2013, Arribas *et al.* 2015, San Sebastián *et al.* 2015a). According to previous literature, δ13C values can provide information on the source of primary carbon, while δ15N values are more useful to infer the trophic level of the organism due to higher fractionation rates (Tieszen *et al.* 1983, Minagawa & Wada 1984, Ambrose & DeNiro 1986, Griffith 1992, Koch 1995, Gannes *et al.* 1997). We sampled ten tadpoles per species and pond, plus a small collection of potential competitors, predators and resources when possible. All animals were euthanized with 70º ethanol, which was some days later substituted with clean ethanol 70º. To avoid biases due to food remains in the digestive tract of tadpoles, these were extracted. Isotopic signatures reflect the diet over the period during which the analysed tissue is formed, which in our case are the whole tadpole – excepting the digestive tract – and their entire life. All samples were dried – in a Memmert heater at 60ºC for three days – and homogenized to fine powder using a manual grinder. As the lipid content of the larvae is low – San Sebastián *et al.* 2015a –, we did not remove the lipids prior to isotope analysis. The powdered samples were then weighed – between 0.7 0.25 and 0.3 mg for tadpoles and insects and between 1.2 and 1.4 mg for algae – and placed in tin capsules for mass spectrometry at the Serveis Científico-Tècnics (University of Barcelona, Spain) using an elemental analyzer (Flash EA 1112) coupled to stable isotope ratio mass spectrometry equipment (CF-IRMS). The laboratory uses international standards – for δ15N and δ13C, atmospheric nitrogen and Pee Dee Belemnite respectively – which are generally run after every 12 samples: IAEA CH7 (87% C), IAEA CH6 (42% C) and USGS 24 (100% C) for δ13C; IAEA N1, IAEA N2 (21% N) and IAEA NO3 (13.8% N) for δ15N. Accuracy was ±0.1% and ±0.2% for δ 13 C and δ 15 N, respectively.

Statistical Analyses

In order to be able to compare the trophic level of both species, we first corrected the isotopic signatures of tadpoles using the trophic fractionation obtained for each of the two species in San Sebastián *et al.* (2015a). Then, we started by exploring pond by pond patterns in both the mean and the dispersion of isotopic signatures for each species. As central tendency measures, we tested for each pond, using the methodologies described in Turner *et al.* (2010), interspecific differences in the mean δ13C, the mean δ15N, and the bivariate mean of the isotopic signature. For dispersion measures, we used SIBER (Jackson *et al.* 2011) to obtain the standard ellipse area (SEAc), plus the mean nearest neighbor (MNN), the mean distance to centroid (MDC), and the total area (TA) (Layman *et al.* 2007) across species and ponds.

Once confirmed widespread differences in the mean isotopic signature of both species in ponds (Table 1), we wanted to test if isotopic niche differences were similar across study areas. For this, we conducted a MANOVA in which δ15N and δ13C formed the matrix of results, and species, study area and pond – allowing for interactions – were the potential explanatory variables. Once confirmed the presence of differences across populations (*i.e.* study areas, see Results), we further examined population-level interspecific differences by fitting, separately for each isotope and study area, linear mixed effects models – LMMs, fitted using lme4 (Bates *et al.* 2015) – with species as explanatory variable and pond as random intercept.

Then, we proceeded to include the habitat information in tests to see if these patterns could be alternatively explained by differences in pond features. Since the presence of some accompanying species often correlate, we first summarized the composition of the vegetal and animal communities in a reduced number of variables. To that end, we created two distance matrices among ponds, one using the simple matching coefficient for the macroscopic vegetal community – thus considering that we did not possibly overlook any of the species –, and one using the Jaccard index for the animal community – thus considering that we could have overlooked some species. Then, we applied a multidimensional scaling to each matrix, and kept two and three axes respectively – representing in total the 69% and the 60% of the variability – for vegetal and animal communities. Then, for each isotope, we fitted a full – the most complex possibility – LMM including species, population, pond sunshine, and the summary variables for vegetal and animal communities as explanatory variables. We allowed for first order interactions among species and the rest of variables, and we always included pond as a random intercept. From this full model, we conducted an exhaustive search for the best model using the corrected Akaike Information Criterion (AICc, Burnham & Anderson 2003) in *glmulti* (Calcagno 2013), allowing for the presence of interactions without the need for both single effects to be also present.

After exploring the patterns of mean isotopic signatures, we also studied the patterns in the mean nearest neighbour distance – MNND, in a two-species community, simply the distance between the centroids of both species – and the niche width of species – using the corrected standard ellipse areas (SEAc) of both species. Since in pond-level measures the ratio among the number of ponds – eleven, for the moment – and the number of variables – eleven as well, even more if we took interactions in consideration – does not allow proper modelling of the dispersion measures, we explored possible relationships among isotopic dispersion running regressions of each measure on the eleven explanatory variables: area, the interspecific abundance ratio, tadpole density, pond surface, pond depth, sunshine on pond, and the summary variables for vegetal and animals communities. To specifically test the possibility that SEAc of each species displays different interpopulation patterns, we also fitted a model with SEAc as the dependent variable and species, study area, and their interaction as explanatory variables. Due to the low number of observations, all regressions were tested using permutation tests implemented in the *lmp* function of the package *lmPerm* (Wheeler & Torchiano 2016). All statistical analyses and figures were done with R (R core team 2015).

Results

The pond by pond exploration revealed that the species differed in all cases in the mean bivariate isotopic signature (Fig. 1, Table 1). The case was the same, with few exceptions, for each isotopic signature taken individually (Table 1). A general trend was evident – with few exceptions commented later –, consisting in higher δ15N but lower δ13C in *D. pictus*. On the other hand, regarding niche widths, differences were less frequent (Table 2) and lacked a common direction, with similar number of cases in which each species presented the widest (Table 2).

There were differences among study areas (Pillai= 1.62, $F_{4,396}$ (approx)= 421, p < 0.001), species (Pillai= 0.64, $F_{2,197}$ (approx)= 177 p < 0.001) and ponds (Pillai= 1.73, $F_{16,396}$ (approx)= 159, p < 0.001) in mean isotopic signature. More interestingly, there were also differences in the interaction among area and species (Pillai= 0.20, $F_{4,396}$ (approx)= 11, p < 0.001) and in the species by pond interaction (Pillai= 0.59, $F_{16,396}$(approx)= 10, p < 0.001). Both δ15N and δ13C had a seemingly opposite tendency across populations, since in the expansion front differences among species were more exaggerated in δ15N than in δ13C, while at the other end – mid expansion area – differences were more exaggerated in δ13C but non-significant for δ15N (Table 3), and results from the far expansion area were found between the extremes in both cases (Table 3).

		***D. pictus*, means**		***E. calamita*, means**		**differences**		**P-values**		
pond	**area**	**δ15N**	**δ13C**	**δ15N**	**δ13C**	**δ15N**	**δ13C**	**δ15N**	**δ13C**	**global**
gi.arg.01	ME	5.39	-26.42	6.44	-24.63	-1.05	-1.79	***0.044***	***<0.001***	***<0.001***
gi.ari.01	ME	7.31	-21.75	6.13	-21.25	1.18	-0.50	***<0.001***	***0.001***	***<0.001***
gi.ver.01	ME	9.15	-23.10	8.12	-21.92	1.03	-1.18	***<0.001***	***<0.001***	***<0.001***
ri.bat.02	FE	0.68	-23.64	-0.95	-22.99	1.63	-0.65	***0.002***	***<0.001***	***<0.001***
ri.gre.02	FE	-4.43	-25.97	-3.82	-24.97	-0.61	-1.00	***<0.001***	***<0.001***	***<0.001***
ri.gre.06	FE	0.22	-25.97	-2.53	-24.63	2.75	-1.34	***<0.001***	***0.003***	***<0.001***
ri.gre.07	FE	2.66	-25.44	-0.65	-24.33	3.31	-1.11	***<0.001***	0.727	***<0.001***
ri.pon.01	FE	1.93	-29.21	0.69	-28.3	1.24	-0.91	***<0.001***	***0.035***	***<0.001***
ho.ari.02	EF	1.10	-25.02	0.27	-23.4	0.83	-1.62	0.288	***<0.001***	***0.019***
ho.duc.04	EF	2.84	-25.50	-0.37	-23.69	3.21	-1.81	***<0.001***	***<0.001***	***<0.001***
ho.mot.08	EF	3.16	-27.37	2.12	-27.8	1.04	0.43	***<0.001***	***0.002***	***<0.001***
ho.mot.10	EF	4.58	-27.10	2.69	-27.16	1.89	0.06	***<0.001***	***0.002***	***<0.001***

Table 1. Mean values for each isotope and species, and p-values for the interspecific differences for δ13C, δ15N and globally (both). Abbreviations: ME= mid expansion area, FE= far expansion area, EF= expansion front. In bold italics, significant differences with alpha=0.05.

Adding habitat information, the best LMM for δ13C included population, pond sunshine – plus its interaction with species – and the second summary variable for the vegetal community – plus its interaction with species as well (Fig S2, Table S2). According to the model averaged importance of terms – that is, the frequency of the presence of each term within the best 100 ponds (Fig S2) – the most important effects were the sunshine on pond, its interaction with species, and the second summary variable for the vegetal community. A second group in importance (Fig S2) comprises the interaction between this variable and species, population, and finally species, in that order and being this last term already below the 50% of support. The rest of terms can be considered to have no support (Figs S2). Thus, there were clear differences among study areas, while the mild tendency of *E. calamita* to display higher δ13C values than *D. pictus* is again present (Fig. S5). Pond shading had a very strong negative effect on δ13C – mainly once there is less than 70% of the pond usually under direct sunlight – and tends to homogenize the δ13C of both species (Fig. S3). On the other hand, the presence of macroscopic flocks of *Nostoc sp.* – in contraposition to the presence of the rest of vegetation types – tends to increase values of δ13C, affecting more clearly *D. pictus* (Fig S4).

Concerning δ15N, the best model included population – *i.e.* study area –, the first summary variable for the macroscopic vegetal community, species, the first summary variable for the animal community, and the interaction between the last two variables (Fig. S6, Table S3). All these terms had high support according to the model-averaged importance of terms, mildly lower for the vegetation (Fig. S7). Curiously, the presence of this variable in the ten best models was limited to the best one (Table S3), and its observable influence on δ15N was extremely mild, consisting in a mild increase with the absence of chlorophytes (Fig. S8). In contraposition, patterns created by the other selected variables were much more clear. Concerning animal communities, δ15N values rise with increasing presence of predators and mosquito larvae – with less cladocerans and ostracods –, being differences more exaggerate for *E. calamita* (Fig. S9). Population – *i.e.* study area – had again a clear effect, and the tendency to find higher δ15N in *D. pictus* was also again detected (Fig. S10). The rest of variables and interactions can be considered to have an extremely poor influence (Fig. S7).

		D.pictus				***E. calamita***				**P-value**			
pond	**area**	**MDC**	**MNN**	**TA**	**SEAc**	**MDC**	**MNN**	**TA**	**SEAc**	**MDC**	**MNN**	**TA**	**SEAc**
gi.arg.01	ME	0.669	0.255	0.705	0.404	1.454	0.786	7.100	5.331	***0.010***	***0.018***	***0.026***	***0.011***
gi.ari.01	ME	0.473	0.268	0.765	0.444	0.497	0.283	1.060	0.589	0.938	0.945	0.639	0.674
gi.ver.01	ME	0.361	0.223	0.410	0.225	0.288	0.158	0.315	0.174	0.489	0.366	0.937	0.964
ri.bat.02	FE	0.900	0.538	0.955	0.515	0.340	0.248	2.785	1.856	***0.005***	***0.035***	0.328	0.158
ri.gre.02	FE	0.369	0.164	10.285	6.223	1.321	0.688	1.425	0.921	***0.002***	***0.005***	0.106	0.154
ri.gre.06	FE	1.071	0.442	1.135	0.673	0.489	0.246	0.490	0.300	***0.001***	0.143	0.056	0.060
ri.gre.07	FE	1.314	0.429	0.960	0.546	0.990	0.399	0.380	0.241	0.387	0.850	0.451	0.546
ri.pon.01	FE	0.757	0.404	2.895	1.598	0.460	0.313	0.580	0.312	0.126	0.431	0.193	0.204
ho.ari.02	EF	0.562	0.277	0.380	0.262	0.931	0.494	4.075	2.130	***0.028***	0.062	0.214	0.267
ho.duc.04	EF	1.720	0.846	3.150	2.039	0.644	0.381	0.675	0.388	***0.003***	***0.039***	0.126	0.178
ho.mot.08	EF	0.596	0.343	1.935	1.265	0.387	0.209	3.400	1.719	0.114	0.180	0.472	0.817
ho.mot.10	EF	0.513	0.290	1.995	1.152	0.368	0.216	0.905	0.590	0.150	0.325	0.582	0.597

Table 2. Values for two dispersion measures (MDC=mean distance to centroid, MNN=mean neighbor distance, TA=Total Area, SEAc=corrected Standard Ellipse Area) for each species, and p-values for the interspecific differences. Area abbreviations: ME= mid expansion area, FE= far expansion area, EF= expansion front. In bold italics, significant differences with alpha=0.05.

Regarding dispersion measures, only two bivariate relationships were significant (Table S4). Concretely, there is a positive relationship between the second summary axis of animal community and the SEAc of *E. calamita*, meaning that the SEAc increases with the presence of cyperaceae and characeae and diminished presence of nostoc (Fig. S11). On the other hand, there is a postive relationship among the first summary axis of vegetation and the SEAc of *D. pictus* (Fig. S12), meaning that the SEAc increases with more diversity of vegetal items in contraposition with the abundant presence of clorophytes. Neither MNND (Table S4, Fig S13) nor values of SEAc show any clear pattern according to study area ($F_{2,16} = 0.351$, p = 0.852), between species ($F_{1,16} = 0.652$, p = 0.293) or in their interaction ($F_{2,16} = 1.231$, p = 0.206).

	difference value		**F**				**P-value**	
Population	***δ13C***	***δ15N***	***δ13C***	***δ15N***	***df (F)***		***δ13C***	***δ15N***
Mid expansion	1.156	0.387	57.89	2.85	1	56	0.0001	0.0972
Far expansion	1.002	1.664	66.98	57.40	1	94	0.0001	0.0001
Expansion front	0.377	1.253	4.93	82.29	1	56	0.0305	0.0001

Table 3: Mean differences in isotopic signature values, and results of test inlcuding pond as random effect:

Discussion

Recent studies in tadpole trophic ecology using stable isotopes analysis (SIA) have been already successful in studying the effects of several factors on the trophic ecology of tadpole guilds, including competitive interactions (Whiles *et al.* 2006, Schiesari *et al.* 2009, Caut *et al.* 2013, Arribas *et al.* 2015, San Sebastián *et al.* 2015a). We here explore possible short-term evolutionary changes in this trophic ecology, studying the same native-invasive competitive relationship across populations with different co-evolutionary history. Interestingly, the trophic segregation between both studied species is clear, and different among populations and ponds (Table 1). While the magnitude of trophic segregation does not seem to vary with time since invasion (Table S3, Figure

S13), its nature does, tending to progressively segregate rather in the carbon signature – *i.e.* type of items consumed – than in the nitrogen signature – *i.e.* trophic level. As it will be discussed nonetheless, with the dataset used hitherto, this interesting pattern can also be alternatively explained under an ecological point of view.

In studies of animal ecology, the carbon signature (δ13C) has been traditionally considered as a suitable indicator of the origin of the organic matter consumed by the study species, both because it has low values of fractionation – meaning few differences due to the trophic level –, and because of important differences in the carbon assimilation patterns across several vegetation typologies and natural habitats (Tieszen *et al.* 1983, Ambrose & DeNiro 1986, Griffith 1992, Koch 1995, Gannes *et al.* 1997). In our study, we found great variation in δ13C (Fig. 1), but the main factor inducing these differences was the pond – and more mildly, the study area, *i.e.* the population – in which tadpoles were collected. This is most probably a combined effect of a different phenology – ponds had to be sampled asynchronously – and different surrounding vegetation, which can be an important source of organic matter in the pond (Syväranta *et al.* 2006, Woodland *et al.* 2012).

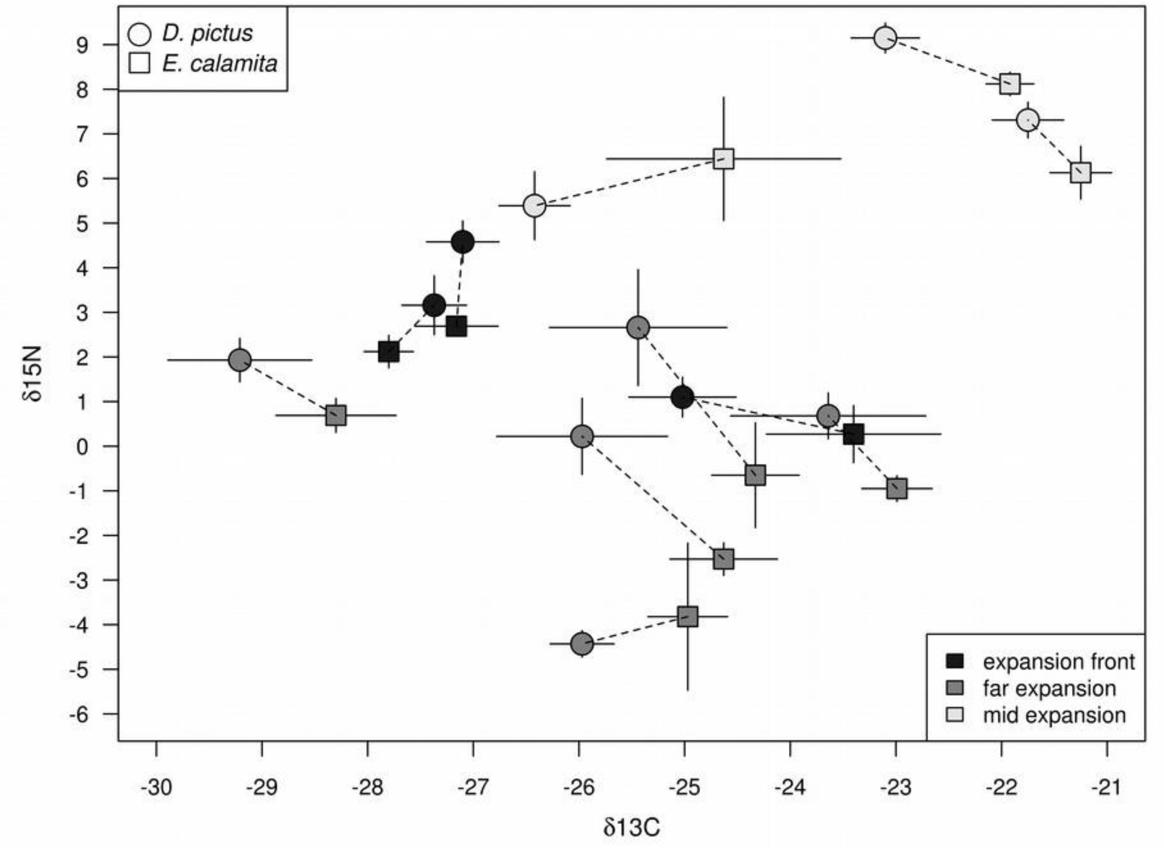

Figure 1. Centroid and standard deviation for both isotopes, for each species in each pond. Dotted lines link tadpole groups from the same pond.

More interestingly, species displayed significant intra-pond differences in δ13C, in all cases except one (Fig. 1, Table 1). In addition, nearly all differences are in the same direction – higher δ13C values in the native toad, coinciding with San Sebastian *et al.* 2015a – and tend to increase as the invasion is more ancient (Table 3). However, two habitat features can alternatively explain these patterns through differential effects across species: the proportion of the pond under direct sunlight, and the composition of the macroscopic vegetal community (Fig. S2, Fig. S4 Table S2). Hence, mean δ13C values tend to drop with increased shading in ponds, at the same time diminishing interspecific differences (Fig. S3). On the other hand, when the macroscopic vegetal community increases its diversity – in contraposition to being mainly represented by *Nostoc sp.* (Fig. S4) – interspecific differences in values of δ13C tend to increase accordingly. Thus, from an ecological point of view, we can interpret this as a dependence of the interspecific segregation in δ13C on the presence of abundant sunlight in the pond – possibly providing richer microscopic algal communities – and a diverse macroscopic vegetal community. From an evolutionary point of view, the examination of the full dataset once it is available will determine if differences indeed correspond only to ecological patterns.

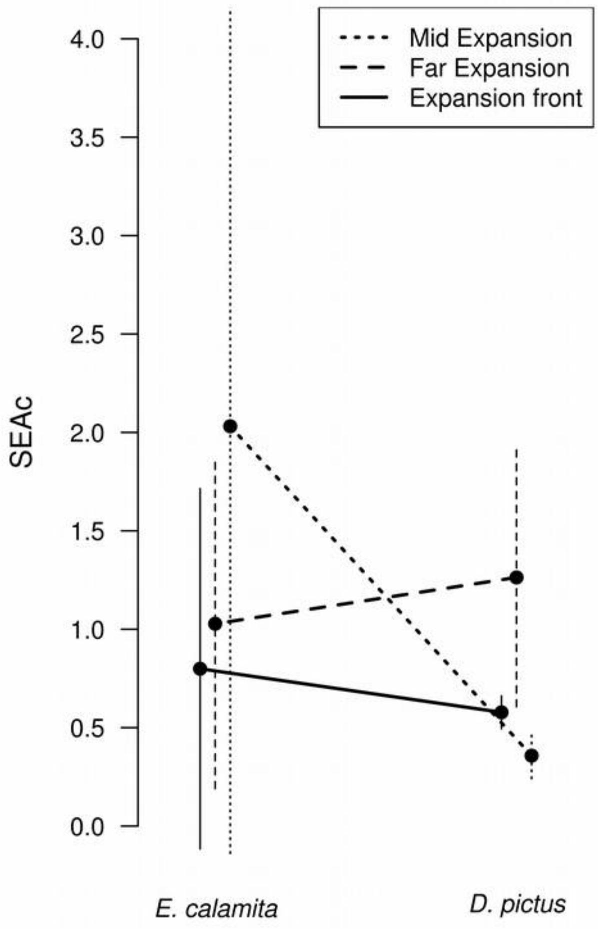

Figure 2. Corrected Standard Ellipse Area (SEAc) by species and area. Means are represented with a dot and vertical lines represent standard deviations. Horizontal lines connect data from the same area of study.

On the other hand, nitrogen signature (δ15N) has been repeatedly found to relate to the trophic level of a consumer, increasing its values from primary consumers to top predators due to high isotopic fractionation (Minagawa & Wada 1984, Ambrose & DeNiro 1986). In this case, we also found great variation in δ15N across populations and ponds (Fig. 1, Fig. S6, Table S2). The possibility that both tadpole species use a more carnivore strategy under some circumstances or in some populations is easily discarded by observing matching variations in δ15N of vegetal items, primary consumers and potential predators (Figs S14 - S16). Therefore, δ15N variation among ponds and areas has to be again considered a consequence of the natural variation of isotopic baselines along time and space (Syväranta *et al.* 2006, Woodland *et al.* 2012). As expected, tadpoles' δ15N usually falls after correcting for fractionation on the raw δ15N values for producers, posing tadpoles as mainly primary consumers which occasionally ingest animal elements (Figs S14 – S16, Diaz-Paniagua 1985, Campeny 2001, Caut *et al.* 2013, Arribas *et al.* 2014). More interestingly, species displayed intra-pond differences in δ15N in all cases but one (Table 1, Fig. 2) and in eight out of ten cases, it was the the invasive species that held a higher trophic level. This tendency (also observed in San Sebastian *et al.* 2015a) is still apparent after incorporating ecological variables in the analyses, and it tends to diminish as the invasion is more ancient (Table 3). In this case, it is the accompanying animal community (Table S2, Fig. S7) that can alternatively explain this pattern. Tadpoles of the invasive *D. pictus* seemingly hold similar δ15N regardless of the animal community present in the pond, while native *E. calamita* increase their δ15N in communities with predators and mosquito larvae – in contraposition to the dominance by ostracods or cladocerans. This translates in mild δ15N differences under increased predation risk (Fig. S9), which could be related to interspecific variation in the stress levels provoked by trorphic competition (San Sebastian et al. 2015a), or in the direct effects of predation (Pujol-Buxó *et al.* 2017). On the other hand, the effects of the vegetal community on δ15N suggested by model selection are suspiciously mild and only occasionally present in the best set of ten models. Thus, although in this case there are also alternative ecological explanations to interpopulation differences, patterns are less open to clear interpretation. This increases the likelihood of evolutionary patterns, but anyhow the addition of the four ponds of the origin area is again totally necessary to discern between possibilities.

Finally, another important aspect that may signal differences across populations in trophic ecology is the isotopic niche width. Interspecific differences in dispersion measures were, in this case, more uncommon and lacking a common direction (Table 2). Therefore, with the present data, we discard the hypothesis by San Sebastian *et al.* [2015] that the invasive species is usually more plastic in terms of niche width. Variations according to years since invasion were also weak or lacking (Fig. 2). We expected wider trophic niches in the expansion front due to greater trophic overlaps (Codron *et al.* 2011, Abbey-Lee *et al.* 2013, San Sebastian *et al.* 2015a), narrowing as competitors progressively adapt to segregate ecologically (Van Valen 1965, Bolnick *et al.* 2010). Thus, the lack of interpopulation differences in niche width could simply match the lack of interpopulation variations in the mean isotopic distance between competitors. Finally, the only instances in which a significant relationship between niche width (SEAc) and habitat features were found were related to the diversity of vegetal community present on the pond, being the SEAc of both species influenced by different summary axes: native *E. calamita* increased its SEAc mainly with the presence of Cyperaceae and Characeae – in contraposition to ponds dominated by *Nostoc sp.* –, while *D. pictus* increased its SEAc in response to a general increase in the diversity of macroscopic vegetation – in contraposition to ponds dominated by filamentous Clorophyta. Thus, regarding isotopic niche width, variations are generally scarce and dominated by ecological factors, with remote possibilities for variations linked to previous evolutionary history.

Conclusions

Invasive *D. pictus* and native *E. calamita* always segregate in their mean trophic position in ponds, regardless of the study area. This is, most probably, signalling an ecological segregation – either through phenotypic plasticity or direct trophic competition – created from the very onset of the invasion. Differences in niche width across species and populations were scarce and seemingly unrelated to co-evolutionary patterns. Both results together discard the hypothesis of a progressive separation and specialization in this competitive system. As an alternative co-evolutionary pattern, not all ponds show the same direction of ecological segregation, with a progressive tendency to segregate more within the same trophic level – δ13C – than in the trophic level itself – δ15N. Nevertheless, with the present dataset, this interesting pattern can alternatively be explained with interspecific differences in the influence of accompanying vegetal and animal communities. The addition of five more ponds in the study will help to clarify the relative importance of ecology and evolution in this system.

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Chapter 7: Supporting Information

Supplementary Figures

Fig S1. Support of the ranked 100 best models explaining δ13C according to the corrected Akaike Information Criterion (AICc):

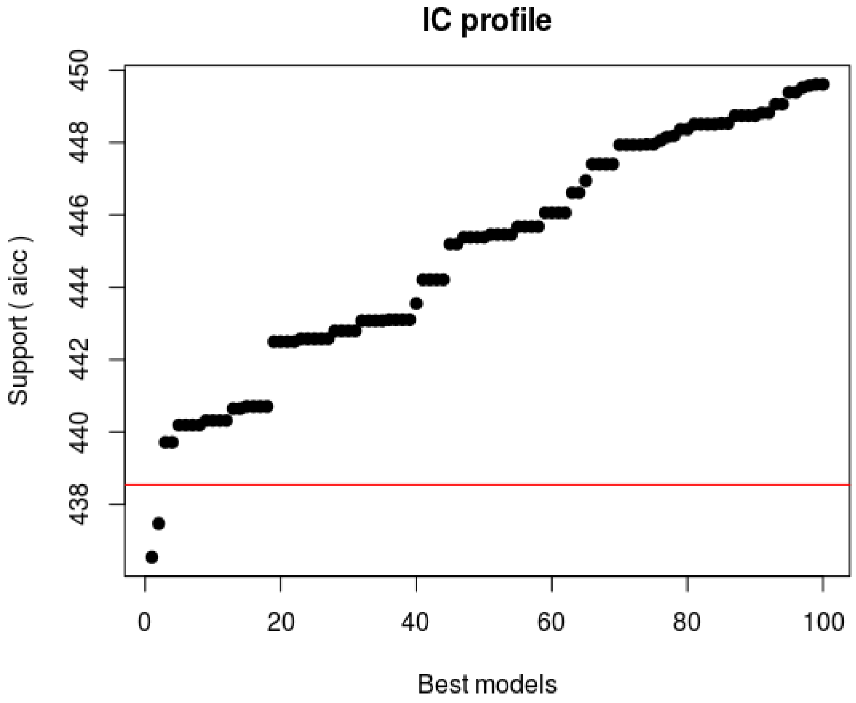


Fig. S2. Model-averaged importance of terms (proportion of presence in the best 100 models) explaining δ13C. Abbreviations: pop=population, study area; PCanimalsX: X summary variables of the animal community, PCresourcX: X summary variables for macroscopic vegetation community.

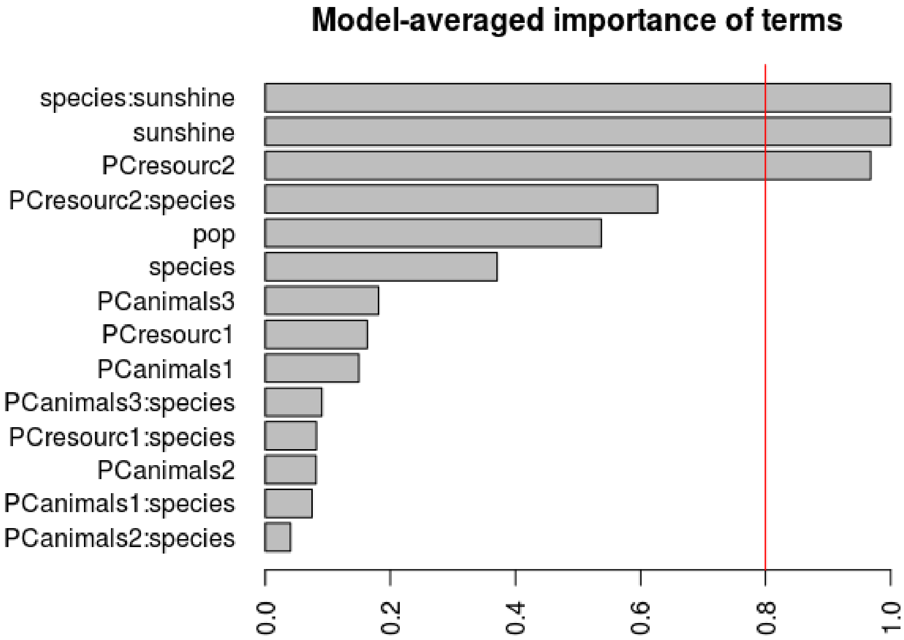


Fig S3. Effect of the sunshine on pond on δ13C of both species. Linear regressions (lines) are added help to ease visualization of the interaction among species and sunlight.

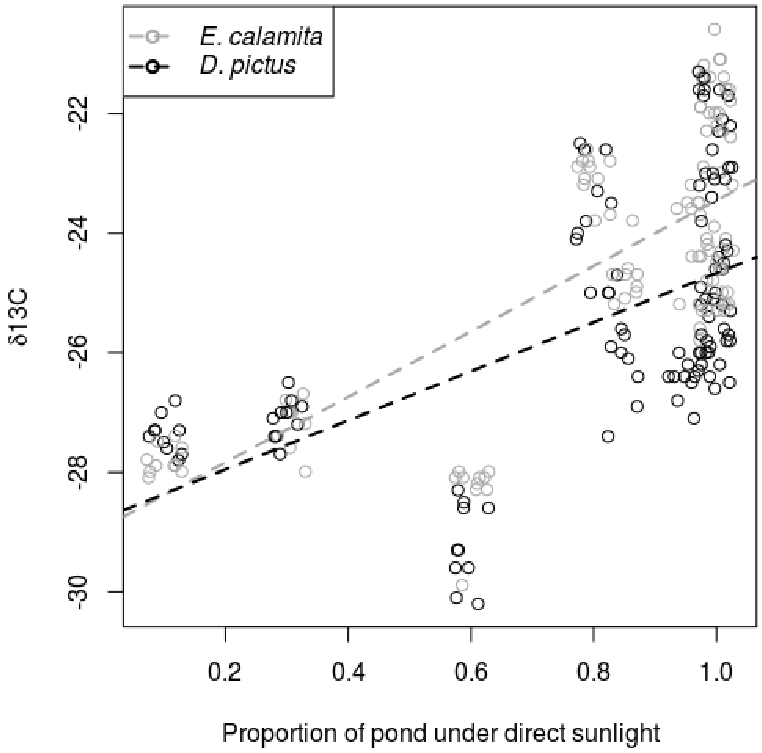


Fig S4. Effect of the second summary variable of vegetation in ponds on δ13C of both species. Linear regressions (lines) are added help to ease visualization of the interaction.

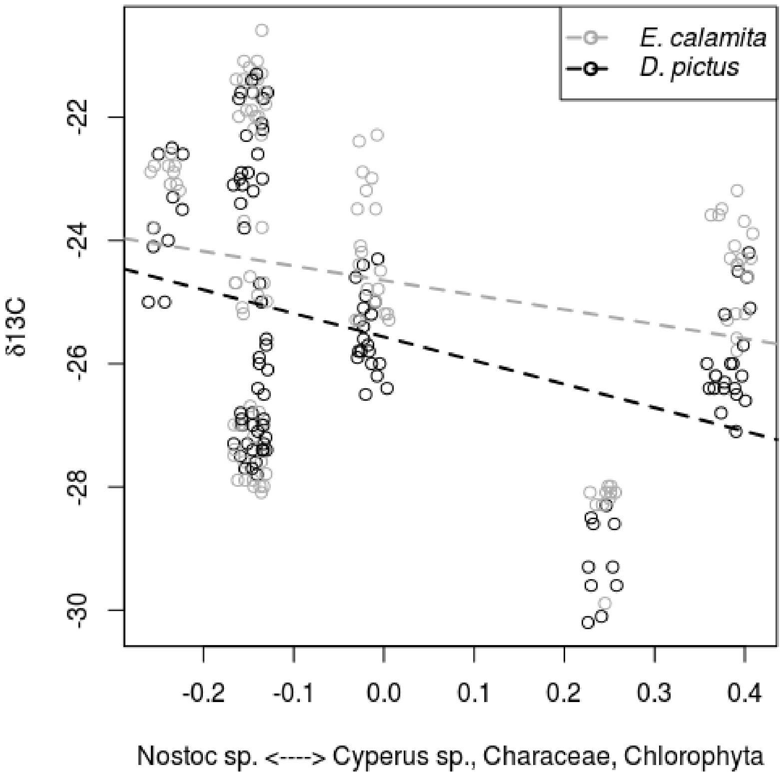


Fig S5. Fitted values of δ13C according to the best model:

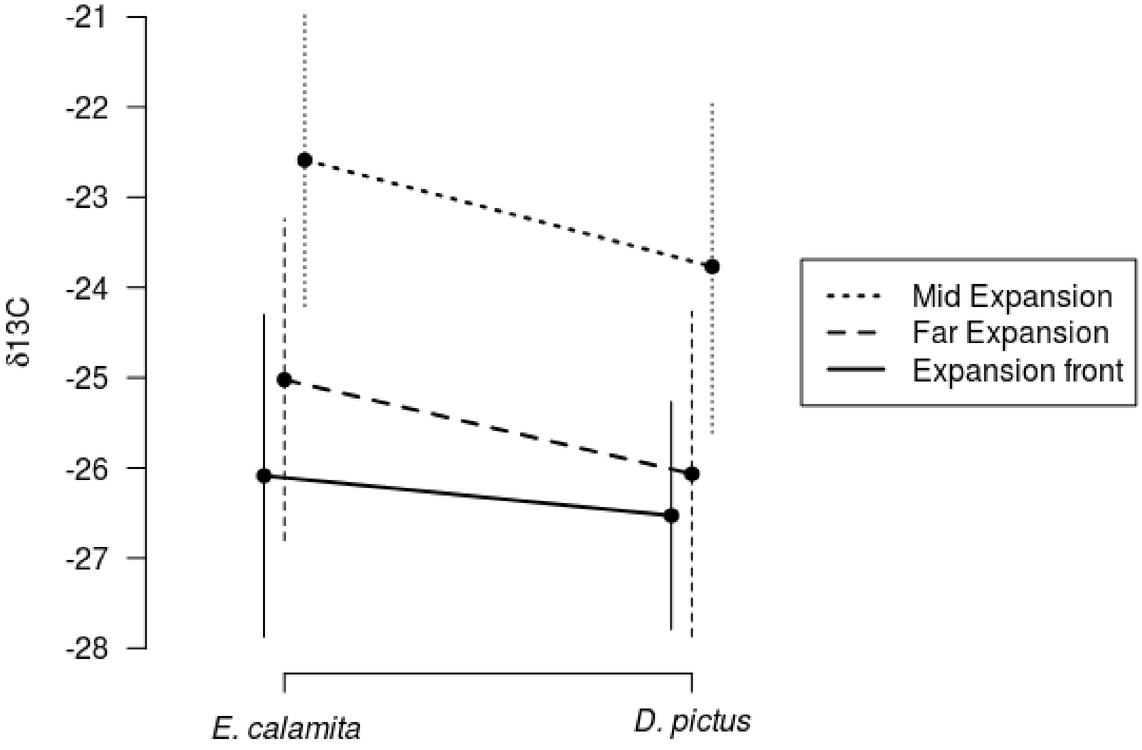


Fig S6. Support of the ranked 100 best models explaining δ15N according to the corrected Akaike Information Criterion (AICc):

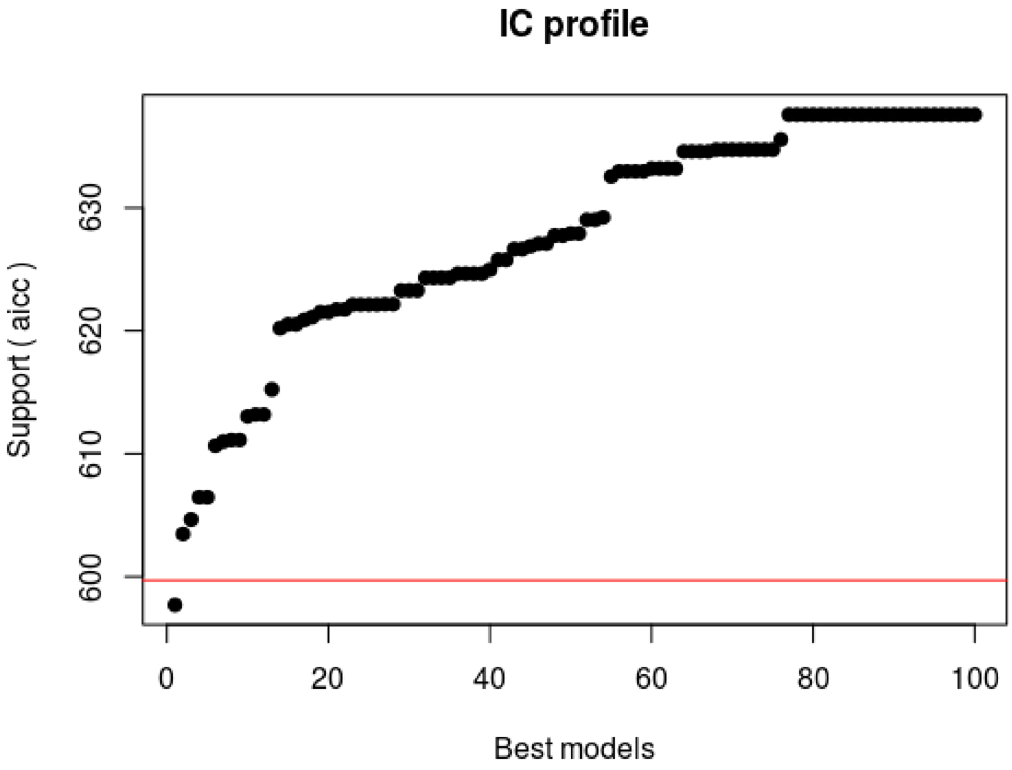


Fig S7. Model-averaged importance of terms (proportion of presence in the best 100 models) explaining δ15N. Abbreviations: pop=population, study area; PCanimalsX: X summary variables of the animal community, PCresourcX: X summary variables for macroscopic vegetation community.

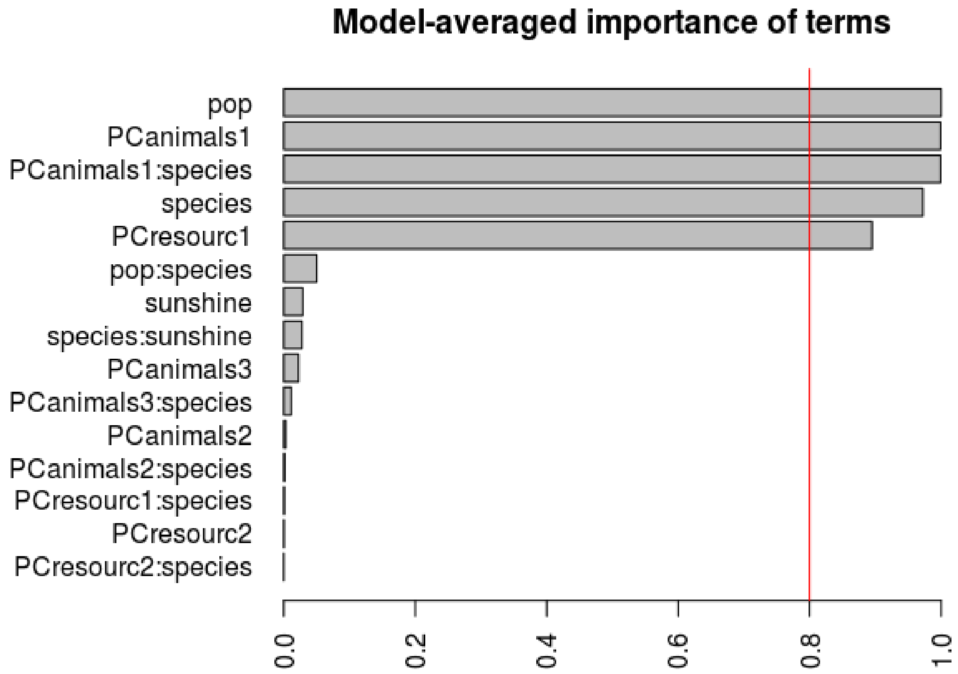


Fig S8. Effect of the first summary variable of vegetation in ponds on δ15N of both species:

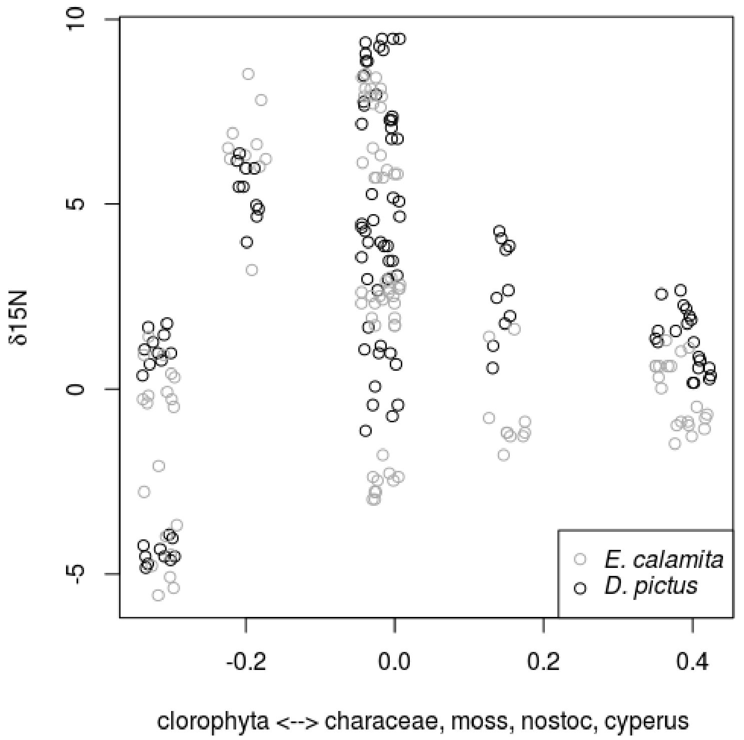


Fig S9. Effect of the first summary variable of the animal community in ponds on δ15N of both species. Linear regressions (lines) are added help to ease visualization of the interaction.

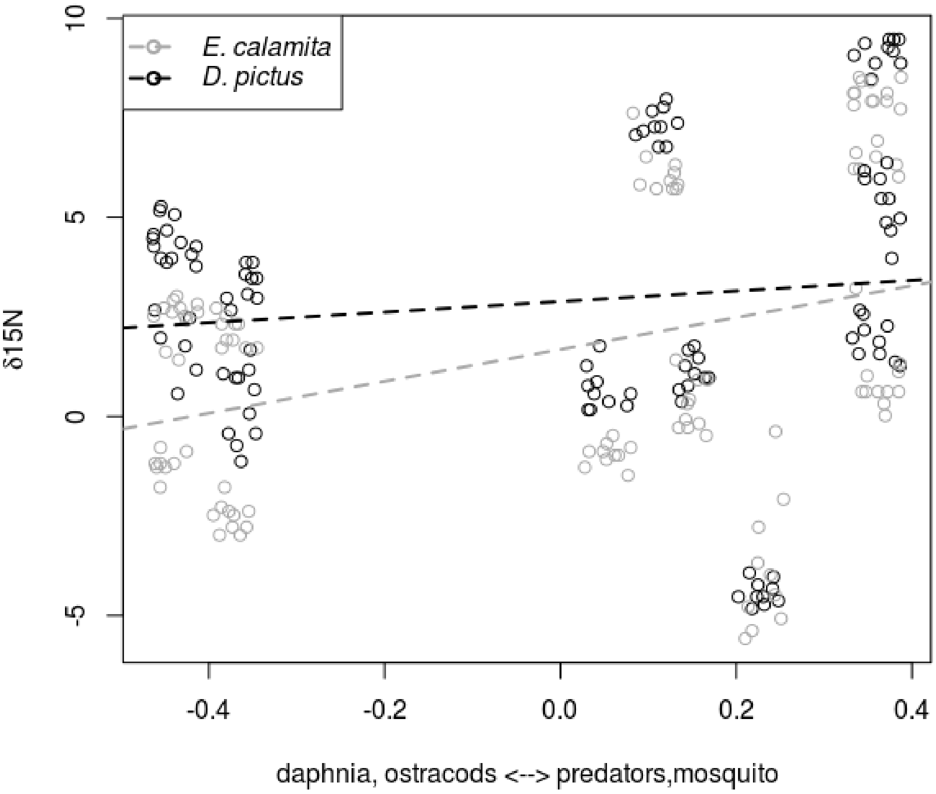


Fig S10. Fitted values of δ15N according to the best model:

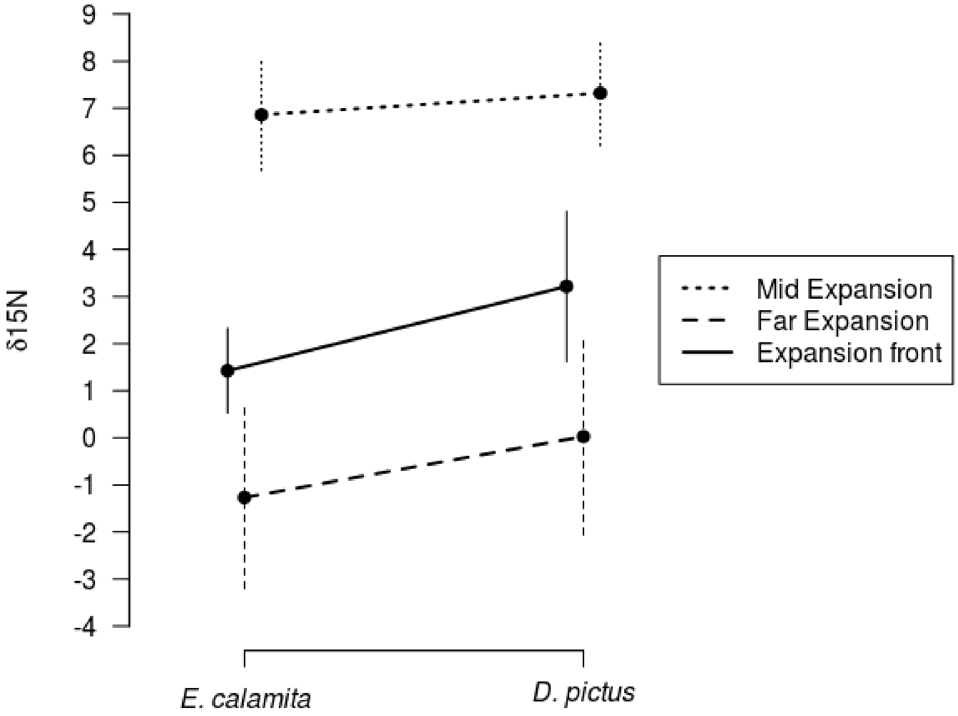


Fig S11. Relationship between the SEAc of *E. calamita* in a pond and the second summary axis of the vegetal community in a pond. The schematic interpretation of the axis is expressed in the x-axis label.

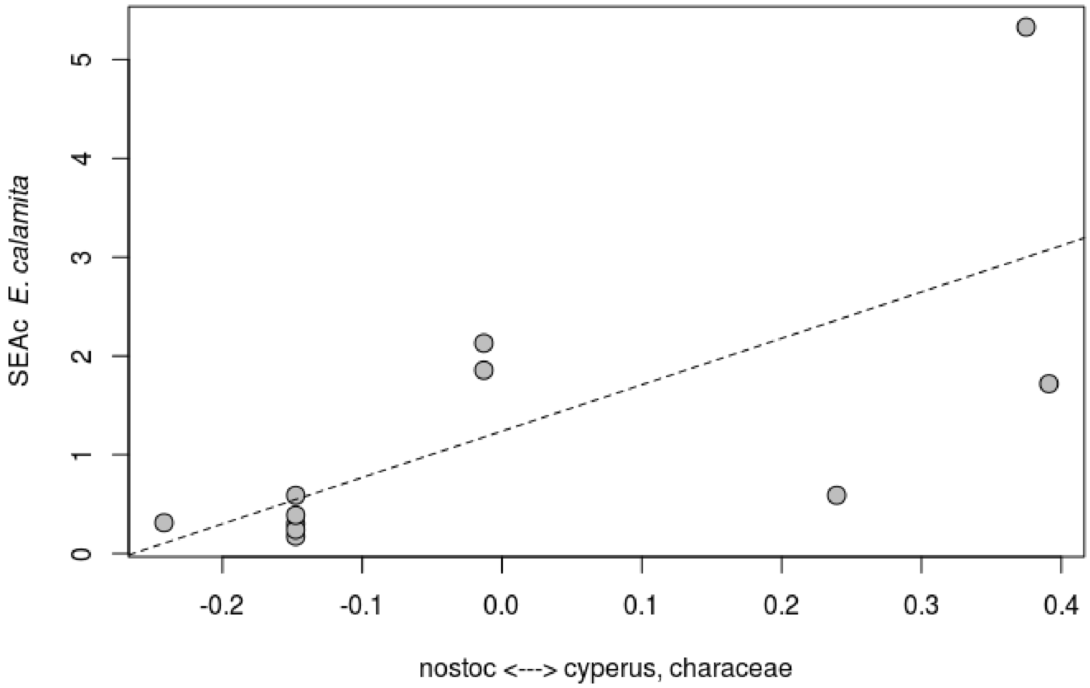


Fig S12. Relationship between the SEAc of *D. pictus* in a pond and the first summary axis of the vegetal community in a pond. The schematic interpretation of the axis is expressed in the x-axis label.

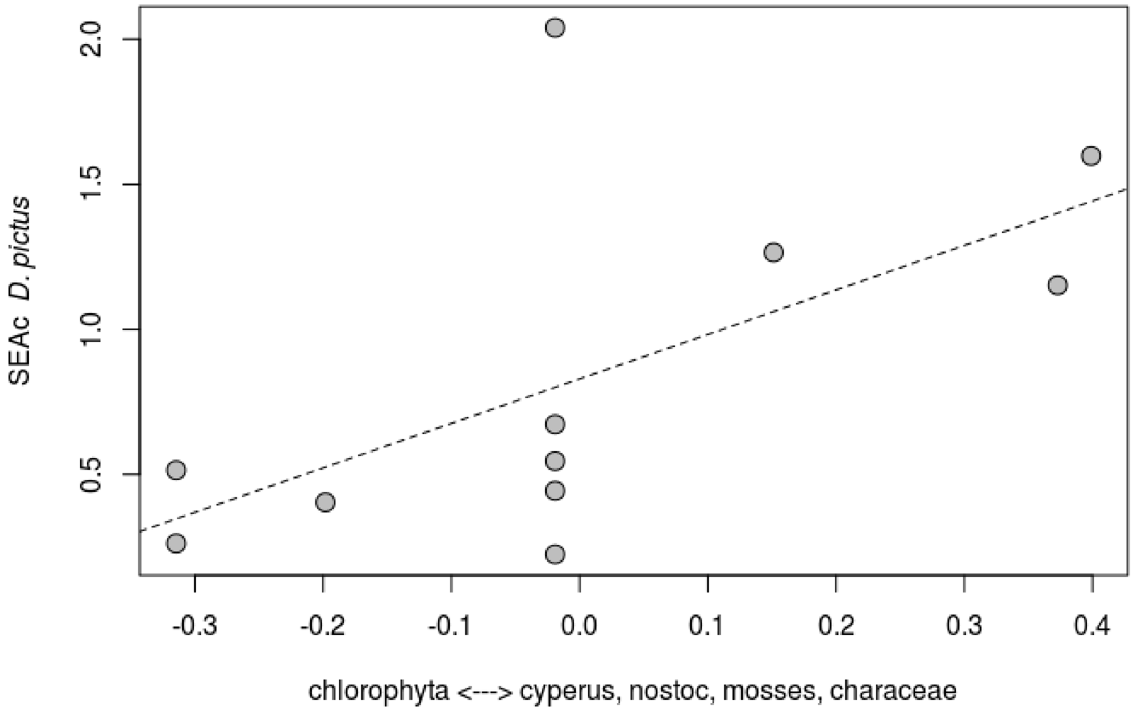


Fig S13. Mean nearest neighbour distance – in this two species community, equivalent to the distance among the centroids of both species – across populations (areas of study). Abbreviations: EF: Expansion front, FE= far expansion, ME= mid expansion.

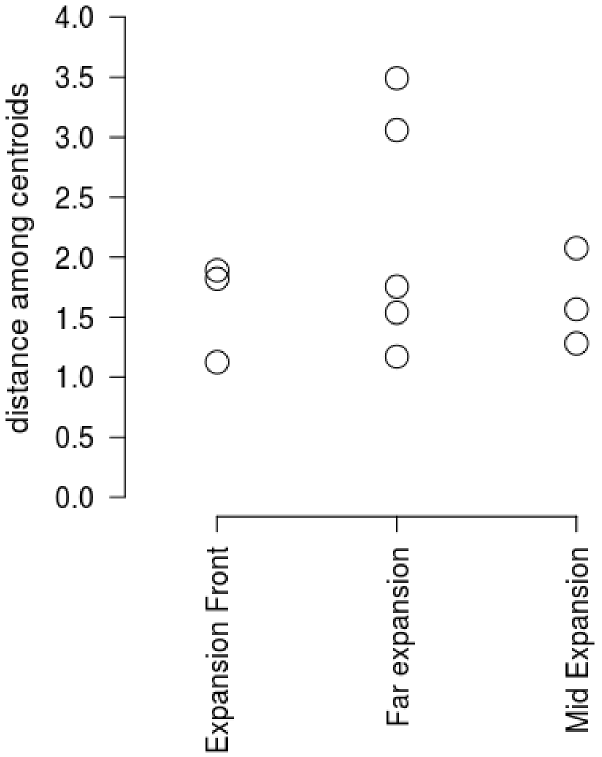


Fig S14. Isotopic values for individuals on the expansion front, and accompanying species.

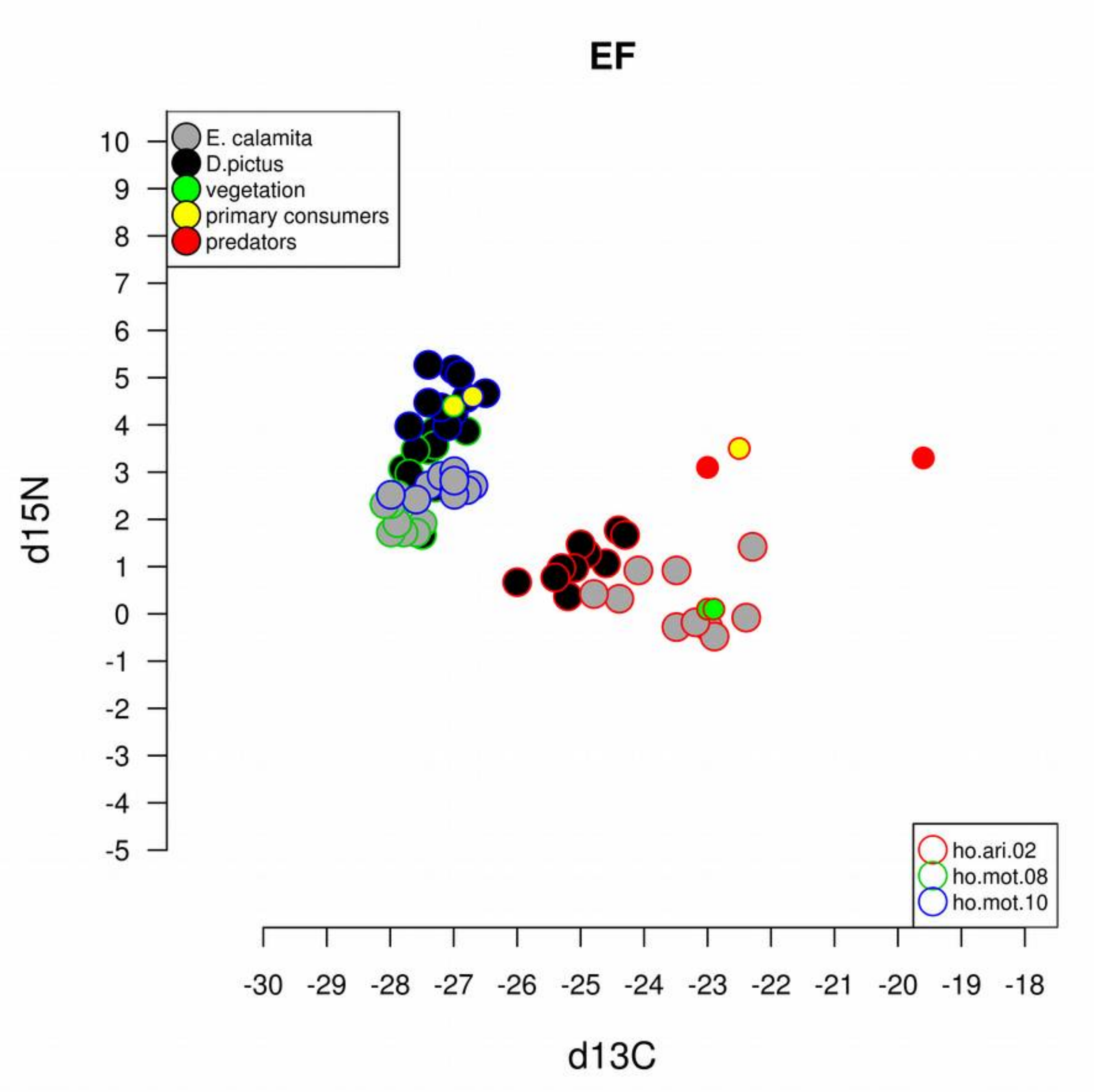


Fig S15. Isotopic values for individuals on the far expansion area, and accompanying species.

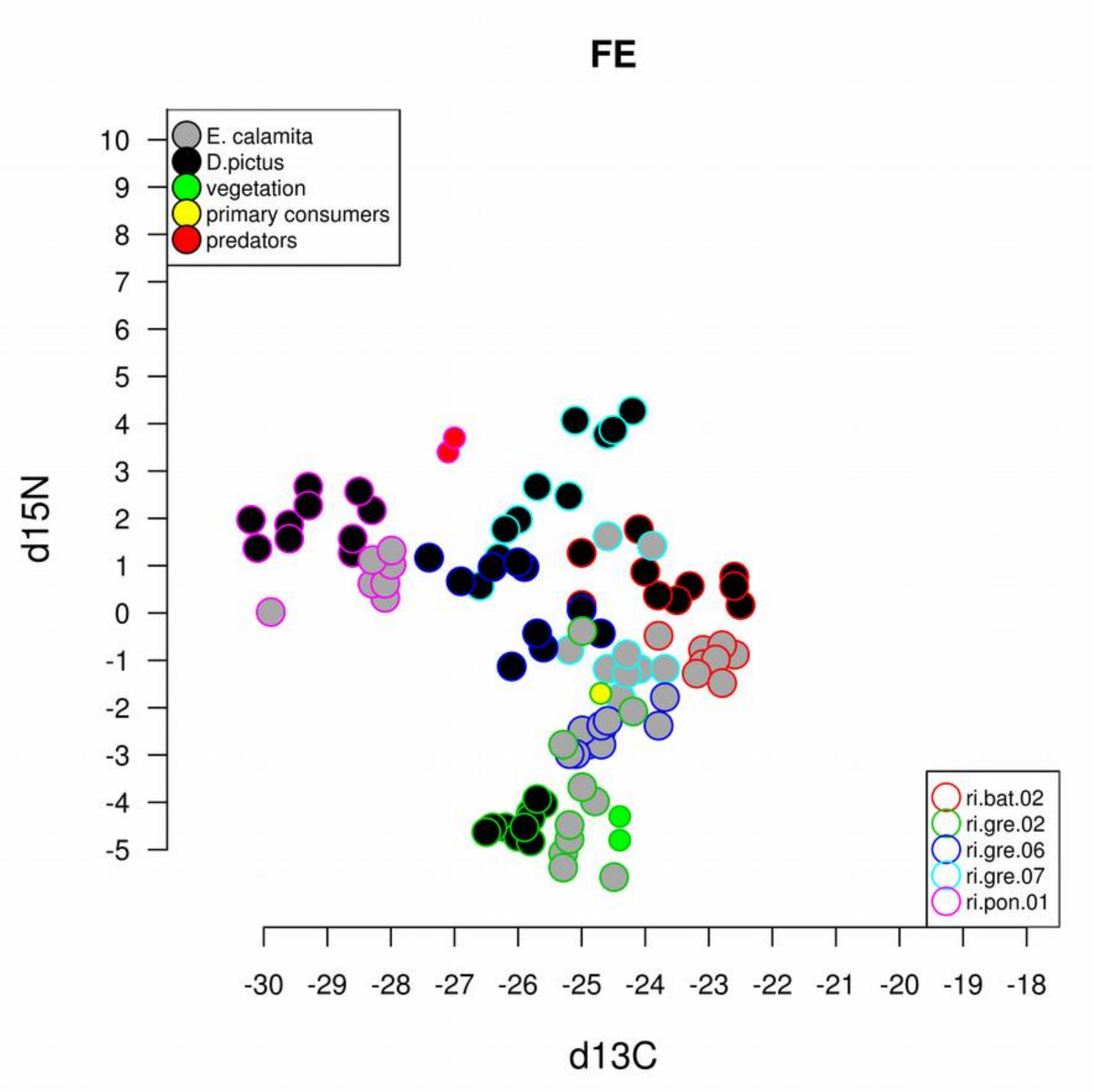
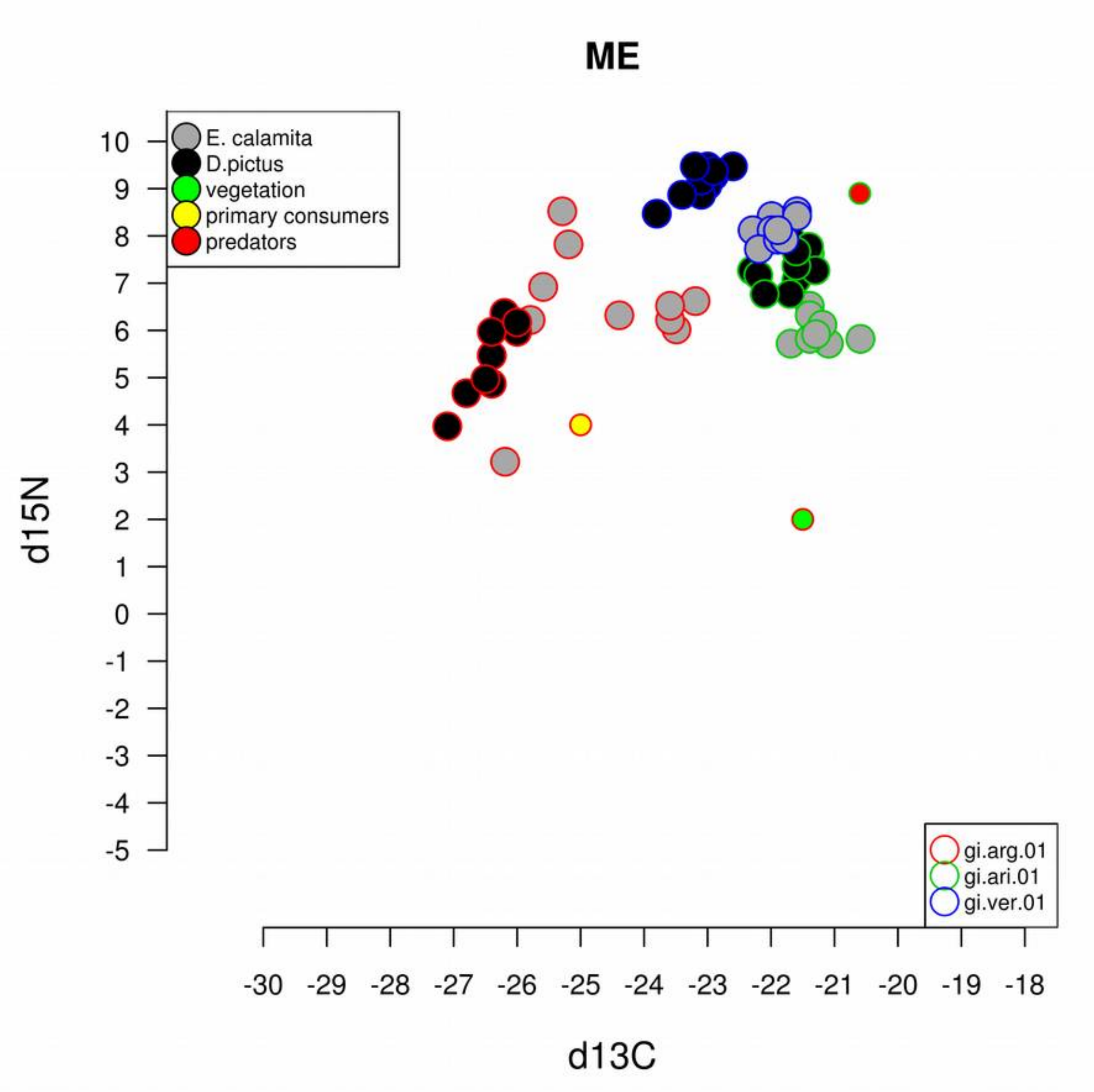


Fig S16. Isotopic values for individuals on the mid expansion area, and accompanying species.



Supplementary Tables

Table S1. Locations and main features of ponds studied hitherto (abbreviations of the accompanying species: cl= clorophyta; ml= mosquito larvae; sym= sympetrum nymphs, sn=snails, daph= daphnia, dy= dytiscus larvae; bra= branchypus):

Pond	Area	Near (town/city)	Latitude (degrees)	Longitude (degrees)	Surface (m2)	Depth (m)	Sunshine (%)	Other species sampled
gi.arg.01	Mid Expansion	Girona	41.9986	2.8441	3.1	0.1	0.95	cl, ml
gi.ari.01	Mid Expansion	Girona, Salt	41.9781	2.7617	4.1	0.2	1	dy,
gi.ver.01	Mid Expansion	Girona, Cartellà	42.0145	2.7534	3.1	0.3	1	
ri.bat.02	Far Expansion	Riudarenes	41.8239	2.6829	4.0	0.2	0.8	
ri.gre.02	Far Expansion	Riudarenes, Sils	41.7965	2.6960	0.9	0.1	1	cl, bra
ri.gre.06	Far Expansion	Riudarenes, Sils	41.8036	2.6871	4.3	0.1	0.85	
ri.gre.07	Far Expansion	Riudarenes, Sils	41.8033	2.6871	6.9	0.15	1	
ri.pon.01	Far Expansion	Riudarenes, Sils	41.7985	2.7061	7.9	0.1	0.6	dy,
ho.ari.02	Expansion Front	Hostalric, St.Celoni	41.7221	2.5647	6.9	0.3	1	cl, sym, sn
ho.mot.08	Expansion Front	Hostalric, St.Celoni	41.7189	2.5506	5.2	0.15	0.1	daph
ho.mot.10	Expansion Front	Hostalric, St.Celoni	41.7192	2.5502	7.4	0.15	0.3	daph

Table S2. Model selection for d13C. Empty cells indicate that the element is not present on the model, written cells indicate the element i present (Abbreviations: sp = species; pop = population, study area; sunsh= sunshine; VegAx1, VegAx2 = first and second axes summarizing the variability in macroscopic vegetal communities [see Methods]; AnAx1, AnAx2, AnAx3= first, second and third axes summarizing the variability in macroscopic animal communities [see Methods]):

model	sp	pop	sunsh	VegAx1	VegAx2	AnAx3	sp:sunsh	sp:VegAx2	sp:AnAx3	AICc	dAICc
1		pop	sunsh		VegAx2		sp:sunsh	sp:VegAx2		436.54	0
2			sunsh		VegAx2		sp:sunsh	sp:VegAx2		437.47	0.93
3	sp	pop	sunsh		VegAx2		sp:sunsh			439.71	3.17
4	sp	pop	sunsh		VegAx2		sp:sunsh	sp:VegAx2		439.71	3.17
5	sp		sunsh		VegAx2	AnAx3	sp:sunsh	sp:VegAx2		440.19	3.65
6	sp		sunsh		VegAx2	AnAx3	sp:sunsh		sp:AnAx3	440.19	3.65
7	sp		sunsh		VegAx2	AnAx3	sp:sunsh	sp:VegAx2	sp:AnAx3	440.19	3.65
8	sp		sunsh		VegAx2	AnAx3	sp:sunsh			440.19	3.65
9		pop	sunsh	VegAx1	VegAx2		sp:sunsh			440.31	3.77
10		pop	sunsh	VegAx1	VegAx2		sp:sunsh	sp:VegAx2		440.31	3.77

Table S3. Model selection d15N. Empty cells indicate that the element is not present on the model, written cells indicate the element i present (Abbreviations: sp = species; pop = population, study area; sunsh= sunshine; VegAx1, VegAx2 = first and second axes summarizing the variability in macroscopic vegetal communities [see Methods]; AnAx1, AnAx2, AnAx3= first, second and third axes summarizing the variability in macroscopic animal communities [see Methods]):

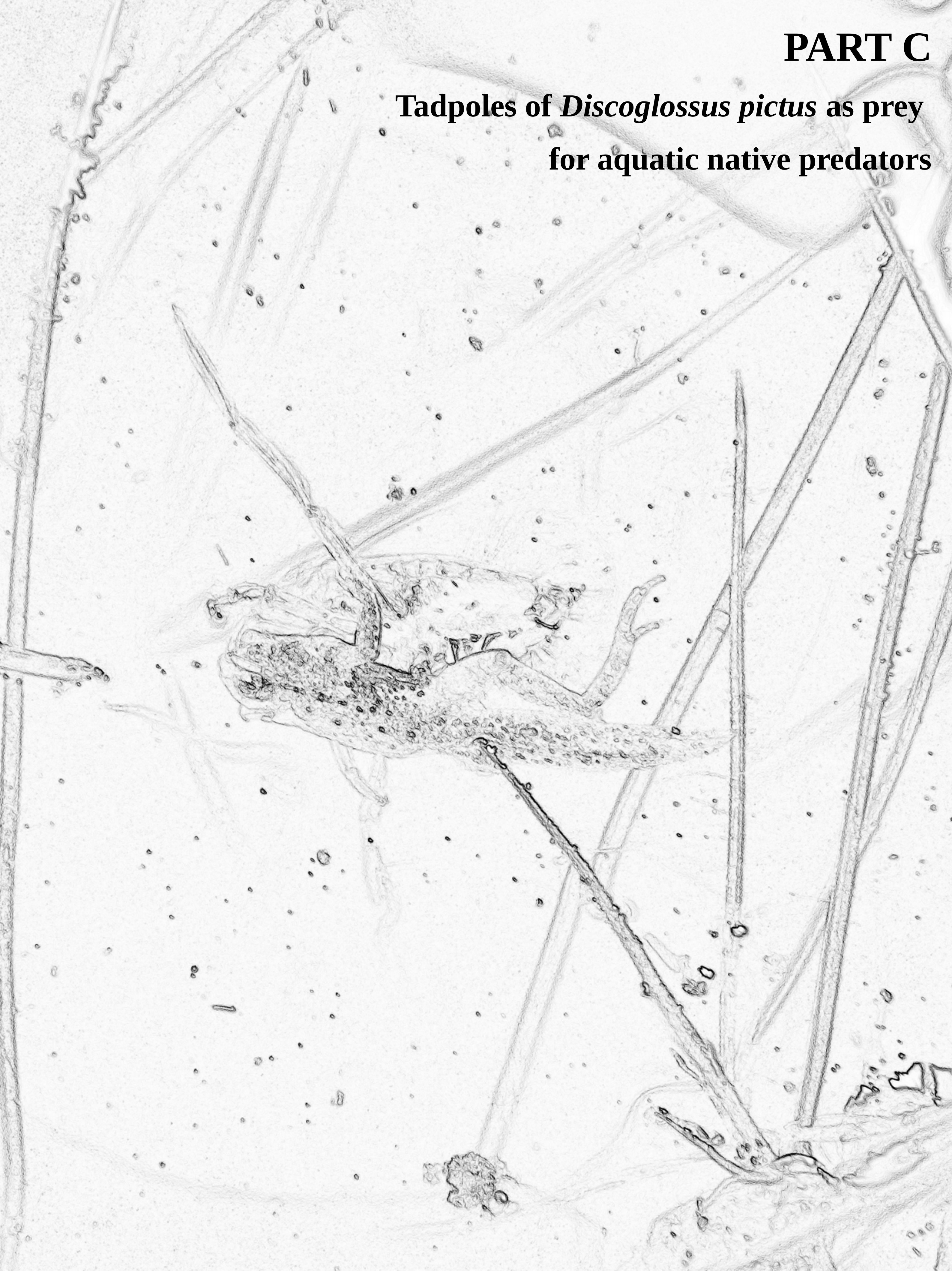
model	sp	pop	AnAx1	sp:AnAx1	other elements		AICc	dAICc
1	sp	pop	AnAx1	sp:AnAx1	VegAx1		597.70	0.00
2	sp	pop	AnAx1	sp:AnAx1	pop:sp		603.47	5.77
3		pop	AnAx1	sp:AnAx1	sunsh	sp:sunsh	604.65	6.95
4	sp	pop	AnAx1	sp:AnAx1	AnAx3		606.46	8.75
5	sp	pop	AnAx1	sp:AnAx1	AnAx3	sp:AnAx3	606.46	8.75
6	sp	pop	AnAx1	sp:AnAx1	sunsh		610.65	12.95
7	sp	pop	AnAx1	sp:AnAx1			610.99	13.29
8	sp	pop	AnAx1	sp:AnAx1	AnAx2		611.12	13.42
9	sp	pop	AnAx1	sp:AnAx1	AnAx2	sp:AnAx2	611.12	13.42
10	sp	pop	AnAx1	sp:AnAx1	VegAx2		613.04	15.34

Table S4. Results of univariate regressions on three variables expression dispersion (top, abbreviations: MNND = Mean nearest neighbour distance, in this case, distance between the centroid of both species; dpi.SEAc: standard ellipse area for *Discoglossus pictus;* eca.SEAc: standard ellipse area for *Epidalea calamita*) on eleven possible explanatory variables (left, abbreviations: Pop = population, study area; RatioSp = ratio of abundance among both species in the pond [see Methods]; Dens = density of tadpoles in the pond; Surf= square root of the pond surface, in m; Depth= pond maximum depth, in m; Sunsh = sunshine; VegAx1, VegAx2 = first and second axes summarizing the variability in macroscopic vegetal communities [see Methods]; AnAx1, AnAx2, AnAx3 = first, second and third axes summarizing the variability in macroscopic animal communities [see Methods]). In bold italic, significant relationships with alpha=0.05:

	Dep.		**MNND**		**dpi.SEAc**		**eca.SEAc**	
Indep.	**df(F)**		**F**	**p**	**F**	**p**	**F**	**p**
Pop	2	9	0.759	0.4991	4.066	0.0605	0.514	0.6166
RatioSp	1	10	2.961	0.1194	1.218	0.2983	0.752	0.4085
Dens	3	8	1.126	0.4014	0.357	0.786	0.677	0.5932
Surf	1	10	0.841	0.3831	0.685	0.4292	0.819	0.3892
Depth	1	10	0.354	0.5667	1.063	0.3294	0.752	0.4083
Sunsh	1	10	0.763	0.4051	0.027	0.8725	1.648	0.2314
VegAx1	1	10	0.225	0.6468	***5.259***	***0.0475***	2.671	0.1366
VegAx2	1	10	1.999	0.191	0.014	0.9085	***8.471***	***0.0173***
AnAx1	1	10	3.021	0.1162	2.18	0.174	1.437	0.2612
AnAx2	1	10	0.946	0.3562	0.576	0.4675	0.818	0.3893
AnAx3	1	10	0.023	0.8829	1.19	0.3037	0.575	0.4679

PART C

Tadpoles of *Discoglossus pictus* as prey for aquatic native predators



PART C

Tadpoles of *Discoglossus pictus* as prey for aquatic native predators

Chapter 8

How does the invasive/native nature of species influence tadpoles plastic responses to predators?

[published in *Oikos*]

Although the purely ecological impacts of biological invasions have been well studied, a less thorough effort has been made in terms of their evolutionary ecology. Previous studies show that anti-predator phenotypic plasticity may be one of the major ecological forces driving survival and rapid evolution of prey facing new predators. In turn, this means that biological invasions embody a perfect case for studying the trade-off s and evolution of phenotypic plasticity *per se*. Here, we studied the plastic responses of native (*Pelodytes punctatus*) and invasive (Discoglossus pictus) anurans facing a native (dragonfly *Anax sp.*) and two invasive (fish *Gambusia holbrooki* and crayfish *Procambarus clarkii*) predators. Marked responses were reported against the native predator from both the native and the invasive anuran, but they both responded mildly to the exotic predators as well. Native *P. punctatus* displayed a morphological reaction to invasive *P. clarkii* after scarcely 30 years of coexistence with this predatory crayfish and responded behaviourally to the invasive fish *G. holbrooki*. Invasive Discoglossus pictus reacted behaviourally to all predators, but unexpectedly only reacted morphologically to native *Anax sp.*. All these results support high prey – predator specificity in these reactions and an evolutionary dissociation between behavioural and morphological plasticity in anurans. Each species displayed a particular set of trade-offs between plastic responses and their costs, which is probably due to differences in ecological niche and evolutionary history, but interestingly we usually detected unexpected patterns in combinations using introduced predators. This suggests that perhaps singular plastic shifts usually occur when tadpoles face recently introduced species. Given the speed in which these evolutionary changes become noticeable and their potential in avoiding predation risk, this study supports that phenotypic plasticity might play an important role in population dynamics during biological invasions.

PART C

Capgrossos de la granota pintada (*Discoglossus pictus*) com a presa per depredadors aquàtics autòctons.

Capítol 8: Com influeix el caràcter autòcton/invasor de les les espècies en les respostes plàstiques – defenses induïbles – del capgrossos davant de depredadors? [publicat a la revista *Oikos*]

Tot i que els impactes purament ecològics de les invasions biològiques han estat ben estudiats, un esforç menor s'ha destinat a fer-ho en termes de la seva ecologia evolutiva. Diversos estudis previs mostren que la plasticitat fenotípica anti-depredador pot ser una de les principals forces ecològiques darrere la supervivència i la ràpida evolució de les preses davant de nous depredadors. Al seu torn, això vol dir que les invasions biològiques encarnen un cas perfecte per a l'estudi dels compromisos i contradiccions – els «trade-offs» – i l'evolució de la plasticitat fenotípica en sí mateixa. En aquest cas vam estudiar les respostes plàstiques – defenses induïbles – del gripauet o granoteta de punts (Pelodytes punctatus, una espècie autòctona) i de la granota pintada (Discoglossus pictus, una espècie invasora) davant d'una sèrie de depredadors, incloent-ne un d'autòcton (la libèl·lula emperador, *Anax sp.*) i dos d'introduïts (la gambússia, *Gambusia holbrooki,* un peix al·lòcton; i el cranc de riu americà, *Procambarus clarkii*). Es van registrar respostes ben marcades contra el depredador autòcton tant en els capgrossos autòctons com – sorpresivament – en els invasors. Per altra banda, tots dos van respondre lleument a la presència dels depredadors exòtics, però només el gripauet mostra una reacció morfològica davant del cranc americà invasor després de tot just 30 anys de convivència amb aquests depredadors. Els capgrossos de la granota pintada invasora van reaccionar a nivell conductual a tots els depredadors, i inesperadament van reaccionar també morfològicament davant la libèl·lula emperador (*Anax sp.)* autòctona. Tots aquests resultats donen suport a la idea de l'especificitat depredador-presa en aquestes reaccions i a la dissociació evolutiva entre la plasticitat del comportament i morfològica en els anurs. Per altra banda, cada espècie mostra un conjunt particular d'equilibris i contrapesos entre les respostes plàstiques i els seus costos posteriors, que és probablement a causa de diferències adaptatives deguts al seu nínxol ecològic i història evolutiva. És interessant remarcar també que en el cas de la granota pintada es van detectar patrons inesperats en combinacions amb depredadors introduïts. En general, es suggereix que canvis singulars en defenses induïbles poden ocórrer quan els capgrossos s'enfronten a espècies recentment introduïdes. Donada la velocitat amb què aquests canvis evolutius es fan evidents i el seu potencial per evitar el risc de depredació, aquest estudi també recolza que la plasticitat fenotípica podria tenir un paper important en la dinàmica de poblacions durant les invasions biològiques.

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How does the invasive/native nature of species influence tadpoles' plastic responses to predators?

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Synthesis

Anti-predator phenotypic plasticity is expected to be one of the major ecological forces driving survival and rapid evolution of prey facing new predators. This implies that biological invasions embody a perfect case for studying the tradeoffs and evolution of phenotypic plasticity. Our manuscript reports on high prey–predator specificity in these reactions and an evolutionary dissociation between behavioral and morphological plasticity in anurans. Each species displayed a particular set of tradeoffs between plastic responses and their costs, but interestingly we also detected mild patterns in combinations using introduced predators. Given the speed at which these evolutionary changes become noticeable and their potential in reducing predation risk, the role of antipredator phenotypic plasticity is expected to be crucial for population dynamics during biological invasions.

Although the purely ecological impacts of biological invasions have been well studied, a less thorough effort has been made in terms of their evolutionary ecology. Previous studies show that anti-predator phenotypic plasticity may be one of the major ecological forces driving survival and rapid evolution of prey facing new predators. In turn, this means that biological invasions embody a perfect case for studying the tradeoffs and evolution of phenotypic plasticity per se. Here, we studied the plastic responses of native (*Pelodytes punctatus*) and invasive (*Discoglossus pictus*) anurans facing a native (dragonfly *Anax* sp.) and two invasive (fish *Gambusia holbrooki* and crayfish *Procambarus clarkii*) predators. Marked responses were reported against the native predator from both the native and the invasive anuran, but they both responded mildly to the exotic predators as well. Native *P. punctatus* displayed a morphological reaction to invasive *P. clarkii* after scarcely 30 years of coexistence with this predatory crayfish and responded behaviorally to the invasive fish *G. holbrooki*. Invasive *D. pictus* reacted behaviorally to all predators, but unexpectedly only reacted morphologically to native *Anax* sp. All these results support high prey–predator specificity in these reactions and an evolutionary dissociation between behavioral and morphological plasticity in anurans. Each species displayed a particular set of tradeoffs between plastic responses and their costs, which is probably due to differences in ecological niche and evolutionary history, but interestingly we usually detected unexpected patterns in combinations using introduced predators. This suggests that perhaps singular plastic shifts usually occur when tadpoles face recently introduced species. Given the speed in which these evolutionary changes become noticeable and their potential in avoiding predation risk, this study supports that phenotypic plasticity might play an important role in population dynamics during biological invasions.

Of all the possible interactions between species in a community, predation often constitutes the major force that affects prey population dynamics, possibly triggering considerable cascade effects (Shurin et al. 2002). Accordingly, prey commonly evolve several ways of avoiding and reducing predation risk, ranging from permanent structures and behavior to morphological and behavioral plasticity (Agrawal 2001). However, introduced predator species may come from remote biotas. In such cases, there is no previous contact with the native community and the naïve prey is initially more vulnerable, what may give invasive predators a considerable advantage. Although various cases of prey extinction have been reported (Gurevitch and Padilla 2004), rapid evolutionary change, which usually involves inducible defenses, can enhance the ability of prey populations to respond to and survive novel predators (Phillips and Shine 2006, Fisk et al. 2007). Consequently, some studies have modeled adaptive phenotypic plasticity as a key evolutionary force opposing species invasions (Peacor et al. 2006, Latta et al. 2007). An assessment of these fast evolutionary changes in novel predator–prey interactions is essential to predict the long-term ecological impact of biological invasions (Strauss et al. 2006). Given the growing presence of invasive species interacting in the same food webs, in this field it is also becoming necessary to work on possibilities such as interaction among invasive species, considering all the evolutionary changes that may be happening at once.

Mediterranean anuran tadpoles represent common prey in ponds and show anti-predator adaptive plastic responses (Richter-Boix et al. 2007). These responses are triggered by chemical cues coming from predators and from injured prey during predation events (Petranka et al. 1987, Schoeppner and Relyea 2005, Fraker et al. 2009). According to previous studies, reducing general levels of activity is a common behavioral change when anuran tadpoles are exposed to known cues from potential predators or from predation events. This shift is usually interpreted as a way to avoid being perceived as prey (Anholt and Werner 1995, Relyea 2001b).

On the other hand, the most commonly reported morphological response to frequent arthropod predators such as dragonfly naiads is an increase in the height of tail fins. Given that these morphological changes do not seem to enhance swimming performance (Van Buskirk and McCollum 2000, Wilson et al. 2005), this can be interpreted as an effective lure to attract predator strikes to body surfaces on which an attack may not be lethal (Van Buskirk et al. 2003). Interestingly, further studies with various predators show that different anti-predator reactions are displayed confronting different threats (Relyea 2001b). Thus, reactions may be predator-specific, depending mainly on the effectiveness of each behavioral–morphological set in reducing predation risk (Wilson et al. 2005, Touchon and Warkentin 2008). However, it is obvious that these advantageous inducible changes must have limits and that they are linked to some costs. Some studies have shown that they can cause tadpoles to grow and develop more slowly (Relyea 2002), and that their costs are not only linked to larval development, but also to subsequent stages in ontogeny (Tejedo et al. 2010).

If we properly consider the responses to different native and invasive predators with different times of coevolution and the positive and negative aspects of these responses, invasion events serve as a perfect occasion to address the costs, limits and all other aspects driving the evolution of phenotypic plasticity. Hence, in recent years, some studies have addressed issues related to tadpoles' inducible defenses against invasive predators, with divergent results. While some studies clearly demonstrate a lack of response to invasive predators in certain anuran species (Smith et al. 2007, Polo-Cavia et al. 2010, Gomez-Mestre and Díaz-Paniagua 2011), other works report varied combinations of behavioral and morphological responses in other species after a relatively short period of coexistence with the invasive predator (Kiesecker and Blaustein 1997, Griffiths et al. 1998, Chivers et al. 2001, Pearl et al. 2003, Cruz and Rebelo 2005, Almeida et al. 2011, Gonçalves et al. 2011). This suggests that no single rule can be made for these events. Thus, when confronting this complex topic, aspects like the frequency of co-occurrence, the recognition and importance of chemical cues, and each species' use of microhabitat or ecological niche need to be taken in consideration.

Here we tested the plastic responses of a native (common parsley frog *Pelodytes punctatus*) and an invasive (Mediterranean painted frog *Discoglossus pictus*) anuran species acting as prey in front of three different predators, two of them invasive (crayfish *Procambarus clarkii* and fish *Gambusia holbrooki*) and the other native (dragonfly larvae *Anax* sp.). We expected an effective plastic behavioral and morphological response when native prey were exposed to native predators and some novel responses in the other combinations. The nature and the degree of these initial responses (and even their sole presence or absence) should be related with the time of coevolution, several ecological factors and the hypothetical effectiveness of each reaction in each case. Finally, we expected a set of reactions to induce a predictable set of costs. Hence, the chosen list of species gives us the opportunity to test native and invasive prey responses in front of native and invasive predators, while indeed testing utterly real interactions that take place in nature.

Material and methods

Study species

Since its recent introduction from Algerian specimens (Francesca and Roberta 2006) in around 1906 in Banyuls-sur-Mer, *Discoglossus pictus auritus* has expanded approximately 130 km northwards, 140 km southwards and 60 km inland. This species now occupies a wide coastal strip from the River Orb in France to the River Tordera in Spain (Montori et al. 2007) and all published material confirms and predicts continuing expansion of this species. These populations show great versatility in their choice of reproduction points, but prefer temporary and ephemeral ponds in open spaces, overlapping niche chiefly with the natives *Bufo calamita* and *Pelodytes punctatus* (Montori et al. 2007). We chose *Pelodytes punctatus* out of these two native species because it is highly plastic and is the most likely to face all three selected invasive species at some point, given its reproductive adaptability using the entire pond permanency gradient (Richter-Boix et al. 2007). As native predator, we chose larval Aeshnid dragonflies (*Anax* sp.). These species are proven predators of tadpoles and commonly occur together with *P. punctatus* and *D. pictus* larvae in ponds within the study area (Richter-Boix et al. 2007), and they are usually used in inducible defenses experiments (Peacor and Werner 1997, Van Buskirk et al. 2003). We chose *Gambusia holbrooki* as an introduced fish predator present in the same area. This species was first reported in the Iberian Peninsula in 1921, when it was introduced to control mosquito populations in order to fight malaria. Since then, the approach became quickly common to cope with mosquitoes all over the peninsula (Elvira 2001), so this species could also be deemed a recent introduction. Some studies have confirmed this species' ability to predate tadpoles and interfere drastically with the reproductive dynamics of amphibian populations in permanent pond communities (Kats and Ferrer 2003). The third predator chosen was the American red swamp crayfish *Procambarus clarkii*. First citations of this invasive species in the same study zone date from the start of the 1980s. It became a widely extended species in the area before 1990 (M. Franch pers. comm.), so it is a very recent introduction. It is well adapted to live in areas with seasonal fluctuations in water levels, where survives the dry season in simple burrows. Therefore, it can potentially colonize most water bodies except the most ephemeral ones (Cruz and Rebelo 2005). Several studies have reported that this species predates the spawn, larvae and adults of local amphibians (Cruz et al. 2006a), and some indicate that *Procambarus clarkii* is a key factor in inducing the decadence of amphibian populations in the Iberian Peninsula and Europe (Cruz et al. 2006b, Gherardi and Acquistapace 2007), which makes an in-depth study of its predatory relationship with larval anurans highly desirable.

General experimental design

The morphological and behavioral anti-predator responses of *Pelodytes punctatus* and *Discoglossus pictus* to the three chosen predators were tested in two equivalent short-term laboratory experiments in consecutive years (2008 and 2009 respectively for *P. punctatus* and *D. pictus*). We reproduced precisely the same controlled conditions for both species, even using exactly the same material and experimental venue, which makes the results completely comparable. The clutches and predators used in each experiment were collected from natural ponds within each species' natural range. We took five clutches of *P. punctatus* from the Garraf massif (Barcelona, northeastern Spain) and four clutches of *D. pictus* from Riudarenes (Girona, northeastern Spain). We collected several larvae of *Anax* sp., median size individuals of *P. clarkii* (8–10 cm body length) and several adults of *G. holbrooki* each year to act as predators. All predators were captured in the same ponds as the clutches or from nearby ponds with registered amphibian reproductive activity. Each collected clutch was brought to the laboratory and placed in an individual container (1.20 × 0.45 × 0.40 m) filled with 30 l of dechlorinated water. Once tadpoles had achieved Gosner stage 25 (Gosner 1960), they were transferred to the treatment tanks. These tanks had the same conditions and dimensions as the hatching containers and contained 20 tadpoles each. According to previous studies, these larval densities cannot trigger plastic anti-competition reactions (Relyea 2004).

To avoid uncontrolled effects due to possible particularities in single clutches, we selected tadpoles in the same proportions from the different spawns. Thus, we consider that possible effects of the presence of predators or competitors prior to our manipulation of the spawns or the deleterious particularities of a concrete spawn were leveled in all tanks. The experiments were performed in a climate room (21–23°C, photoperiod 14L:10D) at Univ. of Barcelona laboratories. After a week, we placed the predator specimens in perforated cylindrical cages (diameter 11 cm) submerged in the tanks, with a double net bottom (mesh size 2 mm). The cages prevented the predators from capturing the tadpoles, but allowed water to flow through (a similar arrangement as in Richter-Boix et al. 2007). Thus, the 20 anuran larvae were in chemical but not physical contact with the chosen predators. The experimental design consisted of four treatments (treatment C, control treatment: 20 tadpoles + 2 empty cages per tank; treatment A: 20 tadpoles + 2 caged larvae of *Anax* sp. per tank; treatment P: 20 tadpoles + 2 caged individuals of *P. clarkii* per tank; treatment G: 20 tadpoles + 4 caged individuals of *G. holbrooki* per tank) each replicated five times, making a total of 20 experimental tanks. We used twice as many predators in the G treatment to avoid extremely mismatching predator biomasses between treatments. Tadpoles were fed ad libitum the same amount of commercial rabbit pellets, three times a week. Predators consumed tadpoles of the same species tested in each running experiment in order to simulate natural predation of siblings in the pond. Since variations in tadpole consumption by predators can affect the availability of chemical cues and alter the responses during the experiment (Schoeppner and Relyea 2005, Peacor 2006, Fraker et al. 2009), each cage of predators received a constant rate of one tadpole per day as food.

Response variables and acquisition of raw data

Throughout the experiment, we checked tadpole survival and stage daily. Survival was defined as the proportion of tadpoles that reached Gosner stage 42 in each tank. Once a tadpole had achieved Gosner stage 42, it was numbered and placed in an individual cage with some water and an absorbent cloth to keep moist until it had achieved Gosner stage 46. Thus, we could report their larval period (days from hatching to attaining Gosner stage 42), tail resorption time (days from reaching Gosner stage 42 to attaining Gosner stage 46), and time to complete metamorphosis (the addition of the two former variables). However, owing to problems in the sampling of juveniles during the *D. pictus* experiment, the only data available in this latter case was the larval period.

We monitored levels of tadpole activity using the proportion of active tadpoles (i.e. larvae that were swimming or feeding, as in Relyea 2004) seen two times per day in each tank at the moment it was first viewed. The proportion of active specimens (level of activity) was the number of active tadpoles divided by the total number of tadpoles observed at first glance (similar to Peacor and Werner 1997, Relyea 2002). These measures were taken on five days spread from the second week of the experiment to the start of extensive metamorphosis.

Three weeks after the experiment was initiated, we measured larval morphologies by tracing a lateral photograph of each larva using Sigma Scan Pro 5.0 software. We took six linear measures (similarly to Relyea 2001b): body length, body height, tail length, fin height, fin length and musculature height (from now on musculature). We obtained values of total larval length by adding body length and tail length. After tail resorption (Gosner stage 46), we took seven measurements of juveniles following the same methodology as in tadpoles. The juveniles' measures were also those normally used in these cases (similar to Relyea and Hoverman 2003): body length, body width, femur length, muscular femur width (from now on femur width), tibia-fibula length, muscular tibia-fibula width (from now on tibia-fibula width) and foot length.

Statistical analyses

Proportions of survival and tadpole activity were all arcsin transformed and analyzed using linear model ANOVAs.

Timing data were non-normal and were analyzed using Kruskal–Wallis ANOVAs. Total length in tadpoles and body length in juveniles were tested using linear model ANOVAs and were taken as individual size indicators in all morphologic analyses that were run. Because all the morphologic reactions displayed by tadpoles are not independent from each other, we explored the multivariate effects on tadpole shape of treatment and interaction between size and treatment using MANCOVAs, including all morphological variables except body length. This variable was excluded to avoid redundancy, given that it is the counterpart of tail length when using tadpole total length as covariate. Once the multivariate effects of interaction were confirmed or rejected by the exploratory MANCOVAs, we ran factorial or one-way MANCOVAs respectively. Multivariate differences in juvenile morphology were tested using the same methodology. Once general multivariate divergences in morphology had been confirmed, we tested the effects of treatment and interaction between size and treatment on single morphologic traits. Once the interaction was confirmed or rejected according to previous exploratory ANCOVAs, we applied respectively separate or homogeneous slopes linear model univariate analyses of covariance (ANCOVAs) with the size indicators as a covariate and followed by Scheffé post hoc tests. As multivariate exploratory techniques we conducted canonical and discriminant analyses using the residuals of regressions of each morphologic measure against total length (body length for juveniles) as size-corrected variables of shape, excluding again tadpole body length to avoid redundancy. Thus we obtained a percentage of classification that was understood as a rough numerical indication of the morphologic divergence of each treatment from the others and a multivariate graphic support (not shown) for interpreting these results. These same size-corrected variables were used to produce the figures in this manuscript. All statistical tests were conducted using Statistica 8 software.

Results

Survival and developmental timing

We found highly significant differences in the overall levels of mortality between species ($F_{1,38} = 76.82$, $p < 0.0001$): *Pelodytes punctatus* had a mean mortality of 46% and *Discoglossus pictus* had a mean mortality of 5%. In addition, each species responded differently to the four treatments ($F_{3,3,32} = 4.42$, $p = 0.0103$). Significant differences occurred among treatments in *P. punctatus* ($F_{3,16} = 4.94$ $p = 0.0129$). Treatment A had a mortality of 66%, while a level of approximately 40% was maintained in the other three treatments (all $p < 0.05$ when compared to the rest of treatments). This was not reported in *D. pictus* ($F_{3,16} = 0.39$ $p = 0.7618$), which had a low and nearly constant level of mortality in all treatments.

In the *P. punctatus* experiment, no significant differences existed between treatments in the time to complete metamorphosis (Kruskal–Wallis $H_{3,211} = 0.69$ $p = 0.8748$) or in the larval period (Kruskal–Wallis $H_{3,211} = 0.46$, $p = 0.9278$). However, we found significant differences in tail resorption time (Kruskal–Wallis $H_{3,211} = 19.06$, $p = 0.0003$): *P. punctatus* larvae that were in chemical contact with *Anax* sp. took two days more on average to reabsorb their tail (between six and seven days in total) than the specimens from the rest of treatments (with very similar durations of four or five days, on average). In contrast, *D. pictus* larvae exposed to *Anax* sp. chemical cues showed a significant lengthening of the larval period (Kruskal–Wallis $H_{3,378} = 23.59$, $p < 0.0001$), as they took approximately four days more to leave the water (roughly 46–47 days) than the rest of individuals (which had very similar results among treatments of 42 days approximately).

Behavior: levels of activity

Overall levels of activity diverged significantly between species ($F_{1,298} = 41.33$, $p < 0.0001$, Fig. 1) and between treatments for both species ($F_{3,96} = 14.58$, $p < 0.0001$ for *P. punctatus*, and $F_{3,96} = 9.16$, $p < 0.0001$ for *D. pictus*). *Pelodytes punctatus* larvae in chemical contact with *Anax* sp. naiads had a noticeable behavioral response in comparison to the control ($p < 0.0001$), with approximately 54% lower general levels of activity. We observed a more moderate reaction in *Gambusia holbrooki* tadpoles ($p = 0.0099$), in which activity levels dropped by approximately 30%. Although mean activity levels were lower in *Procambarus clarkii* tadpoles than in the control, no significant overall differences were reported between these two treatments ($p = 0.4649$). *Discoglossus pictus* larvae displayed a significant behavioral response to all predators in the experiment, with 35 and 40% less activity than the control in all cases ($p < 0.05$ for A, G and P treatments).

Larval morphology of *Pelodytes punctatus*

No significant divergence was observed in total length of *P. punctatus* tadpoles among treatments ($F_{3,190} = 1.395$, $p = 0.2456$), but marked morphological divergence among treatments was found according to multivariate analyses ($F_{15,15,5}$ [Treat × Total length] = 604.57, $p < 0.0001$, and $F_{15,15,5}$ [Treat] = 1.91, $p = 0.0195$). All measures showed significant differences ($p < 0.05$ either in the interaction or the treatment effect, Table 1), except fin length ($p = 0.0537$). Invasive crayfish tadpoles were relatively larger bodied than all other tadpoles and consequently had relatively shorter tails (all $p < 0.0001$ for body height and length, tail length and fin length). All predator treatments displayed an affinity to growing higher fins than control tadpoles, with highly significant differences in native dragonfly and invasive crayfish tadpoles ($p < 0.0001$ in both cases), but no significant differences in *G. holbrooki* tadpoles ($p = 0.1821$). There was also a tendency to grow more musculature in all predator treatments, but differences were again only significant in native dragonfly and invasive crayfish tadpoles in post hoc analyses ($p = 0.0041$ and $p < 0.0001$ when compared to control). Fins tended to be longer in native dragonfly and invasive crayfish tadpoles, but only invasive crayfish tadpoles had significantly longer fins according to post hoc analyses ($p < 0.0001$ when compared to the control). Discriminant and canonical analyses indicate that native dragonfly treatment was the

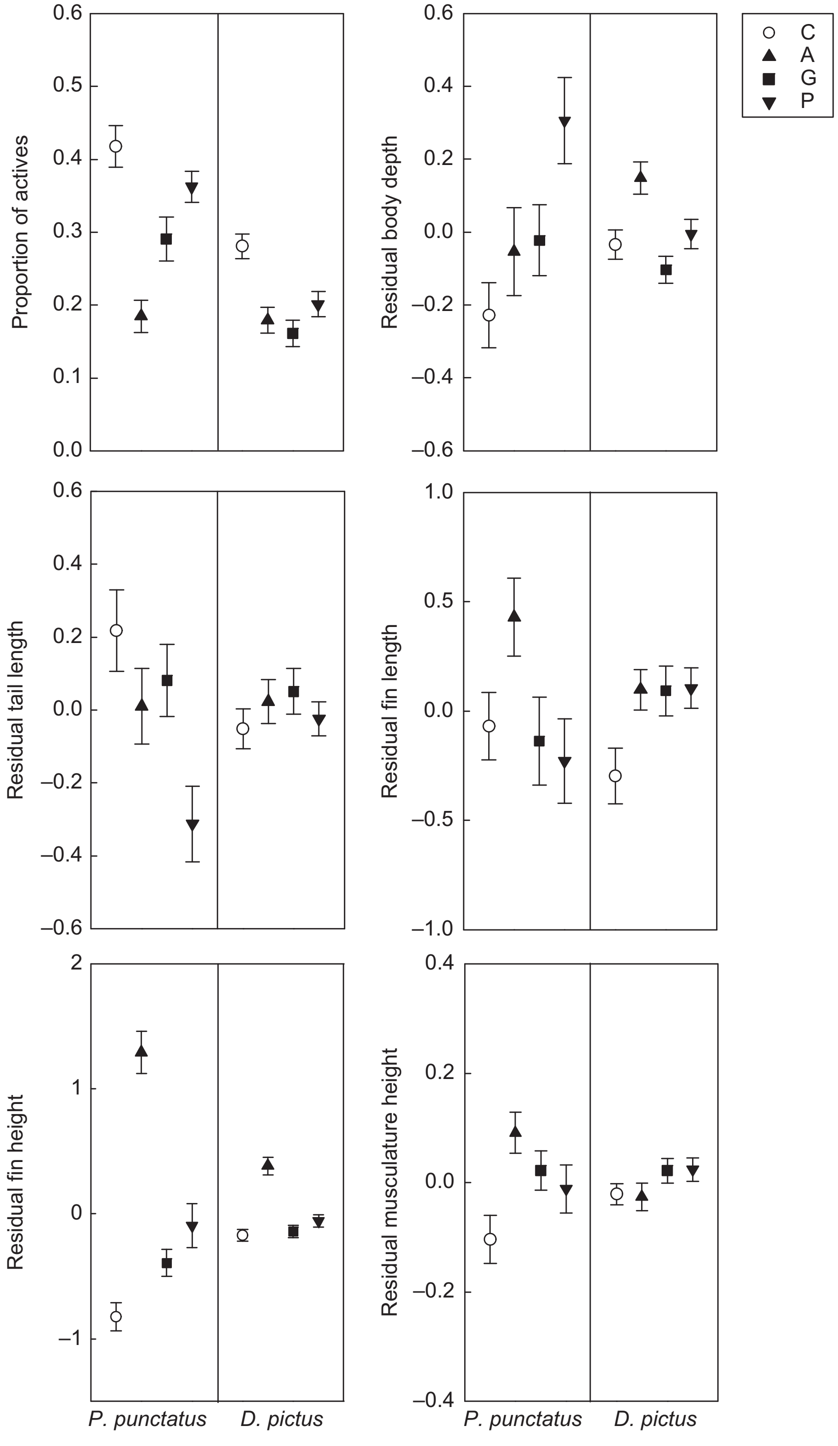

Figure 1. Larval behavior and morphology (according to the size-corrected variables used to run multivariate tests) in both species. C = Control treatment, A = *Anax* sp. treatment, G = *Gambusia holbrooki* treatment, P = *Procambarus clarkii* treatment. Results of predator treatments are positioned more rightwards in the figure as predators' presence is more recent in the study zone. Error bars refer to standard error.

only to use a clearly different morphological space: while *Anax* sp. was visibly separate from the rest of the treatments (71.4% of correct classifications), these remain mixed and unclassifiable in multivariate analyses (correct classification percentages of 45.8% in the control treatment, 38.8% in *G. holbrooki* treatment and 45.8% in *P. clarkii* treatment).

Larval morphology of *Discoglossus pictus*

Differences in total length of *D. pictus* tadpoles ($F_{3,193} = 3.2672$, $p = 0.0224$) lacked a clear pattern and all disappeared in post hoc analyses. However, clear morphologic divergences occurred according to multivariate analyses

Table 1. Linear model ANCOVAs (with and without the interaction term) for effects of treatment on tadpole morphology in both species.

	MS (Treat × Size)	MS (Treat)	$F_{4,3,86}$ (Treat × Size)	$F_{4,3,186}$ (Treat)	$F_{1,3,189}$ (Treat)$^{\Delta}$	p (Treat × Size)	p (Treat)
P. punctatus							
Body length	819,7974	0,9024	1593,1170	1,7540		0.0000	0.1576
Body height		2,4319			4,3650		0.0053
Tail length	2818,2760	0,9020	5476,7710	1,7540		0.0000	0.1576
Fin length		4,2500			2,5980		0.0537
Fin height	408,9896	2,6580	420,8731	2,7353		0.0000	0.0450
Musc. height	39,3042	0,3720	533,7756	5,0521		0.0000	0.0022
D. pictus							
Body length	50,9408	0,6406	340,0057	4,2754		0.0000	0.0060
Body height	12,9486	0,2984	171,3419	3,9492		0.0000	0.0092
Tail length	224,0040	0,6406	1495,1190	4,2750		0.0000	0.0060
Fin length		1,9320			3,3600		0.0199
Fin height	20,5759	0,3092	150,0024	2,2254		0.0000	0.0835
Musc. height		0,0357			1,5055		0.2144

Δ, tests using models without interaction.

($F_{15,15,5}$ [Treat × Total length] = 614.53, $p < 0.0001$ and $F_{15,15,5}$ [Treat] = 1.74, $p = 0.0402$) (Table 1). Body height differences were insignificant in post hoc analyses, but each treatment displayed a singular ratio between body and tail length (all $p < 0.05$ tail length post hoc analyses), having control tadpoles the relatively shortest tails. All predator treatments had also longer fins than the control treatment (Fig. 1), but according to post hoc tests these differences were only significant in *G. holbrooki* ($p < 0.05$) and *Anax* sp. ($p < 0.0001$) tadpoles when compared to control treatment. Interestingly, only *Anax* sp. tadpoles displayed markedly higher fins than control ($p = 0.0093$). Discriminant and canonical analyses showed that treatment *Anax* sp. treatment was the only one that was distinctive from the others, with a correct classification of 70.8%, while the rest remain largely unclassified (correct classifications of 51.0% for control, 42.0% for *G. holbrooki* tadpoles, and 24.0% for *P. clarkii* tadpoles).

Juvenile morphology of *Pelodytes punctatus*

Although significant differences were found in absolute body length of *P. punctatus* juveniles, these were only held between *G. holbrooki* and *P. clarkii* treatments ($p = 0.0048$), and no clear pattern was observed. However, there was a highly marked divergence among treatments according to multivariate analyses ($F_{18,6}$ [Treat] = 568.99, $p < 0.0001$) and significant divergence was found in all univariate morphologic analyses (Table 2). Variation in relative body width showed a seemingly clear pattern (Fig. 2), decreasing along predator treatments as predators were more recently introduced, falling to the lowest values in *P. clarkii* juveniles. In this case, all combinations gave significant post-hoc results ($p < 0.001$), except differences between *Anax* sp. treatment and control ($p = 0.9961$) and differences between *P. clarkii* and *G. holbrooki* treatments ($p = 0.2604$). The model for the hind limb seems clear as well: while juveniles from both invasive treatments tended to have slightly more powerful hind limbs than the control treatment, native dragonfly juveniles had clearly weaker (both shorter and thinner) hind limbs than the rest. Discriminant analysis classified quite correctly control (62.3%) and *P. clarkii* (65.5%) juveniles, but performed feebly in *Anax* sp. (48.4%) and incorrectly in the *G. holbrooki* treatment (31.1%). However, despite the correct classifications, canonical analysis showed a high overlap between all treatments.

Table 2. Linear model ANCOVAs (with and without the interaction term) for effects of treatment on juvenile morphology in both species.

P. punctatus	MS (Treat × Size)	MS (Treat)	$F_{4,3,203}$ (Treat × Size)	$F_{4,3,203}$ (Treat)	$F_{1,3,206}$ (Treat)$^{\Delta}$	p (Treat × Size)	p (Treat)
Body width		3,2193			30,1505		0.0000
Femur length		4,2097			22,9893		0.0000
Femur width		0,1543			2,7931		0.0415
Tibiofibula length		0,8611			5,0999		0.0020
Tibiofibula width		0,1099			5,3105		0.0015
Foot length		0,0004			6,2629		0.0004

D. pictus	MS (Treat × Size)	MS (Treat)	$F_{4,3,369}$ (Treat × Size)	$F_{4,3,369}$ (Treat)	$F_{1,3,372}$ (Treat)$^{\Delta}$	p (Treat × Size)	p (Treat)
Body width		0,0459			1,3087		0.2712
Femur length		0,0148			0,2234		0.8801
Femur width		0,1183			4,3753		0.0048
Tibiofibula length	19,0972	0,2064	310,2306	3,3525		0.0000	0.0191
Tibiofibula width	2,1361	0,0860	90,7082	3,6519		0.0000	0.0128
Foot length		0,1418			1,0764		0.3590

Δ, tests using models without interaction.

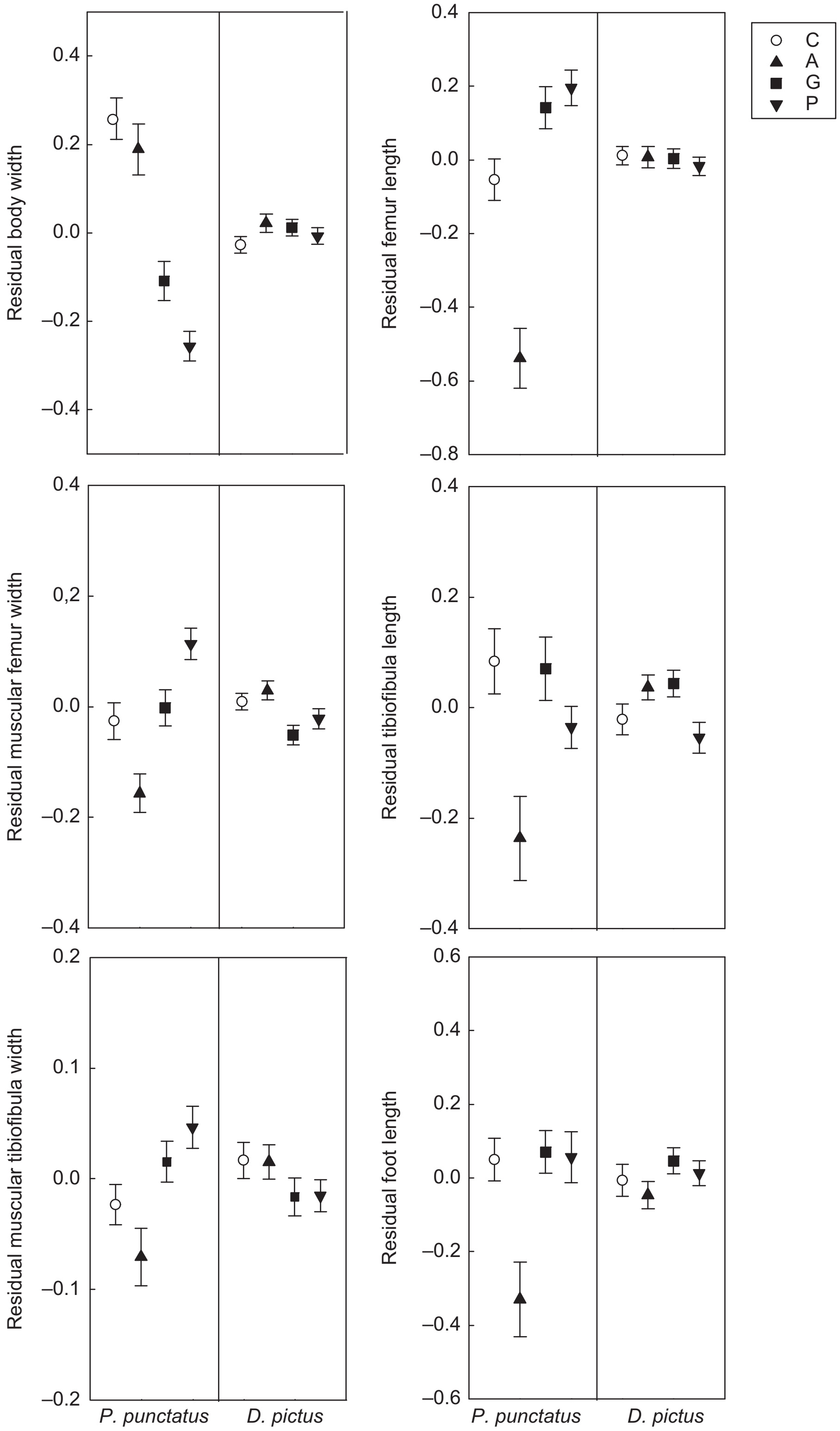

Figure 2. Juvenile morphology in both species according to the size-corrected variables used to run multivariate tests. C = Control treatment, A = *Anax* sp. treatment, G = *Gambusia holbrooki* treatment, P = *Procambarus clarkii* treatment. Results of predator treatments are positioned more rightwards in the figure as predators' presence is more recent in the study zone. Error bars refer to standard error.

Juvenile morphology of *Discoglossus pictus*

Clear multivariate differences ($F_{18,18,6}$ [Treat × Total length] = 41.81, $p < 0.0000$ and $F_{18,18,6}$ [Treat] = 2.31, $p = 0.0014$) and highly significant variation in absolute body length ($F_{3,373} = 10.12$, $p < 0.0001$) was found in *D. pictus* juveniles. All predator treatments yielded significantly longer juveniles than the control ($p < 0.0001$ for native dragonfly tadpoles, $p = 0.0244$ for invasive fish tadpoles, $p = 0.0029$ for invasive crayfish tadpoles), and the variation among predator treatments was not significant ($p > 0.15$). Although there were some differences in the relative femur width and tibia–fibula length and width (Table 2), there was no clear pattern in hind limb morphology in this case (Fig. 2). Discriminant and canonical analyses support the lack of a clear pattern of relative morphologic divergence among treatments, since no treatment achieved the threshold of 50% correct classification (correct classifications of 30.2% for control, 42.6% for *Anax* sp. tadpoles, 48.4% for *G. holbrooki* tadpoles, 28.3% for *P. clarkii* tadpoles).

Discussion

As expected from the literature (Richter-Boix et al. 2006, 2007), local dragonfly predators clearly triggered behavioral and morphological responses in *Pelodytes punctatus* larvae. However, we observed only behavioral responses against the invasive fish and only morphological responses against the invasive crayfish, in both cases milder than the reactions against the local predator. This opposite pattern of reactions from *P. punctatus* to both introduced predators reminds the results observed in some similar other studies (Wilson et al. 2005, Touchon and Warkentin 2008) and strongly supports that the expression of morphological and behavioral responses are not necessarily linked (Van Buskirk and McCollum 2000).

This means, in turn, that it must be possible to evolve each response separately as well. While reduced activity is usually considered a way to avoid detection (Anholt and Werner 1995, Relyea 2001b), induced higher fins diminish the probability of a lethal attack by growing fragile surfaces that act as a lure and can easily be torn off without fatal consequences (Van Buskirk et al. 2003). Given that amphibian prey can detect the chemical cues emitted by specific predators (Relyea 2003, Pearl et al. 2003), a precise combination of these responses in front of each predator could be easily favored (Van Buskirk 2001).

Invasive fish *Gambusia holbrooki* normally attacks in group and stalks tadpoles until they are totally consumed, this fish being much faster than tadpoles (unpubl.). As only a slight chance exists of a tadpole escaping once it has been detected by fish, high fins might be useless against this type of predator (Wilson et al. 2005, Touchon and Warkentin 2008), and a more effective plastic reaction would be to avoid recognition, that is, behavioral changes. Therefore, the mild behavioral reactions we observed in *P. punctatus* could be a microevolutionary midpoint to more drastic avoidance tactics against introduced *G. holbrooki.* This results, however, could also be explained as a generalized moderate response to fish cues (Ferrari et al. 2007), or even as an indicator of a still unspecific response to an unknown but detected predatory risk (Schoeppner and Relyea 2005).

On the other side, invasive crayfish *Procambarus clarkii* is an active seeker that mainly uses the bottom of water bodies (Cruz and Rebelo 2005). It does not usually pursue its victims, relying on explosive attacks and a firm grip once prey have been detected, even adopting a sit-and-wait strategy (Gherardi et al. 2001). The lack of a clear behavioral reaction of *P. punctatus* to *P. clarkii* may indicate that is not useful, being inactive tadpoles at the bottom of ponds equally or even more at risk of predation than active individuals. This is the case in Cruz and Rebelo (2005), where this reaction was detected in some anuran species, but failed to increase survival. However, survivors escaping crayfish's first attack might not be stalked and hence not consumed, which could explain why morphological reactions similar to that displayed against local dragonflies can potentially lead to increased survival confronting *P. clarkii* (Gomez-Mestre and Díaz-Paniagua 2011). Although we clearly observed significant morphological reactions in a substantial subset of individuals of *P. punctatus*, they didn't occur in a large proportion of tadpoles, which may indicate that more time of coevolution is needed for a clear and general reaction. This contrasts with the lack of reactions in southwestern Iberian Peninsula green frogs against the same invasive crayfish (Gomez-Mestre and Díaz-Paniagua 2011). Given the ancient presence of a similar crayfish species (*Austropotamobius pallipes*) in our area (being southwestern Iberian Peninsula devoid of native crayfish), the presence of mild reactions after scarcely 30 years of coexistence could be explained arguing that cues have been co-opted or generalized (Ferrari et al. 2007) in our area. The different predatory habits and forced allopatry of the two crayfish species (Gherardi et al. 2001, Gil-Sánchez and Alba-Tercedor 2002), in addition to the importance of chemical communication within each species of crayfish (Rose 1986, Zulandt Schneider et al. 1999, Zulandt Schneider and Moore 2000) discards that *P. punctatus* tadpoles take invasive crayfish as an identical predator, but at the same time makes the possibility of co-option slightly remote. Hence, probably the best explanation could be superior plastic abilities in *P. punctatus* (Richter-Boix et al. 2007).

Invasive *Discoglossus pictus* responded similarly to all predators reducing its activity and increasing fin and tail length, but only tadpoles confronting native dragonfly displayed the typical antipredator high fins as well. Known that several Aeshnidae dragonfly species inhabiting northern Algeria are shared with the European areas occupied by *D. pictus* (Aguilar and Dommanget 1998), these results imply that similar encounters in its original biota already triggered a fin height increase, being dragonfly cues co-opted when introduced *D. pictus* populations met local Aeshnidae. This should explain why invasive *D. pictus* reacts so clearly against native *Anax* sp. individuals, being an interesting example of co-option of cues in anti-predator plasticity. Assuming that invasive *D. pictus* recognized only *Anax* sp. as a known predator, the observed pattern of reactions in this species matches with the results in Schoeppner and Relyea (2005), where only a full combination of alarm and consumption cues and known predator kairomones induced the full suite or magnitude of antipredator traits, while other mild responses were observed when some of these cues lacked. Hence, it clearly seems that the similar responses observed against all predators were triggered by alarm or consumption cues, while only the addition of known predator kairomones could induce the extra reaction (increase of fin height) in the tadpoles confronting local dragonflies.

Interestingly, despite this morphological reaction is clear, it is also markedly less exaggerate than the response observed in native *P. punctatus* tadpoles exposed to the same dragonfly predator. Similar studies with known Algerian predators are required to know whether the milder nature of these morphological reactions in *D. pictus* are the result of a still improving co-opted response or due to poorer morphological plasticity in this invasive anuran.

Similar is the case in all behavioral reactions of this invasive anuran, which are proportionally similar to *P. punctatus* reactions confronting the invasive fish *G. holbrooki*. Although it could seem that the lower levels of control activity may limit *D. pictus*' behavioral reactions, being tadpoles obviously constrained to feed over sustainable minimums, this option should be discarded because *D. pictus* juveniles from the predator treatments arose significantly larger than control individuals. Thus, concerning behavior the best explanation clearly seems to be the existence of a particularly exhaustive response from native *P. punctatus* to native *Anax* sp. larvae.

We attribute the widespread high mortality rates observed in *P. punctatus* to a reduced viability in one of the collected spawns. Thanks to the initial balanced merging of clutches, we consider that this fact does not alter any interpretation of the results because all treatments had the same basal mortality. Hence, the additional mortality observed in native dragonfly *P. punctatus* tadpoles seems a noticeable cost of higher stress levels caused by detecting the known predator. This stress was manifestly important and even synergistically lethal in several studies (Relyea 2005, Relyea and Mills 2001, Relyea and Hoverman 2003). Albeit the high mortalities in this treatment (66%), we discard any predatory risk differences due to differential density because all initial densities were already low and all other treatments had a high mortality as well (40%). We observed noticeable metamorphosis delays in this same native versus native combination (longer resorption time) and when *D. pictus* was confronted to local dragonflies. These were the combinations with more exaggerate behavioral or morphological responses in both anurans, supporting the tradeoff between manifest anti-predator reactions and diminished growth and developmental rates in tadpoles (Relyea 2002). Neither mortality nor timing costs were observed confronting *P. punctatus* to introduced predators, confirming this linkage and a differential state of strain in larvae detecting known predator cues.

Concerning juveniles, body length and width are closely related to total mass, which in turn is an important character defining individual fitness in this developmental stage of anurans (Smith 1987). *Pelodytes punctatus* juveniles arose equally longer, but were significantly thinner as the predator was more recently introduced. This unusual pattern may account for a delayed cost that emerged after reacting to unknown predators, which may trigger responses that still require improvement and thus lead to uncommon costs. Conversely, invasive *D. pictus*' juveniles from all predator treatments were longer than control juveniles. Given the lower levels of activity in all predator treatments and the ad libitum feeding, the outcome might have been that these less active tadpoles had more energy to employ in growth. However, in natural conditions, such as in temporary or ephemeral ponds (commonly inhabited by this species), this unexpected additional gain could lack due to limited food supplies, what seems to be a crucial point to consider in this species (Enriquez-Urzelai 2011).

It is known that relatively shorter hind limbs decrease jumping performance and consequently affect the fitness of juveniles (Wassersug and Sperry 1977, Walton 1988). This statement is even more reasonable in *P. punctatus* if we add that native dragonfly juveniles' hind limbs were not only shorter but also thinner. This results further support a possibly widespread tradeoff between immediate benefits of anti-predator plasticity in larvae and linked costs as relatively shorter hind limbs in juvenile stages (Tejedo et al. 2010). The advantageous sizes of hind limbs in *G. holbrooki* and *P. clarkii* tadpoles when compared to the control could add to the fact that the mild novel reactions against less known predators lead to atypical consequences for juveniles, even creating advantageous morphologies. However, perhaps these beneficial consequences are not restricted to reactions against invasive species (Relyea 2001a). The relative divergences in *D. pictus* juveniles did not show any clear pattern, suggesting that clear morphologic costs might be unimportant or lack in invasive *D. pictus*, at least when faced with these three predators.

Thus, the usual tradeoffs between anti-predator responses in larval stages and juvenile fitness are only reported in native *P. punctatus*. Having already mentioned that all responses were milder in invasive *D. pictus*, we can hypothesize that these reactions may not yet have achieved the threshold for developing delayed costs. The other possibility is that *D. pictus* escapes such costs by naturally displaying more limited larval anti-predator plasticity. Some studies suggest that a particular use of the pond permanency gradient shapes the possibilities of evolving different plastic responses (Richter-Boix et al. 2007), so it is fairly likely that the marked use of temporary and ephemeral ponds with few predators fails to select strong anti-predatory adaptations in *D. pictus*. The use of several typologies of pond, regardless of low or high predator densities, could explain why *P. punctatus* exhibits more exaggerated anti-predator induced morphologies. Hence, as already mentioned, even though it seems that *D. pictus* is a globally less plastic species compared to *P. punctatus*, studies confronting *D. pictus* with Algerian predators are needed to confirm or reject this possibility.

On the other hand, the consistency of our estimations regarding gains and costs of all listed responses is limited because we could not evaluate the real effectiveness in reducing predation of all the observed anti-predator responses. For example, predation on tadpoles by invasive predators has been described as potentially catastrophic (Cruz and Rebelo 2005). Therefore, probably most tadpoles from our invasive predator treatments would not have had the chance to become 'advantageous' juveniles in nature. Besides, once accepted the high predator–prey specificity, some important features of tadpoles in the less studied predator–prey combinations may be misinterpreted or overlooked to some extent. This suggests that a better assessment of the real effectiveness of plasticity could be very interesting in future experiments that address the evolution of these reactions against invasive species. Although a lot of drawbacks of plasticity indeed exist, it is widespread in anuran lineages. It follows that gains must usually surpass costs, and rapid changes observed affirms once again the adaptive nature of these traits, suggesting an important influence of phenotypic plasticity in the ecological and evolutionary outcomes of biological invasions.

Conclusions

This study supports that tadpoles' plastic responses can be highly prey–predator specific and that each reaction may evolve singularly, with particular benefits and costs. Discordance among behavioral and morphologic reactions was widespread, and the observed combinations of reactions in several cases seem to match the different requirements facing each predator species according to the biology of each prey species. Responses were strongest against the native predator for both anurans, although they both responded mildly to the exotic predators. Interaction with the less known predators usually led to unusual or unexpected patterns of responses and costs. These mild and uncommon patterns of reactions and costs could be a rule when plastic responses against novel predators are still being improved or unspecific. Given the speed at which these evolutionary changes become noticeable and their potential in reducing predation risk, we believe that the role of antipredator phenotypic plasticity might be crucial in the population dynamics during biological invasions.

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PART C

Tadpoles of *Discoglossus pictus* as prey for aquatic native predators

Chapter 9

Alien versus predators: effective induced defenses of an invasive frog in response to native predators

[published in *Journal of Zoology*]

Inducible defences enhance fitness of prey living in environments with unpredictable predation risk, but these plastic reactions depend on the detection of the threat by the prey. To anuran larvae confronted with unknown predators, only prey-borne cues are noticeable, generally triggering either an incomplete set of reactions or no response at all. Thus, we should expect a certain disadvantage for establishing invasive anurans if tadpoles are unable to recognize local predators. Here, we test the presence and effectiveness of inducible defences in tadpoles of the invasive frog Discoglossus pictus confronting two native predators. Using both lab and mesocosm experiments, we also evaluate the effects these predators may exert on the invasive frog populations. Interestingly, although Discoglossus pictus has been introduced from another continent, its native (African) and invasive (European) ranges are included in the same ecoregion (Mediterranean Basin), sharing several genera and species of aquatic predators. In both experiments and using both invertebrates, tadpoles responded to the native predators, and our results match those usually reported in similar predator-prey systems using combinations of native species. Moreover, these reactions seem clearly effective in reducing mortality and injury rates of Discoglossus pictus tadpoles. We believe that the introduced frog is highly benefiting from a previous knowledge of populations of these or similar predator species. Therefore, even though native and invasive ranges of Discoglossus pictus are in different continents, the similarity of predator communities of both areas may be advantageous for its establishment and expansion.

PART C

Capgrossos de la granota pintada (*Discoglossus pictus*) com a presa per depredadors aquàtics autòctons.

Capítol 9

Alien vs. Predators: efectivitat de les defenses induïbles d'una granota invasora davant de depredadors autòctons

[publicat a la revista *Journal of Zoology*]

Les defenses induïbles milloren les probabilitats de supervivència de les preses que viuen en entorns amb un risc de depredació impredictible o variable, però aquestes reaccions plàstiques depenen de la detecció de l'amenaça per part de la presa. Per les larves d'anurs – els capgrossos – que s'enfronten a depredadors prèviament desconeguts, s'espera que només les senyals derivades de les preses depredades siguin detectades, fet que en general provoca un conjunt incomplet de reaccions induïbles o cap resposta en absolut. Per tant, hauríem d'esperar un cert desavantatge per a l'establiment d'espècies invasores d'anurs si els capgrossos són incapaços de reconèixer els depredadors locals. Aquí, posem a prova tant la presència com l'eficàcia de les defenses induïbles dels capgrossos de la granota pintada invasora (*Discoglossus pictus*) confrontant-los a dos depredadors autòctons molt comuns, les larves del pixaví estriat (un tipus de libèl·lula, *Sympetrum striolatum*) i els nedadors d'espatlles (*Notonecta glauca*). Amb l'ús tant d'experiments de laboratori com de mesocosmos, també avaluem els efectes que aquests depredadors poden exercir sobre les poblacions de la granota invasora. Com a detall important, tot i que la granota pintada ha estat introduïda des d'un altre continent, la seva distribució nativa (a l'Àfrica) i la seva distribució invasora (a Europa) s'inclouen en la mateixa bioregió (la conca mediterrània occidental), compartint diversos gèneres i espècies de depredadors aquàtics. En tots dos experiments i davant d'ambdós invertebrats, els capgrossos de la granota invasora van respondre correcta i efectivament als depredadors autòctons. Així doncs, els nostres resultats coincideixen amb els reportats en general en sistemes similars de depredador-presa entre espècies autòctones. Aquestes reaccions semblen un mètode clarament eficaç per la reducció de les taxes de mortalitat i de lesions dels capgrossos de granota pintada. Anant més enllà dels resultats, creiem que la granota introduïda es beneficia d'un coneixement previ d'aquests depredadors o d'espècies similars en la seva àrea de distribució nativa. Per tant, tot i que les àrees de distribució autòctona i invasora de *D. pictus* estan en diferents continents, la similitud de comunitats de depredadors de les dues àrees pot ser clarament avantatjós per al seu establiment i expansió.

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Alien versus predators: effective induced defenses of an invasive frog in response to native predators

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Abstract

Inducible defenses enhance fitness of prey living in environments with unpredictable predation risk, but these plastic reactions depend on the detection of the threat by the prey. To anuran larvae confronted with unknown predators, only prey-borne cues are noticeable, generally triggering either an incomplete set of reactions or no response at all. Thus, we should expect a certain disadvantage for establishing invasive anurans if tadpoles are unable to recognize local predators. Here, we test the presence and effectiveness of inducible defenses in tadpoles of the invasive frog *Discoglossus pictus* confronting two native predators. Using both lab and mesocosm experiments, we also evaluate the effects these predators may exert on the invasive frog populations. Interestingly, although *D. pictus* has been introduced from another continent, its native (African) and invasive (European) ranges are included in the same ecoregion (Mediterranean Basin), sharing several genera and species of aquatic predators. In both experiments and using both invertebrates, tadpoles responded to the native predators, and our results match those usually reported in similar predator-prey systems using combinations of native species. Moreover, these reactions seem clearly effective in reducing mortality and injury rates of *D. pictus* tadpoles. We believe that the introduced frog is highly benefiting from a previous knowledge of populations of these or similar predator species. Therefore, even though native and invasive ranges of *D. pictus* are in different continents, the similarity of predator communities of both areas may be advantageous for its establishment and expansion.

Introduction

Inducible defenses are a key feature enhancing fitness and survival of prey living in environments with unpredictable predator density or composition (Tollrian & Harvell, 1999; Miner *et al.*, 2005). For anuran tadpoles, reported changes confronting invertebrate predators mainly include reduction or shifts of activity levels (Skelly & Werner, 1990; Skelly, 1994), increase of the surface and height of tail fins (McCollum & Leimberger, 1997; Touchon & Warkentin, 2008) and coloration changes (Van Buskirk *et al.*, 2004; Touchon & Warkentin, 2008). While the first example is interpreted as a mechanism to avoid detection by predators (Skelly, 1994; Relyea, 2003), the second and third are usually interpreted as ways to lure the predator to non-lethal surfaces in case of attack (Van Buskirk *et al.*, 2003, 2004; Johnson, Burt & DeWitt, 2008).

However, these plastic reactions depend on the detection of the threat by the prey. In tadpoles, this detection is mediated through a combination of predator- and prey-borne cues (Hettyey *et al.*, 2010, 2015). Thus, when these are confronted with unknown predators, only prey-borne cues are noticed (Schoeppner & Relyea, 2009), triggering either an incomplete or milder set of reactions (Almeida *et al.*, 2011; Pujol-Buxó *et al.*, 2013; Nunes *et al.*, 2014) or even no response (Gomez-Mestre & Diaz-Paniagua, 2011). Hence, when inducible defenses play an important role in predator-prey interactions, we could expect certain disadvantages for invasive – and therefore naive – prey in their initial stages of introduction (Peacor *et al.*, 2006) if they are unable to generalize cues.

Even in largely studied organisms in terms of phenotypic plasticity like tadpoles, a consensus about their ability to generalize cues seems difficult. Some experiences show that tadpoles are able to use learning skills to alter their behavior confronting unknown predators (Mirza *et al.*, 2006; Gonzalo, López & Martín, 2007; Polo-Cavia & Gomez-Mestre, 2014), but in other cases, tadpoles react only to predatory cues from individuals belonging to particular populations (Griffiths *et al.*, 1998) or species (Van Buskirk, 2001). Other studies point at different degrees of cue generalization among similar species (Ferrari *et al.*, 2009). In general terms, a full set of inducible defenses (behavior, morphology, coloration) is more likely to appear as predators are more similar to the previously known (Ferrari *et al.*, 2009) and the co-occurrence of prey and predator is more ancient (Nunes *et al.*, 2014). This, in turn, should mean that invasive species with native and invasive ranges within the same ecoregion – with a co-evolutionary past with similar predators – could be able to generalize cues more easily.

In a previous study, Pujol-Buxó *et al.* (2013) reported a pattern of inducible defenses of tadpoles of the invasive Mediterranean Painted Frog *Discoglossus pictus* (Otth, 1837) more expected for a native species. The species lacked clear inducible defenses confronting invasive predators (crayfish *Procambarus clarkii* and mosquito fish *Gambusia affinis*) while showing clear plastic reactions in front of a native predator (dragonfly *Anax imperator*). Both invasive (Europe) and native (Northern Africa) ranges of *D. pictus* are included in the Western Mediterranean Basin ecoregion, possibly meaning that invasive tadpoles are not suffering from a shift in predator communities. Here, we test the presence and effectiveness of plastic reactions of this anuran confronting two common natural predators from its invasive range (the Notonectid *Notonecta glauca* and the Libellulid *Sympetrum striolatum*) and assess the possible effects of these reactions on its population dynamics. We used mesocosms – including competitor species to better assess the realism of the outcomes – and controlled lab experiments to this end. Although there are certain differences between both areas in terms of predator communities (Samraoui & Corbet, 2000; Askew, 2004; Annani, Alfarhan & Samraoui, 2012) and populations (Pilgrim & Von Dohlen, 2007), we hypothesized that tadpoles should be able to generalize cues and display clear inducible defenses in front of the chosen predators. Moreover, we expected this plasticity to be clearly adaptive, effectively reducing mortality and injury rates. To test these hypotheses, we combined common garden experiments – simulating natural ponds – and controlled lab experiments.

Materials and methods

Study species

The Mediterranean painted frog *D. pictus* is native to Northern Africa and Sicily (Zangari, Cimmaruta & Nascetti, 2006). It was introduced from Algerian specimens – subspecies *D. pictus auritus* (Zangari *et al.*, 2006) – around 1906 in Banyuls de la Marenda, France (Wintrebert, 1908). From then, it has expanded northwards up to Montpellier (SE France) and southwards to Vilassar de Mar (NE Spain) occupying now a continuously increasing (Llorente, Montori & Pujol-Buxó, 2015) coastal strip of approximately 250 km.

As the species mainly breeds in temporary and ephemeral ponds, based on observation we selected the common backswimmer *N. glauca* (Notonectidae, Heteroptera) and nymphs of the common darter *S. striolatum* (Libellulidae, Odonata) as predators for the study. *Notonecta glauca* can be found in a wide range of ponds (Svensson, Tallmark & Petersson, 2000), and it is the most common notonectid in the area. It is a visual predator, diving from the water surface or from a perch to capture prey with its legs (Giller & McNeil, 1981; authors pers. obs.). *Sympetrum striolatum* is the commonest *Sympetrum* species over much of Western Europe (Askew, 2004) and in the area of study. The long-legged nymph of this species is not a good swimmer, ambushing prey among the algae and detritus from the bottom of ponds (authors pers. obs.). Although conforming very different predator assemblages in terms of relative abundances, both genera are present also in the invasive range of *D. pictus*, including these two species (Askew, 2004; Annani *et al.*, 2012).

We chose two usual competitors of *D. pictus* (Richter-Boix *et al.*, 2012) with divergent features: the Natterjack Toad *Epidalea calamita* and the Stripeless Treefrog *Hyla meridionalis. Epidalea calamita* larvae are bottom dwelling and have low food consumption rates (Diaz-Paniagua, 1985; San Sebastián *et al.*, 2015b; Pujol-Buxó *et al.*, 2016) while *H. meridionalis* larvae use the water column and have intermediate food consumption rates (Diaz-Paniagua, 1985; Pujol-Buxó *et al.*, 2016).

Experimental procedures

Mesocosm experiment

Here, we tested the presence and effectiveness of *D. pictus'* inducible defenses confronting the chosen predators in an environment as realistic as possible, including two competitor species and several other organisms. Along two consecutive rainy nights from 5th to 7th of April of 2012, we took nine amplexing couples of *D. pictus,* eight amplexing couples of *E. calamita* and six amplexing couples of *H. meridionalis* from several natural ponds near Cassà de la Selva (UTM: 31T 49128 463531), NE Catalonia (Spain). Each pair was brought to the lab and placed in a container with water and perches to breed. Once the clutches were released, parents were freed in the capture points and clutches were taken care in 30L tanks. To avoid biases, tadpoles were randomly selected keeping the same proportions from different spawns, being transferred to experimental mesocosms species-wise as they reached Gosner stage 25 (Gosner, 1960). Mesocosms (90*60*40 cm) contained 150 L of water, including one sapling of *Ranunculus aquatilis* and *Cyperus eragrostis*, similar numbers of *Daphnia* spp., *Branchypus schaefferi*, Ostracods, Copepods and Freshwater Snails, plus leaf litter of *Quercus* spp., all taken from the study ponds. Roughly emulating natural proportions, we included 36 *D. pictus*, 36 *E. calamita* and 18 *H. meridionalis* tadpoles in each mesocosm. Two days after the addition of the last species (*H. meridionalis*), we introduced free-ranging predators, also collected from the same ponds: three individuals of *N. glauca* or *S. striolatum* were included in *Notonecta* and *Sympetrum* mesocosms, respectively, none to control mesocosms. Although mesocosms were protected with a net to avoid the entry of unwanted fauna, local predators sneaked into a number of them and these had to be discarded, leaving an unbalanced design: *Notonecta* treatment had five mesocosms, but we left only two for *Sympetrum* and three for control (five initially intended for both). This experiment was performed outdoors at Pedro Pons facilities (University of Barcelona, UTM: 31T 42589 458425).

We monitored activity levels of *D. pictus* counting the number of active (i.e. larvae that were swimming or feeding) and visible tadpoles in each mesocosm at the moment it was first viewed. We took activity measures thrice per day (morning, noon and afternoon) on five different days from the second week of the experiment until the onset of first metamorphosis. When tadpoles were 1-month-old, we randomly collected 10 individuals from each mesocosm and photographed them laterally for the morphological analyses. We checked the mesocosms daily. Once we spotted a tadpole in Gosner stage 42, it was removed from the experiment and placed with water and mud in a numbered individual cage until it achieved Gosner stage 46 (total time to metamorphosis). When there were no individuals of *D. pictus* left, we finished the experiment. Therefore, while survival of *D. pictus* corresponds to the proportion reaching complete metamorphosis, for *E. calamita* and *H. meridionalis* corresponds to the proportion of live tadpoles when the experiment ended, counting the few cases of metamorphosis of these two species as survivors as well.

Lab experiments

During the autumn-winter of 2013–2014, we conducted a series of predation trials to test the effects of the inducible defenses, detected in the previous mesocosm experiment, on the individual survival and injury probability. We collected predators and six amplexing couples of *D. pictus* from the same area (20th October 2013) and clutches were treated as in mesocosm experiments. Once they reached Gosner stage 25, we initiated the potential induction of the different morphologies of tadpoles by rearing them with chemical and visual contact with predators: two individual predators were kept and fed *D. pictus* tadpoles inside cylindrical transparent cages (8 cm diameter and 15 cm height, with the bottom replaced by a net in order to allow full chemical contact) immersed in the rearing tanks (35*20*20 cm). Each tank contained 10 tadpoles, which were fed *ad libitum*. Non-induced tadpoles environment was manipulated in exactly the same way as the induced tadpoles but without any predator inside the cages. Based on previous experiences, we considered that we could use tadpoles for predation trials after at least 2 weeks of induction. Predation trials consisted in keeping three induced and three non-induced tadpoles of matching sizes during 24 h with a free-ranging predator and some refugia (natural stones and plastic plants). Tanks used for predation trials had the same measures as the rearing tanks. In order to identify which individuals survived the test, we took side photographs of all tadpoles before and after – if survived – each test.

Statistical analyses

Mesocosm experiment

Survival data had very low n and did not meet the necessary assumptions for parametric or rank tests, so we tested differences between treatments applying pairwise non-parametric randomization ANOVAs (NP-R-ANOVAs), using *adonis* from the package *vegan* (Oksanen *et al.*, 2015).

Activity was treated as a binomial response variable (1 = active, 0 = inactive). We selected the best generalized linear mixed model (GLMM) using AIC, BIC and likelihood ratio tests (LRTs). As potential explanatory variables, we included treatment and time (morning, noon, afternoon) as factors, plus age (days) as a covariate, allowing for first-order interactions. We always included 'tank' as a random effect to account for the lack of independence of the data from each mesocosm.

Total time to metamorphosis was analyzed fitting linear mixed models (LMMs). We created a full model including treatment as fixed effect and 'tank' as random effect, and a null model including only 'tank', testing the significance of treatment comparing both models using a LRT.

To analyze morphology of tadpoles, we digitized five landmarks and 19 semi-landmarks in their lateral photographs (Fig. S1). Injured tadpoles had to be discarded and thus we digitized 30 control tadpoles (no injuries), 48 tadpoles from *Notonecta* treatment (two injured) and 18 tadpoles from *Sympetrum* treatment (two injured). In order to neutralize the effect of the different position a live tadpole may adopt – different angles between tail and body – we first made a Procrustes superimposition, followed by the standardization of the angles (using *fixed.angle* from *geomorph*), finally superimposing the specimens for a second time. We then tested the effects of treatment, size and their interaction on tadpole morphology applying pairwise NP-R-ANOVAs using 999 replications. To compare the effects of morphological induction among venues and under different predators, we applied a trajectory analysis (Collyer & Adams, 2013) which tests separately the amount and direction of the morphological change. As a surrogate of size, we always used the centroid sizes obtained from a Procrustes superimposition excluding landmarks from tail fin (Fig. S1). We tested size differences between treatments fitting LMMs and applying LRTs between a null model (including only tank as a random effect) and a full model (including also treatment as a fixed effect).

Lab experiments

Before testing the effects of the inducible defenses, we needed to prove that our tadpoles had indeed changed their morphology. Therefore, we first tested for morphological differences between groups of tadpoles using the same routines described for the mesocosm experiment. In order to acquire a linear classifier representing the degree of induction of each tadpole (useful for subsequent analyses), we projected morphological data on the axis defined between the multivariate mean shapes of control and induced tadpoles ('between groups PCA' *sensu* Mitteroecker & Bookstein, 2011).

Using the final status of each tadpole (alive, wounded or dead), we compared the probability of surviving/surviving unwounded between induced and control tadpoles. To test these results, we fitted GLMMs using binomial response variables (1 = dead/dead or wounded, 0 = alive/alive unwounded). As potential explanatory variables, we included tadpole size, rearing treatment (induced vs. non-induced tadpoles), and degree of morphological induction of each tadpole. To correct for lack of independence, we included predation trial as a random effect in the model.

We fitted and selected models using the lme4 package (Bates *et al.*, 2014) using *glht* from *multcomp* package (Hothorn, Bretz & Westfall, 2008) for post hoc comparisons when necessary. We did all geometric morphometrics analyses using *geomorph* (Adams & Otarola-Castillo, 2013). All analyses and figures of this paper were done using (R core team 2015).

Results

Mesocosm experiment

Survival in mesocosms under *N. glauca* predation risk was significantly lower for all species (Fig. 1): survival of *E. calamita* diminished up to 80.5% relative to control levels ($F_{1,6} = 73.49$, $P < 0.001$), *H. meridionalis'* diminished 81.6% ($F_{1,6} = 109.8$, $P = 0.017$) and *D. pictus'* 30.3% ($F_{1,6} = 6.73$, $P = 0.036$). Survival in mesocosms under *S. striolatum* predation risk was significantly lower for *E. calamita* (70% decrease, $F_{1,3} = 21.57$, $P = 0.008$), but survival variations in the other two species were not significant (15.4% decrease for *H. meridionalis,* $F_{1,3} = 1.44$, $P = 0.308$), unexpectedly mildly increasing 28.2% in the case of *D. pictus* ($F_{1,3} = 7.10$, $P = 0.200$).

The best model for activity (Fig. 2) of tadpoles included treatment, age and their interaction (Table S1). This meant a globally diminished activity both under *N. glauca* ($z = -3.995$, $P < 0.001$) and *S. striolatum* ($z = -2.398$, $P = 0.016$) predation risk, particularly during the early period of experimental induction (days 24–27).

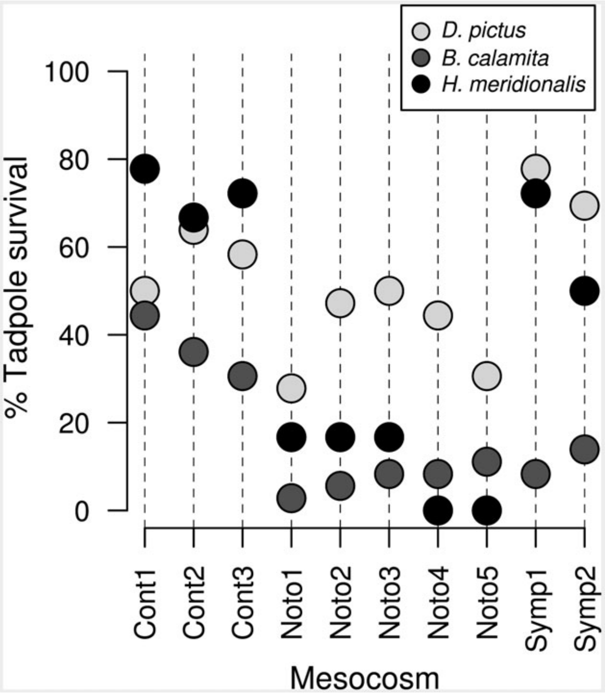

Figure 1 Survival of each species in each mesocosm. Control mesocosms: Cont1 to Cont3, *Notonecta* mesocosms: Noto1 to Noto5, *Sympetrum* mesocosms: Symp1 and Symp2.

Treatment significantly affected total developmental timing ($\chi^2_1 = 7.42$, $P = 0.025$). Control tadpoles lasted 45.24 ± 0.68 days (mean ± SE) to achieve Gosner stage 46, while *Notonecta* tadpoles lasted 49.27 ± 0.64 days and *Sympetrum* tadpoles 46.30 ± 0.62 days. Therefore, presence of *N. glauca* delayed development in 4.03 days in mean ($z = 2.56$, $P = 0.028$), while the presence of *S. striolatum* delayed it 1.06 days. However, in the latter case, differences were not significant ($z = 0.487$, $P = 0.877$).

In the mesocosm experiment, treatment had no significant effect on tadpole size ($\chi^2_1 = 1.87$, $P = 0.391$). There were significant differences in mean tadpole shape ($F_{2,90} = 8.20$, $P < 0.001$) and shape allometry ($F_{2,90} = 2.95$, $P = 0.005$) among mesocosm treatments: *Notonecta* tadpoles changed their mean shape ($P = 0.002$) compared to control tadpoles, while *Sympetrum* tadpoles changed their shape allometry ($P = 0.045$). There were significant differences in mean body shape ($F_{2,354} = 31.11$, $P < 0.001$) and shape allometry ($F_{2,354} = 3.26$, $P = 0.004$) among treatments in lab as well: *Sympetrum* tadpoles changed their mean shape ($P = 0.002$) compared to control tadpoles while *Notonecta* tadpoles changed their shape allometry ($P = 0.002$). Responses were always similar in response to both predators (Fig. 3): tadpoles confronting predators had shorter and deeper bodies with deeper tail fins. However, in lab, reactions to both predators had a significantly different direction ($z_{1,456} = 2.24$, $P = 0.003$), being more exaggerated in the presence of *N. glauca* ($z_{1,344} = 4.34$, $P = 0.001$). In mesocosms, these differences were not detected ($F_{1,117} = 1.549$, $P = 0.160$).

As venue-linked differences, mesocosm tadpoles displayed bigger heads and less muscular tails than lab tadpoles ($F_{1,211} = 12.66$, $P < 0.0001$ for Control tadpoles, $F_{1,107} = 11.28$, $P < 0.0001$ for *Sympetrum* tadpoles and $F_{1,140} = 36.69$, $P < 0.0001$ for *Notonecta* tadpoles). Interestingly, trajectory analysis revealed no differences among venues in direction ($z_{1,344} = 1.793$, $P = 0.141$) or amount ($z_{1,344} = 0.218$, $P = 0.848$) of morphological change linked to the presence of

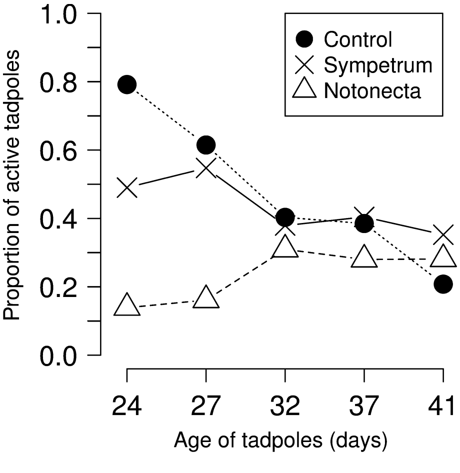

Figure 2 Mean proportion of active *Discoglossus pictus* tadpoles in mesocosms of each treatment and day in which data was taken. Control = control treatment. *Sympetrum* = *Sympetrum* treatment, *Notonecta* = *Notonecta* treatment.

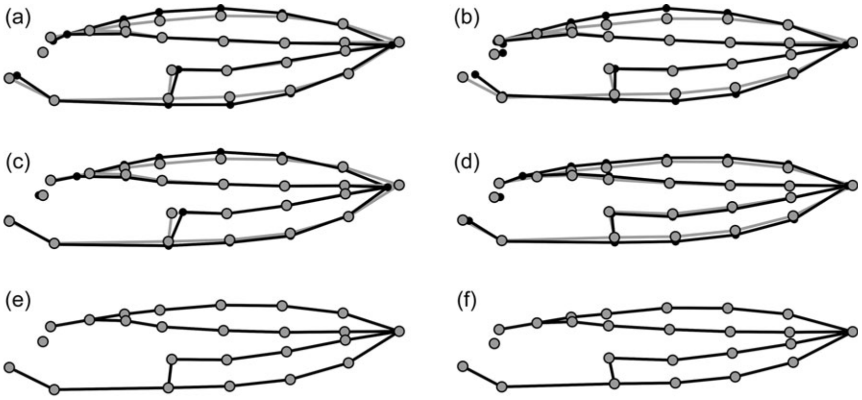

Figure 3 *Discoglossus pictus* tadpole mean morphologies for each treatment (black) in relation to their control morphology (gray), differences magnified 2× in order to ease visualization. (a) *Notonecta* treatment, mesocosm, (b) *Notonecta* treatment, Lab, (c) *Sympetrum* treatment, mesocosm, (d) *Sympetrum* treatment, Lab, (e) Control treatment, mesocosm, (f) Control treatment, Lab

S. striolatum. The direction of morphological changes confronting *N. glauca* was also the same in both venues ($z_{1,344} = 1.4$, $P = 0.102$), but the amount of change was slightly exaggerated in lab ($z_{1,344} = 2.46$, $P = 0.012$).

Lab experiments

Experienced tadpoles significantly decreased injury rates (Tables S2 and S3, Fig. 4) confronting both *N. glauca* (84% decrease compared to control, $\chi^2_1 = 5.11$, $P = 0.024$) and *S. striolatum* (55% decrease, $\chi^2_1 = 9.06$, $P = 0.003$). Interestingly, in the first case, tadpole induction seems better expressed by the treatment as a factor, but for *S. striolatum*, the degree of induction of each tadpole was the selected variable. In both cases, tadpoles were more likely to survive unwounded as they were larger ($\chi^2_1 = 4.03$, $P = 0.047$ for *N. glauca*, $\chi^2_1 = 9.04$, $P = 0.003$ for *S. striolatum*).

More importantly, deaths of induced tadpoles were reduced (Tables S4 and S5, Fig. 4), compared to control tadpoles, up to 87% confronting *N. glauca* ($\chi^2_1 = 4.21$, $P = 0.040$) and up to 40% confronting *S. striolatum* (in this case, however, not significant: $\chi^2_1 = 1.11$, $P = 0.262$). Treatment as a factor was the variable selected in the first case – not the degree of induction of each tadpole. Larger tadpoles were less likely to be predated ($\chi^2_1 = 3.755$, $P = 0.053$ for *N. glauca*, $\chi^2_1 = 7.06$, $P = 0.008$ for *S. striolatum*). Overall, *N. glauca* predated 15% of control tadpoles but 3% of the induced tadpoles, while *S. striolatum* predated 6% of the control tadpoles but 3% of the induced tadpoles.

Discussion

Although this study confronts invasive prey with native predators, we predicted similar inducible defenses – and their costs – to those usually reported in similar native predator-prey systems. Results follow our predictions, as *D. pictus* tadpoles decreased their activity (Fig. 2), increased tail fin area (Fig. 3) – considered the main tadpole nonlethal surface in case of attack (Van Buskirk *et al.*, 2003, 2004; Johnson *et al.*, 2008) –, and developed slower in turn (Tejedo *et al.*, 2010) in the presence of both native predators. Morphological changes from both venues largely match, being only exaggerated in magnitude when confronting *N. glauca* in lab. Therefore, even though the morphological starting point may be different – mesocosm tadpoles displayed bigger heads -, lab experiments seem a valid surrogate for outcomes in more natural-like environments.

Concretely, tadpoles under predation risk from *N. glauca* displayed the most effective tandem of behavioral and morphological responses (Fig. 4): induced tadpoles largely reduced rates of predation and injury in lab trials, statistically equaling survival of control treatment in mesocosms. Tadpoles confronting *S. striolatum* displayed milder responses, but positive outcomes nevertheless in terms of survival. Most probably because in this case reduction of death rates seems mainly mediated through diminishing the number of attacks (Fig. 4), these benefits were clearer in complex environments like mesocosms (Fig. 1), where avoidance tactics are easier to implement. In any case, tadpoles developing under predation risk from both predators were not exempt of costs, like delays in development. Developmental delays are repeatedly reported in similar studies, and most probably related to the induced defenses and behavior (Tejedo *et al.*, 2010). Tadpoles under predation risk diminished their activity mainly in the first stages of development (similarly as in Relyea, 2003), suggesting that behavior-linked costs may originate in these stages in which maintaining high activity levels incurs in high survival costs (Relyea & Werner, 1999; Laurila *et al.*, 2002).

Since survival of competitors generally decreased more markedly than *D. pictus'* in the presence of predators (Fig. 1), this competitive decrease could be an additional positive factor indirectly improving outcomes for the invasive tadpoles (San Sebastián *et al.*, 2015a,b). Given that inducible defenses were most probably present in the competitors as well, different survivorship among species is most probably related to higher growth rates in the invasive tadpoles (García-París, Montori & Herrero, 2004): predator attacks are more common and lethal

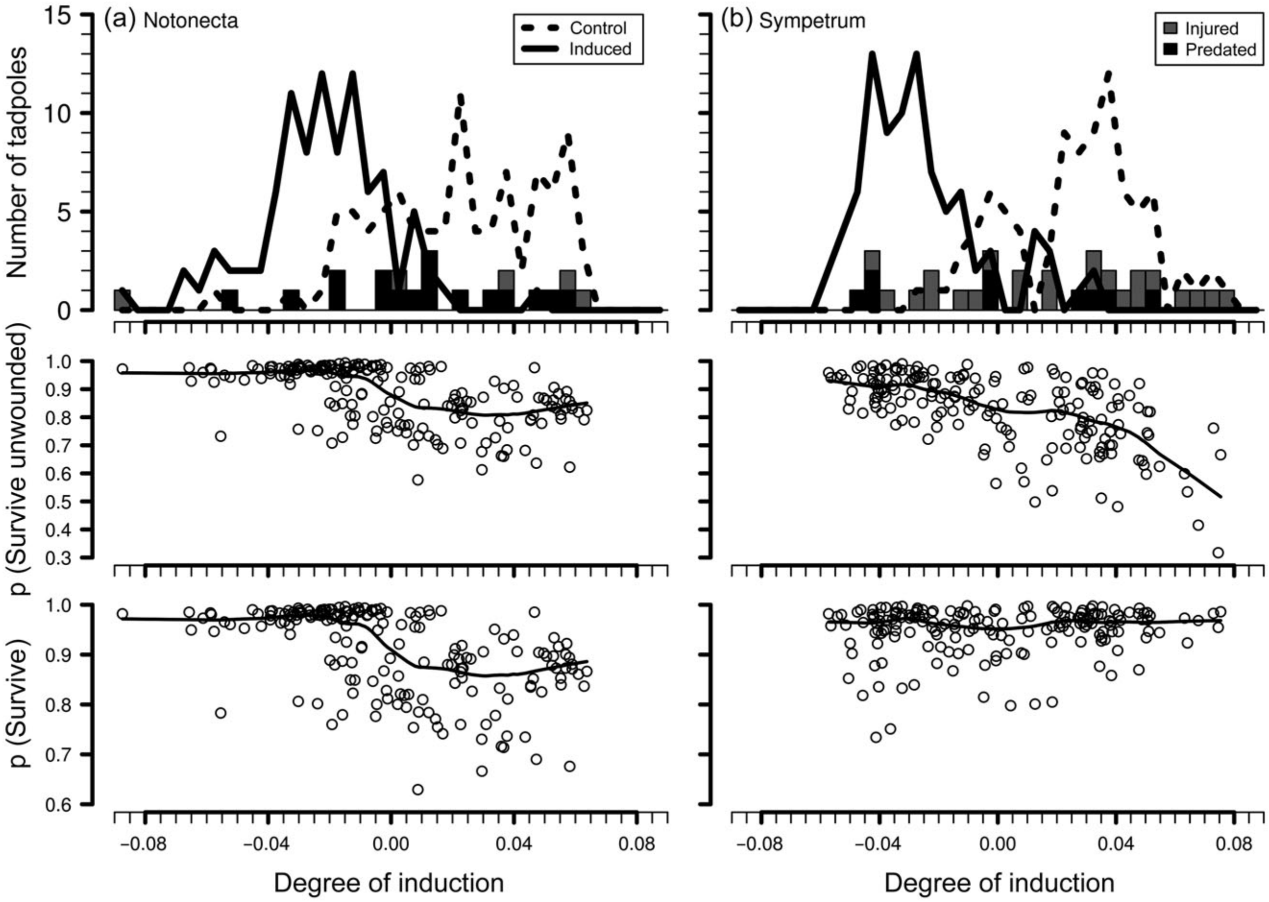

Figure 4 Survival and injuries of tadpoles under direct predation from each predator. Upper row: number of total (lines), injured (gray columns) and dead (black) tadpoles along the morphological induction axis (left = more predator-induced, right = less predator-induced). Mid row: probability of surviving unwounded the lab predation test (according to binomial models) and general tendency (line). Lower row: probability of surviving the lab predation test (according to binomial models) and general tendency (line).

on smaller individuals (see Results), so during first weeks after the onset of the temporary ponds, faster growth in *D. pictus* may help to direct predatory pressure to its competitors. Finally, other factors could interact in a natural-like environment, like habitat use: for instance, tadpoles of *H. meridionalis*, which are water column dwellers, clearly suffered more from the water column predator (*Notonecta glauca*). Further experiments are needed to disentangle the relative importance of all these factors. Although the loss of mesocosm replicates weakened this part of our study in terms of available data, the great similarity of results among tanks of the same treatments makes the authors think that all these outcomes are not spurious.

Both predators used in the experiments are also present – or in very similar forms (Samraoui & Corbet, 2000; Annani *et al.*, 2012) – in the native origin (i.e. Northern Algeria, Zangari *et al.*, 2006) of the invasive European populations of *D. pictus*. Despite possible specific/subspecific differences between predator populations and assemblages, invasive tadpoles seem able to generalize their cues and display an effective set of reactions. Lacking or mild inducible defenses of *D. pictus* when confronted to previously unknown predators (Pujol-Buxó *et al.*, 2013) makes us discard that the observed full set of reactions was triggered only by prey-borne cues. As some studies highlight the presence of different reactions matching particularities of each predator (Van Buskirk, 2001; Hettyey *et al.*, 2015), even suggesting ‘fine-tuned phenotypes’ (Relyea, 2004), more marked reactions confronting *N. glauca* should be unrelated with the detection of the threat by the prey, simply matching a higher lethality of the Notonectid.

Conclusions

Tadpoles of the invasive populations of *D. pictus* effectively reduce predation rates from their common native predators by means of inducible defenses. Our results clearly match those usually reported among similar native predator-prey systems, suggesting a lack of disadvantages for invasive tadpoles, and a generalization of cues from the native – North African – to the invasive – European – range.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Generalized linear mixed model selection table for tadpole activity in the mesocosm experiment.
Table S2. Generalized linear mixed model selection table for «surviving unwounded» undergoing *Notonecta glauca* predation risk the mesocosm experiment.
Table S3. Generalized linear mixed model selection table for «surviving unwounded» undergoing *Sympetrum striolatum* predation risk the mesocosm experiment.
Table S4. Generalized linear mixed model selection table for «surviving» undergoing *Notonecta glauca* predation risk the mesocosm experiment.
Table S5. Generalized linear mixed model selection table for «surviving» undergoing *Sympetrum striolatum* predation risk the mesocosm experiment.
Figure S1. Landmarks and semi-landmarks used in the study. Definitions of each landmark are described below. Landmarks are 1, 2, 5, 10, 24. The rest of points were statistically treated as semi-landmarks.

Supplementary material for:
Eudald Pujol-Buxó, Cristian García-Guerrero, and Gustavo A. Llorente (2016) Alien vs. Predators: Effective induced defenses of an invasive frog in front of native predators .

Table S1. Generalized linear mixed model selection table for tadpole activity in the mesocosm experiment. Treat: treatment at which the mesocosm was adscribed (control / notonecta / sympetrum); Age: age of tadpoles at the moment the activity measure was taken; Time: time of the day (morning / noon / afternoon); (tank): number of the mesocoms, random effect to account for autocorrelation. Best model according to AIC, BIC and logLikelihood ratio test is highlighted in italics. All models tested against best model («no» when test not applyable):

model	Df	AIC	BIC	logLik	X^2	dfX^2	p(X^2)
actives ~ (tank)	2	404.32	410.34	-200.1	51.69	14	<0.001
actives ~ treat + (tank)	4	395.09	407.13	-193.5	38.47	12	<0.001
actives ~ treat + age + (tank)	8	386.39	410.48	-185.2	21.77	8	0.005
actives ~ treat + age + time + (tank)	10	386.32	416.42	-183.1	17.69	6	0.007
actives ~ treat + age + treat:age + (tank)	*16*	*380.62*	*428.79*	*-174.3*	-	-	-
actives ~ treat + age + treat:age + time + (tank)	18	380.24	434.43	-172.1	4.382	2	0.112

Table S2. Generalized linear mixed model selection table for «surviving unwounded» undergoing *Notonecta glauca* predation risk the mesocosm experiment. Csize: centroid size of tadpole (excluding tail fins); indAXIS: degree of induction of each tadpole along the induction axis (see Methods); treat: rearing treatment (induced / control); (test): test in which each tadpole was tested (random effect, always included). Best model according to AIC, BIC and logLikelihood ratio test is highlighted in italics.All models tested against best model («no» when test not applyable) :

model	Df	AIC	BIC	logLik	X^2	dfX^2	p(X^2)
Attacked ~ (test)	2	139.22	145.67	-67.61	18.57	2	0.093
Attacked ~ Csize + (test)	3	127.76	137.43	-60.88	5.11	1	0.024
Attacked ~ indAXIS + (test)	3	138.29	147.97	-66.15	15.65	1	0.001
Attacked ~ treat + (test)	3	126.68	136.36	-60.34	4.03	1	0.045
Attacked ~ Csize + treat + (test)	*4*	*124.65*	*137.55*	*-58.32*	-	-	-
Attacked ~ treat + indAXIS + (test)	4	127.67	140.57	-59.84	no	no	no
Attacked ~ Csize + indAXIS + (test)	4	129.15	142.05	-60.57	no	no	no
Attacked ~ Csize + treat + indAXIS + (test)	5	126.09	142.22	-58.04	0.56	1	0.455

Table S3. Generalized linear mixed model selection table for «surviving unwounded» undergoing *Sympetrum striolatum* predation risk the mesocosm experiment. Csize: centroid size of tadpole (excluding tail fins); indAXIS: degree of induction of each tadpole along the induction axis (see Methods); treat: rearing treatment (induced / control); (test): test in which each tadpole was tested (random effect, always included). Best model according to AIC, BIC and logLikelihood ratio test is highlighted in italics.All models tested against best model («no» when test not applyable) :

model	Df	AIC	BIC	logLik	X^2	dfX^2	p(X^2)
Attacked ~(test)	2	171.47	177.85	-83.74	16.23	2	0.000
Attacked ~ indAXIS +(test)	3	166.30	175.86	-80.15	9.06	1	0.003
Attacked ~ Csize +(test)	3	166.28	175.84	-80.14	9.04	1	0.003
Attacked ~ treat +(test)	3	167.48	177.04	-80.74	10.24	1	0.001
Attacked ~ indAXIS + treat +(test)	4	167.94	180.69	-79.97	no	no	no
Attacked ~ Csize + indAXIS +(test)	*4*	*159.24*	*171.99*	*-75.62*	-	-	-
Attacked ~ Csize + treat +(test)	4	162.63	175.38	-77.31	no	no	no
Attacked ~ Csize + treat + indAXIS +(test)	5	161.24	177.17	-75.62	0.01	1	0.991

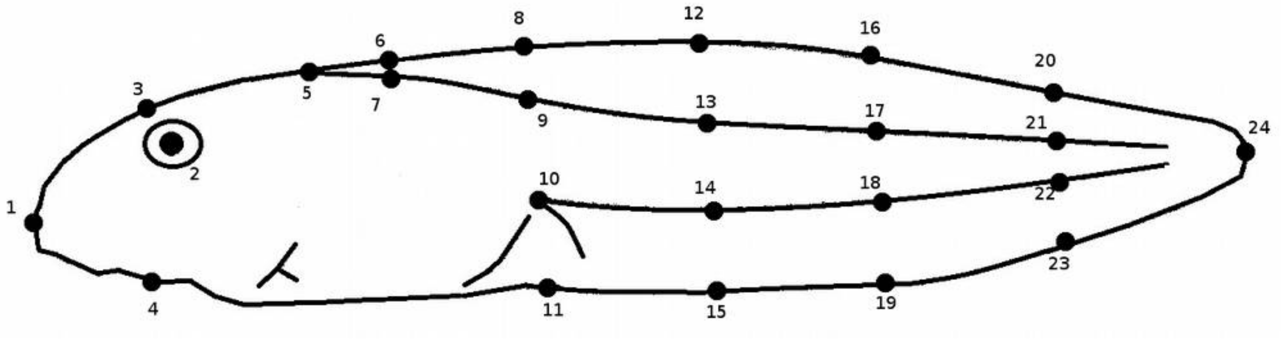
Table S4. Generalized linear mixed model selection table for «surviving» undergoing *Notonecta glauca* predation risk the mesocosm experiment. Csize: centroid size of tadpole (excluding tail fins); indAXIS: degree of induction of each tadpole along the induction axis (see Methods); treat: rearing treatment (induced / control); (test): test in which each tadpole was tested (random effect, always included). Best model according to AIC, BIC and logLikelihood ratio test is highlighted in italics. All models tested against best model («no» when test not applyable):

model	Df	AIC	BIC	logLik	X^2	dfX^2	p(X^2)
Deaths ~ (test)	2	117.74	124.19	-56.87	16.01	2	0.001
Deaths ~ Csize + (test)	3	107.94	117.62	-50.97	4.21	1	0.040
Deaths ~ indAXIS + (test)	3	117.97	127.65	-55.99	no	no	no
Deaths ~ treat + (test)	3	107.49	117.17	-50.74	3.76	1	0.053
Deaths ~ Csize + treat + (test)	*4*	*105.73*	*118.64*	*-48.87*	-	-	-
Deaths ~ indAXIS + treat + (test)	4	108.07	120.97	-50.03	no	no	no
Deaths ~ Csize + indAXIS + (test)	4	109.75	122.65	-50.88	no	no	no
Deaths ~ Csize + treat + indAXIS + (test)	5	106.80	122.93	-48.40	0.93	1	0.334

Table S5. Generalized linear mixed model selection table for «surviving» undergoing *Sympetrum striolatum* predation risk the mesocosm experiment. Csize: centroid size of tadpole (excluding tail fins); indAXIS: degree of induction of each tadpole along the induction axis (see Methods); treat: rearing treatment (induced / control); (test): test in which each tadpole was tested (random effect, always included). Best model according to AIC, BIC and logLikelihood ratio test is highlighted in italics. All models tested against best model («no» when test not applyable):

model	Df	AIC	BIC	logLik	X^2	dfX^2	p(X^2)
Deaths ~ (test)	2	75.36	81.74	-35.68	7.06	1	0.008
Deaths ~ Csize + (test)	*3*	*70.31*	*79.87*	*-32.15*	-	-	-
Deaths ~ indAXIS + (test)	3	77.33	86.89	-35.67	no	no	no
Deaths ~ treat + (test)	3	76.25	85.82	-35.13	no	no	no
Deaths ~ Csize + treat + (test)	4	71.20	83.95	-31.60	1.11	1	0.292
Deaths ~ indAXIS + treat + (test)	4	77.31	90.06	-34.66	no	no	no
Deaths ~ Csize + indAXIS + (test)	4	71.96	84.71	-31.98	0.35	1	0.556
Deaths ~ Csize + treat + indAXIS + (test)	5	73.10	89.03	-31.55	1.21	2	0.546

Figure S1. Landmarks and semilandmarks used in the study. Definitions of each landmark are described below. Landmarks are 1, 2, 5, 10, 24. The rest of points were statistically treated as semilandmarks.



Landmark 1 - Most anterior point of the headbody

Landmark 2 - Center of the eye

Semilandmarks 3 & 4- Respectively, dorsal and ventral points of the headbody at one quarter of the distance between landmarks 1 and 10

Landmark 5 - Intersection between the headbody and the dorsal edge of the tail fin.

Semilandmarks 6 & 7 - Respectively, dorsal and ventral points of the headbody at three quarters of the distance between landmarks 1 and 10.

Landmark 10 - Intersection between the headbody and the lower edge of the tail muscle

Semilandmarks 8, 9 & 11 - Respectively, dorsal side of the tail fin, dorsal side of the the tail musculature and ventral side of the tail fin at the same vertical line as of landmark 10.

Semilandmarks 12, 13, 14 & 15 - Respectively, dorsal side of the tail fin, dorsal side of the the tail musculature, ventral side of the tail musculature and ventral side of the tail fin at half the distance between landmarks 10 and 24.

Semilandmarks 16, 17, 18 & 19 - Respectively, dorsal side of the tail fin, dorsal side of the the tail musculature, ventral side of the tail musculature and ventral side of the tail fin at half the distance between landmarks 10 and 24.

Semilandmarks 20, 21, 22 & 23 - Respectively, dorsal side of the tail fin, dorsal side of the the tail musculature, ventral side of the tail musculature and ventral side of the tail fin at three quarters of the distance between landmarks 10 and 24.

Landmark 24 - Tip of the tail fin

GENERAL DISCUSSION

GENERAL DISCUSSION

To ease the organization of the treatment of the several topics, I divided the discussion in five sections, first discussing each part of the thesis separately – therefore, adding up to three parts – with two additional sections to wrap up the reflections produced from the work here presented. Although the idea is to present an organized discussion, the aim here is to give an global view of the results as well. Hence, even though divided in sections, in order to enhance the integrative nature of the text, I am not here excessively strict with the topics discussed within each of them.

1. Phenotypic and genetic evolution in the invasive populations of the Mediterranean Painted Frog in Europe

All previous studies involving *D. pictus* had hitherto overlooked the possibility that its invasive populations might indeed have phenotypic and genetic differences among them. Thus, comparisons across populations had been either absent or restricted to differences between native and invasive ranges (e.g. Escoriza & Boix 2014). This indirectly assumes that the invasive populations of *D. pictus* are, at least in general terms, a more or less homogeneous unit. However, literature on similar cases of biological invasions (Phillips & Shine 2005, Phillips *et al.* 2006, Rollins *et al.* 2009 2011, Xuan *et al.* 2010, White *et al.* 2013, Ducatez *et al.* 2016) generally supports the opposite possibility, reporting marked genetic and phenotypic structure within invasive ranges. This structure is found to be linked either to local adaptations or to the expansion process itself. Interestingly, depending on the trait and the invasive species under study, either one factor – local adaptations – or the other – evolutionary effects of the expansion itself – dominates. As an example for the first possibility, invasive populations of the American Bullfrog *Lithobates catesbeianus* in China (Xuan *et al.* 2010) exhibit geographical variation in morphological and life-history traits, fitting predictions of response to climatic differences across locations. On the other hand, the case of *Rhinella (Bufo) marina* in Australia is highly relevant for the other possibility: individuals belonging to the expansion front of the species are exaggeratedly differentiated in external and internal morphology, behaviour, and immune function (Phillips & Shine 2005, Phillips *et al.* 2006). Strikingly in this case, although genetic structure among populations is fairly low – probably due to an already meagre genetic diversity inherited from the introduction event itself – the toad is able to to create prominent phenotypic differences mainly through different degrees of genic expression, possibly involving other processes like epigenetics (Rollins *et al.* 2015).

So, does *D. pictus* display similar differences? Concerning phenotypic variations in adult specimens explored hitherto, the main conclusion is that populations indeed display phenotypic differences among them. These are found to be possibly linked to local climatic factors, concretely, to mean annual precipitation. Thus, the correlation between mean adult body size and mean annual precipitation is, according to the data gathered hitherto, the cause behind the small adult sizes in the surveyed native populations from northern Algeria. Matching the variations in mean body size, mean growth rates and reproductive age distributions also varied between the native (1-5 years, dry area) and invasive populations (2-4 years, rainy area) analysed. Unluckily, the number of populations studied to test these phenotypic differences can still be considered too low to be conclusive. Hence, the sampling of additional populations in both ranges should elucidate if this pattern still holds along the whole range of the frog.

At any rate, perhaps here it is more interesting to note that the recent divergence among populations of the invasive range – decades – does not prevent to observe phenotypic differences among them. Obviously, it is important to remark that these differences may be created through several mechanisms – from plasticity to epigenetics – that we did not try to ellucidate. On the other hand as well, it is interesting to note that, after years of sampling and surveying in several parts of the range of the frog, I could not observe any striking differences – meaning easily visible on bare eye – among frogs in the edge of the expansion front and the rest of populations. This does not mean that there are not any differences at all, since the phenotype extends much further from what we see or measure, but anyway it is important to remark here that there are no expansion-front dispersing phenotypes that become easily detectable – thus differing from the case of *Rhinella marina*. For the moment, we could say that *D. pictus* resembles more the pattern found in *L. catesbeianus* in terms of phenotypic divergence, where local adaptation most probably overruns the selective pressures of the expansion and colonization events. This could be related either to the speed and dynamics of each expansion – *R. marina* clearly surpasses the other two species in terms of speed of invasion – or to other factors possibly affecting microevolution, like rates of migration among populations or the initial evolutionary potential – non-neutral genetic diversity – in the invasive range. Finally, in the case of *D. pictus*, it has yet to be explored if local adaptations – which create larger or smaller individuals in the expansion front depending on the climate – can influence the expansion dynamics of the species. This would be significant for future predictions of the rates of expansion across the dryer areas present to the south and west of its present distribution in the Iberian Peninsula, for example.

On the other hand, we could find evidence of genetic structure linked to the expansion history of the invasive populations. In this case, tests to detect local adaptations or expansion-linked genetic differences were not yet conducted, posing an interesting future direction (see Section 5 of this discussion). We found a clear loss of genetic diversity along both expansions of the frog – *i.e.* northwards and southwards from Banyuls –, while exploratory and clustering analyses suggest largely independent genetic histories for each colonizing direction. Interestingly for the **PART B** of this thesis, the near total lack of population structure among the locations from the tip of the southern expansion front, even if separated up to 20 or 40 km, may imply that patterns of phenotypic variation should be difficult to observe in short distances, or at least near the southern limits of the invasive distribution. Thus, the apparent contradiction between the lack of evolutionary patterns found in Chapters 6 – and 7, hitherto – , and the positive results found in Chapter 5, might correspond to the poor genetic isolation among the populations analysed in the first pair of chapters – which explored the southern tip of the expansion front –, and much greater genetic differences between the two populations analysed in Chapter 5. In this second case (Chapter 5), it is interesting to note that the individuals from La Jonquera, even though being from the southern side of the Pyrenees, are always grouped with northern populations and the origin area, that is, Banyuls. Thus, individuals sampled in La Jonquera were correctly considered beforehand to be near enough the original point of introduction to represent data from the "origin area", and to be genetically different enough from the populations of the southern expansion front. However, although genetic structure can easily shape adaptive or phenotypic structure, and therefore helps to explain the pattern of results observed in **PART B**, it is important to remind that the case of *Rhinella marina* highlights that striking phenotypic change can also happen with few genetic differences (Rollins *et al.* 2015) .

On the other hand, the genomic analyses of populations also helped to detect and confirm historic particularities of the northern expansion of the species. In this sense, individuals from Jacou, which belong to a group of populations originating from either a translocation or to a very swift invasion of the Hérault department in France, display markedly low intrapopulation genetic diversity and have greatly differentiated genetically from the other populations on the northern expansion front. In this case, it is very interesting to note that Geniez & Cheylan (2012) comment that frogs from that group of populations are all phenotypically similar in terms of colouration, making very interesting to conduct further tests of phenotypic variability and reduced evolutionary potential in the Hérault populations. This possible link among phenotypic variation in colouration and genetic diversity is the main possibility I regret not having had time to explore numerically in this part of the thesis. Thus, similarly in the southern expansion, it seems, at bare eye, that adults display higher diversity of colouration in the origin area – where the frog present all three possibilities, *i.e.* red uniform, a striped, and a "painted" design – than in the expansion front – where adults display mainly the "painted" design. This could be the only phenotypic difference I have observed – or I believe to have observed, I insist, this has to be tested seriously and numerically – which could relate to the expansion history itself.

Summing up, at least with the data gathered hitherto, it seems that the species can be affected by local adaptation at the phenotypic level, and has an underlying genetic structure related to the expansion itself. Concretely, the mean size and life-history of the species is seemingly affected by local mean annual precipitation, while the underlying genetic structure among populations is visibly affected by the paths of historic colonizations of new territories. As already commented, this pattern totally opposes the observed in the most studied anuran expansion to date – *Rhinella marina* in Australia –, where main phenotypic differences are found in relation to the expansion history and genetic structure is largely absent.

2. Possible impacts on native competitors

Apart from the community wide study in Chapter 3, in this thesis we have repeatedly compared the Mediterranean Painted Frog – with or without being the main focus on competition – with its two main competitors: the Natterjack toad *Epidalea (Bufo) calamita*, and the Parsley Frog *Pelodytes punctatus* (Montori *et al.* 2007, Escoriza & Boix 2012, Richter-Boix *et al.* 2013). A general summary of results, including competitive abilities and other interesting data for discussion, is exposed on Table 1. Concerning the competitive interaction most studied in the **PART B** of this thesis, most results of this thesis, and previous studies (Table 1), clearly point that *D. pictus* is a very good competitor – and a good survivor under other situations, like predation risk – at the individual level, clearly performing much better than *Epidalea calamita*. The invasive frog has a clearly consumption-based growth (Chapter 3) and accordingly, has great behavioural abilities to improve its consumption rates and food quality to achieve it (Chapter 4). This tandem is probably behind the competitive impacts observed in this thesis and similar studies (Richter-Boix *et al.* 2013, San Sebastian *et al.* 2015), as the invasive species clearly surpasses the main competitor, *E. calamita*, in all aspects related to food acquisition. So, how do both species coexist? How does *E. calamita* survive the invasion of such a powerful invasive competitor?

Table 1. Comparison of several features of importance among *D. pictus* and its two main native competitors

character	*Discoglossus pictus*	*Epidalea calamita*	*Pelodytes punctatus*	data from
Niche overlap in pond use				
Pianka's index (overlap ranking)		0.31 (1)	0.2 (2)	Escoriza & Boix 2012
Pianka's coefficient (overlap ranking)		0.994 (2)	0.997 (1)	Richter-Boix et al. 2013
Growth physiology of tadpoles				
Mean growth rate	High	Low	Moderate	Chapter 3
Mean consumption rate	High	Moderate / Low	Low	Chapter 3
Mean assimilation efficiency	Moderate / Low	Low	Moderate / Low	Chapter 3
Mean growth allocation	Moderate	Low	Moderate	Chapter 3
Allocation – Consumption	Consumptive growth	Consumptive/Allocation	Allocation growth	Chapter 3
Feeding abilities				
Activity according to food quality	yes	no	yes*	Chapter 4, *Richter-Boix et al. 2007
Food choice abilities reported	yes	no	yes*	Chapter 4, *Richter-Boix et al. 2007
Effect of Interspecific competition				
Survival	no visible effects	no visible effects		Chapter 6
	losses, significant (vs. both)	losses, significant	losses, not significant	Richter-Boix et al. 2013
Growth rate	losses, *only in origin area*	losses, significant		Chapter 6
	no visible effects (vs. both)	losses, significant	losses, significant	Richter-Boix et al. 2013
Developmental rate	losses, significant	losses, significant		Chapter 6
	no visible effects (vs. both)	losses, significant	no visible effects	Richter-Boix et al. 2013
Changes with age since invasion	To worse (vs. *E. calamita*)	To better		Chapter 6
Predation				
Plastic reaction to introduced predators	no	no*	mild	Chapter 8, *Nunes et al. 2014
Mean survival to *Notonecta glauca*	30.3% loss	80.5% loss		Chapter 9
Mean survival to *Sympetrum striolatum*	no significant differences	70% loss		Chapter 9
Reproductive Output				
Estimated per spawn	819 to 1072	1.500 to 5.620	Up to 1600	Boulenger 1897, Knoepffler 1962, Beebee 1983, Marangoni et al. 2008
Estimated per female and year	4.917 to 5.636			

Although there is indeed a mild fine ecological and phenological segregation between both species (Chapter 6), it seems difficult to explain the survival of the native species only in this terms. The diet segregation observed within ponds (Chapter 7) might also by an important factor, but given the results in lab (Richter-Boix et al. 2013, and Chapters 4 & 5) it may also be a competitive exclusion from preferred food sources (San Sebastian et al. 2015, Chapter 4). Evolutionary patterns (Chapter 5, more extensively discussed in the section 4 of this discussion) can explain, and possibly ensure, long-term survival of the native competitor, but do not account for the lack of observable declines during the first stages of invasion of the Painted Frog. There is not seemingly a possible single factor to account for the lack of observable declines in the native *E. calamita*. Nevertheless, in a list of combined possibilities and hypotheses, I would include the following:

→ A. The growth physiology of *E. calamita*, which is comparatively more focused in assimilation/allocation (Chapter 3), might prevent disastrous effects of increased competition pressure. This physiology might be related to the great intraspecific densities that the species can often attain in small ephemeral ponds, now helping – in a “sort of” exaptation – to survive also interspecific competition.

→ B. Population explosions in habitats free of interspecific competition. Although in Chapter 6 is clearly stated that ecological segregation cannot totally account for the lack of native declines, some interesting field observations might actually shed some additional light to this discussion. Thus, although invasive species are usually considered as the most benefited from human interference in natural systems, in this case, the most pioneering competitor is the native. The Natterjack Toad is amazingly effective colonizing newly created ponds which are totally devoid of vegetation (Chapter 6). For instance, in an “off-the-record” survey near Girona, we could observe how nearly forty ponds inside a mineral extraction area, with nothing more than mud and ten centimetres of water, were literally overrun with tadpoles of the native toad. Matching the results of Chapter 6, the invasive frog was only and exclusively present in the less disturbed sections of the mineral extraction area, where some vegetation around the ponds was present. This was an important and interesting confirmation of a pattern that is already hinted with the data in the study, but it is also an observation that must be noted in terms of population dynamics: the destructive human interference – in terms of general natural habitat – in that location created in turn the perfect habitat to allow a population explosion for – only – the native species, totally free of interspecific competition of any type. In a world where human interference is continuously increasing in natural habitats, this might paradoxically benefit the native species in this case.

→ C. Micro-macrohabitat synergies favouring the native competitor: the native competitor mainly chooses sunlit ponds for reproduction (Chapter 6), a fact most possibly related to its high tolerance of heat (Gutiérrez-Pesquera *et al.* 2016) and the relationship among water temperature and development rates. Interestingly, in sunlit ponds – in opposition to shaded ponds – both species can segregate more easily in the type of items consumed (Chapter 7), so usual pond choice by *E. calamita* might be now in fact doubly selected in the invasion range. In the long run, if this interaction holds, and native species is indeed improving its competitive abilities at the individual level (Chapter 5) this could lead to ecological segregation displacing the invasive species more often to shaded ponds where interspecific competition is more probably absent. Testing both thermal tolerance limits (CTmax and CTmin) and the effects of shading on tadpole growth of the three usual competitors in ephemeral ponds would be an interesting future study in this sense.

→ D. Sheer reproductive output and adult survival: this is another possibility that, regrettably, had to be left for future studies. Individual competitive and survival abilities, in fact, are an incomplete picture of the population-level outcomes of a competitive interaction. That is, individual capacities are irrelevant if they are not discussed under the light of carrying capacity and reproductive output of the population. So, invasive tadpoles clearly surpass *E. calamita* tadpoles in all aspects at the individual level but, how many excess tadpoles of *E. calamita* are there to compensate for that? The possibility of a "numeric war", even though difficult to work with – how to reliably assess the carrying capacity, mean lifelong reproductive output, and adult survival at a population level? – could be a key factor for coexistence. In this sense, perhaps it would be *Pelodytes punctatus* the most menaced native competitor (Table 1). In the "future directions" section of this discussion this possibility is further discussed.

Thus, several possibilities might help to explain the lack of observable – at least, easily observable – declines of the native toad in the invaded area, but no definitive answer is yet totally convincing, at least in my opinion. In the long run, given the encouraging improvement – from the native point of view – of competitive outcomes observed in Chapter 5, I believe the survival of the native toad is ensured, at least regarding the effects of invasive competition. In "future directions" I discuss what could be the next steps for a deep study of other impacts of this invasion from an invasion biology point of view.

3. Predatory impacts from native and invasive species on tadpoles of *Discoglossus pictus.*

Starting in this case with a global summary of results, in **PART C** of this thesis we exposed the invasive frog to real or simulated predation risk from a total of five predators, with varied results:

1. Native dragonfly nymphs. Concretely, larval *Anax sp.* and *Sympetrum striolatum*. They are voracious predators of tadpoles (e.g. Relyea 2001, Van Buskirk 2001, Richter-Boix *et al.* 2007, Gomez-Mestre & Díaz-Paniagua 2011) which are present in both the northern and southern side of the Mediterranean Sea, in the same – *Anax sp.* – or similar – *Sympetrum striolatum* – abundances and forms (Askew 2004, Dijkstra & Lewington 2006). Both predators are very common in the study area during the autumn reproduction of the painted frog, being the first present in permanent and temporary ponds, and the second from permanent to highly ephemeral habitats. The invasive frog displayed inducible defences against both species (Chapters 8 and 9), with nearly undetectable effects of the benthic nymph of *Sympetrum striolatum* in terms of survival. Although similar effectiveness confronting *Anax sp.* is difficult to expect – prey-predator size relationship, deadly predatory abilities of the Aeshnid –, comparing the results among induced and non-induced tadpoles (Chapter 9) clearly gives support to the idea that the inducible defences observed are indeed useful to reduce predation risk of the tadpoles. However, it has also to be noted that non-consumptive effects, which greatly affect individual fitness (Nunes *et al.* 2010), were often present (Chapter 9).

2. Common Backswimmers (*Notonecta glauca*). These native predators can be found in a wide range of ponds (Svensson *et al.* 2000), being arguably the commonest tadpole predator observable in a bare eye survey of temporary ponds. The species used – as implied by its name – seems to be the most abundant backswimmer in the study area, and it is also present on the southern side of the Mediterranean. These are visual predators of tadpoles (Van Buskirk 2001) that happened to be much more deadly than expected, even preventing us from transporting them with other predators that easily became their victims, like *Sympetrum* nymphs. These predators dive from the water surface or a perch to capture prey with its legs (Giller & McNeil 1981). Consequently, in comparison to benthic predators (*Sympetrum*), they were clearly disastrous for the survival of the nektonic *Hyla meridionalis* tadpoles in mesocosms. Although significantly reducing the survival of invasive *D. pictus* tadpoles, they were much more deadly to the the native *Epidalea calamita*. This happened even though being *Epidalea calamita* considered a more strictly benthic tadpole than *Discoglossus pictus*. This might be an effect of the clearly marked behavioural and morphological defences

displayed by the invasive tadpoles in front of *Notonecta glauca*. As previously stated, at the individual level, invasive tadpoles seem to surpass the native larvae of *E. calamita* in every single aspect.

3 & 4. *Gambusia holbrooki* and *Procambarus clarkii*, as introduced fish and crayfish, respectively. Neither of these predatory species has yet had its first century anniversary in the Iberian Peninsula. The Mosquito Fish (*Gambusia holbrooki*) is first reported in 1921 (Elvira 2001), introduced to control mosquito populations. Exact dates for the study area are therefore later than 1921, but largely unknown. On the other hand, the American red swamp crayfish (*Procambarus clarkii*) was most probably introduced in the study area during the 1980s, becoming widely extended just before 1990, embodying a very recent introduction. It is well adapted to live in areas with seasonal fluctuations in water levels, and is an omnivorous species that has great predation abilities on tadpoles (Cruz *et al.* 2006, Gomez-Mestre & Díaz-Paniagua 2011). The invasive species did not react to neither of these introduced species morphologically, and displayed the same behavioural reaction as in front of native predators.

Globally, the pattern of inducible defences of the invasive frog might suggest two important ideas: (1) that behavioural reactions in the invasive species are more generalizable than morphological ones. (2) that, similarly as a native species, the invasive frog will be mostly affected by alien predators. In the second case, given that mild morphological reactions are observed in *Pelodytes punctatus*, it is possible to suggest that the invasive species might be, in fact, more affected by allochthonous predation than the native competitor. As it will be further discussed in the following section, from an evolutionary point of view, the observation of an almost complete match of the patterns of the invasive frog with the patterns observed in native species might not be surprising at all.

4. The role of evolution in the ecology of invasive populations of *Discoglossus pictus*

In a global sense, the system studied here is nicely fit to discuss the impacts of evolution on the present ecology of species. As will be now discussed, there are solid reasons to believe that previous evolutionary history of both the invasive species and the native biota have shaped the predatory and competitive outcomes studied here.

Starting with long-term effects of co-evolution, we learn in **Part C** of the thesis that, although the frog is geographically allochthonous, “it might not be so” from a predator-prey point of view. Thus, if we examined, blind at the prey species, the results of the several predation experiments and trials done with the frog here in this thesis, we would most probably state that the prey is native, because it displays inducible defences effectively in front of all native species but not in front of the allochthonous predators. Hence, with the exception of mild responses against introduced fish and crayfish, the pattern is exactly the same as the one we observe in *Pelodytes punctatus*. How is that possible? As stated in the introduction, several important geological and climate changes (Rage & Roček 2003) have shaped the current distribution of all amphibians from the western Mediterranean Basin, disappearing and reappearing the Discoglossidae, Pelodytidae, and Bufonidae, for example, several times in Europe during the tertiary (Rage & Roček 2003), and representing the lack of a native species of *Discoglossu*s in the study area more an exception than a common biogeographical situation. This is most probably the same for most native arthropod predators, whose flight capacities might further increase the possibilities for connectivity, being the presence of the same species at both sides of the Mediterranean more common than in amphibians (Askew 2004, Dijkstra & Lewington 2006). This commonly shared previous evolutionary history in the same biogeographic area among predator and prey is most probably the key to ease – or directly make unnecessary – the cooption by the frog of chemical cues from northern African to European populations of predators. If we go further, it even becomes difficult to think of a common predator in the invasive range of the frog which is totally absent from its native range, if we except the introduced ones. In a freshwater environment, like ponds, where inducible defences and chemical communication are such an important asset for survival, the lack of previous evolutionary history with native predators could have been a clear disadvantage for the invasion of the Mediterranean Painted Frog. This is now a discarded possibility and a first example of the complexity to define “allochthonous” if we were to do this from an evolutionary point of view.

The other second important evolutionary discussion is for competition, mainly with the native competitor *Epidealea calamita* – by extension, some ideas might also apply for to *Pelodytes punctatus*. It is clear that the invasive frog can clearly affect fitness of both native competitors (Chapters 4, 5 & 7, Richter-Boix *et al.* 2013), but how these species, which are usually syntopic to the invasive frog, have avoided competitive exclusion similar to that observed in other alien-native competitive relationships like squirrels or minks (Bertolino *et al.* 2014, Santulli et al. 2014) is still unclear – several hypotheses have been discussed for *E. calamita* in part 2 of this discussion, none totally convincing. Once discussed the predator-prey interactions and the recent geological and biogeographical history of the area, the obvious question arises: what if they were preadapted to the presence of a Discoglossinae-type competitor? Or what is the same, what if there was a niche ready to be occupied – in a sort of “ghost of competition past” (Connell 1980, Pritchard & Schluter 2001)? Results on loss of community structure reported in Richter-Boix *et al.* (2013) imply that, if indeed there was an empty “ghost niche”, the re-occupation has not been thoroughly placid. It is important to remark however, that although some community structure results in this study were different among invaded and non-invaded areas, others – like the overlap differences between areas – although showing the same tendency, were not significant. In another study, Escoriza & Boix (2014) examined reproductive preferences in the North African and Iberian subset of *Discoglossus* and highlighted that all species could reproduce in very small water bodies with high tolerance for other parameters like salinity, conductivity, etc. hence implying a similar niche for all the examined species. In this study, results on community structure also give conflicting views, since both invasive – *D. pictus* – and native – *D. galganoi* – populations correlate their presence in a pond with species richness in the tadpole guild, suggesting that both species tend to be agents of increase in pond use overlap. This, together with the fact that Richter-Boix *et al.* (2013) could not compare the invaded community before and after the invasion, leaves open the possibility that the observed overlap is indeed “normal” for an Iberian anuran community comprising a *Discoglossus* species. Finally, glacial refugia for *Pelodytes* and *Epidalea* is indeed the Iberian Peninsula, which is shared with a local species of *Discoglossus* with similar ecological features as the invasive, a fact that adds up for the “ghost niche” possibility. So, what can we learn from the results in this thesis that may help to shed light in this discussion?

In this thesis we did not attempt to make a third community structure assessment – probably leading to similar results as the previous two –, but we can add pieces of evidence in support, and opposition, to the ghost niche theory. Firstly, Chapter 3 poses *Discoglossus pictus* as a physiologically unique species in the local guild of tadpoles, thus possibly speaking in favour of the empty niche. The species is highly particular in terms growth physiology, having the highest consumption rates in the local tadpole guild, with a fast and clearly consumption-oriented growth (in this sense, it is interesting to wonder if the strong effects of food deprivation on developmental plasticity observed by Enriquez-Urzelai et al. [2013] would have been the same using any other tadpole species). Anyhow, these physiological traits combine perfectly with the pond preferences of the invasive frog (Chapter 6): small and short-lived ponds which are not devoid of a certain diversity and abundance of food. That is, the invasive frog avoids reproducing in ponds which are devoid of surrounding – and inner, due to a present correlation between both variables – vegetation.

Accordingly, both isotopic (Chapter 7) and laboratory data (Chapter 4) show that the invasive tadpoles are able to diversify its diet when this is possible. Globally, all these results delineate a tadpole with great survival abilities, not only simply part of a massive numerical strategy for adult recruitment. In other words, ecologically comparing with the other two competitors, the invasive tadpoles are perhaps more in the side of K tadpoles – more similar to the larvae of *Pelodytes punctatus* – than r tadpoles – larvae of *Epidalea calamita,* and most Bufonids (Van Boxclaer *et al.* 2010). Given the reproductive tactics and output, the overlap – which could argue against the "ghost" empty niche theory – is perhaps greater with *P. punctatus* in this sense, whose individual losses of fitness are possibly more important at the population level. This opens again the possibility that the Parsley Frog is perhaps more menaced by the invasive competition than signalled by the community analyses presented hitherto.

On the other hand, microevolutionary patterns detected in Chapter 5 clearly point that populations of *E. calamita* are adapting – or readapting? – to the presence of an additional tadpole species in the guild. Thus, the invasive species seems to lose its "competitive immunity" in the origin area, showing there clear signs of distress in the presence of Natterjack toad tadpoles, even resembling this native species in some aspects of inferred individual fitness. If this is indeed a general and long-held pattern in wild populations, this could even put the invasive species in an inferior position to *E. calamita* – similar individual competitive abilities but probable numeric inferiority in ponds (Table 1) – and could lead to unexpected final outcomes like an ecological displacement not of the native species (as hinted by Richter-Boix *et al.* 2013) but of the invasive: in this case, as discussed previously, shaded ponds are probably a suitable refugium for the invasive species. Given that, among the populations sampled for isotopic data, genetic differences are the greatest for La Jonquera – which is the only population that could not be sampled for isotopic data in time for this thesis –, we cannot yet tell here if data on trophic ecology corroborates the microevoultionary patterns hinted in Chapter 5. This would arguably contradict the ghost niche theory. Contrarily, if results of trophic ecology do not match evolutionary tendencies in Chapter 5, this could picture an even more complex scenario.

5. Future directions and management perspectives

As stated in "Aims and Objectives", if we were to pinpoint this PhD thesis in the hypothetical space where all biological areas of study lie, we should find it on the multivariate mean point among the areas of zoology, ecology, and invasion and evolutionary biology. Hence, this thesis has become an amazingly interesting maze – at least for me – with several ways out. Concretely, I see three main directions in which interesting – and useful, let us not forget it needs also to be useful – research could be conducted.

5.1. From a "fundamental principles of evolution" point of view: deeper study of evolutionary patterns during range expansions.

Biological invasions are an erotic bombshell for evolutionary biologists which like to experiment and see evolution "happen in front of their eyes". The link between past or present evolution and the naturally present ecological and phenotypic patterns, is perhaps never more apparent than in a system under an ecological disequilibrium. Examples like the Cane Toad in Australia, a genetically depauperate species that, thanks to a lack of previous co-history with local predators (Phillips & Shine 2004) and the evolutionary creation of an expansion front heavily adapted for invasion (Phillips & Shine 2005, Phillips *et al.* 2006), has overcome all possible barriers to become a national plague, ask for the study of similar cases to see the extent at which we are overlooking similar cases worldwide. In this sense, I believe that a path similar to that explored by White *et al.* (2013) should be followed for *D. pictus*: the use of the GBS libraries to detect loci under differential selection, try to find correspondences with genes or regulatory regions, and – that is a important addition, and perhaps the most important part – design lab or mesocosm experiments to test the several predictions drawn from this genetic studies. This functional study to prove that candidate genes under selection are indeed having a real effect on phenotypes or ecology, if it is indeed possible, would be highly motivating since it is usually overlooked in similar studies.

In these sense, it is important to remark that the undertaking of the experimental and field work from Chapter 5 was perhaps a little bit too risky without having underlying evidences to back our hypotheses. This is not to suggest that we did not have a clear hypothesis – we clearly had one – but to state instead that I really recommend that similarly huge amounts of work demanded by projects like Chapter 5 should be done with some evidences to back the effort if it is possible to have them. Finally, other – already commented – evolutionary studies that were left for future projects are the detection of fine-scale patterns of phenotypic divergence in adult frogs, like for example in adult colouration patterns – possibly related to patterns of genetic diversity in populations – or patterns of mean body size.

5.2. From a “fundamental principles of ecology” point of view: including reproductive output and carrying capacity in the equation.

From a purely ecological point of view, the enigma left to solve – more mysterious as we gather more data – is that of the “peaceful” coexistence of *E. calamita* (and *P. punctatus*?) with *D. pictus*. As hinted in the corresponding section of this discussion, I would very much like to put numbers – I mean, numbers of specimens in a population dynamics context – and other functional data to this competitive relationship. That is, we now already know that, at an individual level, tadpoles of *E. calamita* are clearly subordinate competitors at least during the first stages of invasion. In terms of predation they are also clearly inferior to the invasive species (even predated upon as eggs by the invasive species! see Annex), being much more predated when they are put together (Chapter 9). How does the native species keep its populations so healthy in recently invaded areas? Several hypotheses have been presented, and the least explored is the differential reproductive output and possibly, differences in adult survival and longevity (but see Oromi *et al.* 2012). A deeply functional study including also adults could signal important overlooked similarities and differences among *D. pictus* and the rest of species in the guild.

5.3. From a management, applied ecology, and invasion biology point of view: it's time for *Pelodytes punctatus* and *Discoglossus galganoi*.

Finally, from the point of view of the study of this biological invasion *per se* and a management perspective, there is still work to do as well. According to the framework presented in the introduction of this thesis to classify the impacts of allochthonous species on native communities (Blackburn *et al.* 2014), we should classify the invasion of *Discoglossus pictus* as a “minor impact”: “*Competition affects fitness (e.g., growth, reproduction, defence, immunocompetence) of native individuals without decline of their populations*”. Our recommendations for management are therefore limited to monitoring and study (see Annex), not even including containment plans. However, this categorization is obviously done only with the information available up to date, that is, the lab-tested impacts on co-occurring native species, mainly *E. calamita*.

In this sense, a first item in a hypothetical to-do list relates to my believe that we are making an important assumption that should indeed be finally tested and confirmed at some time: that there are not declines of native species attributable to the presence of the invasive frog. That is, given that no native species possibly affected by the invasive frog is absent from the origin area, and that there are still abundant native populations in it, we have long assumed that their present abundance is similar to that previous to the invasion or that is totally uncorrelated with the abundance of the alien competitor. In this sense, a planned survey to assess the abundance – mainly – of the three main competitors in ephemeral and temporary ponds would clearly be needed to discard a possible overlook of local declines along the invasion area. Obviously, if possible, a guild-wide community would be even better. A complementary option is to survey areas which are now ready to be invaded (the Vallès area, in central Catalonia, for example), not only to gather presence/absence data of each species in a long list of water bodies, but to see also possibly changing patterns in their abundance before and after invasion.

A second item in this list would be the exploration of possibly overlooked effects on *Pelodytes punctatus*. Although mild competitive impacts at the individual level are already reported, as repeatedly hinted, in this case the kind of studies undertaken should also use a population level point of view – reproductive output, adult longevity, etc. – from the start in the assessment of competitive impacts. That is, not only detect individual effects of competition, but also to assess the importance of these effects at a population level.

A third item in this to-do list would be related to the globally spread amphibian pathogen fungus *Batrachochytrium dendrobatidis*, the agent of chytridiomycosis. The presence of the fungus is known in the area, and a serious assessment of the dynamics and possibilities that the invasive frog may create for this pathogen would be highly important to be studied.

Finally, last but not least, another possibility is to focus on the possible clash between native and invasive *Discoglossus*. That is, for biologists who have worked with Iberian *Discoglossus*, the most obvious and important question yet to be explored is: what will happen when *Discoglossus pictus* gets in contact with the easternmost populations of the Iberian Painted Frog *Discoglossus galganoi*? In this case, the impacts could easily travel from “minor” to much higher impact classes (Blackburn *et al.* 2014): possibility for hybridization, very similar ecological niches and, in some cases, already isolated and scarce populations of the Iberian species which could be literally overrun by abundant invasive populations of *Discoglossus pictus*. At the present pace of expansion, there are still decades to come before the first contact, but recent mid- and long-distance dispersal events in Catalonia (Franch *et al.* 2007) show that a predictable periodicity of expansion is not guaranteed at all. On the optimistic side of things, the invasion abilities of *D. pictus* in more arid and continental areas – e.g. south and west of Catalonia – have yet to be proven, and the species seems nowadays limited in its expansion westwards and inland, possibly due to climatic and geographical barriers (Escoriza *et al.* 2014). But on the other hand, its arrival to densely populated areas surrounding Barcelona will multiply its contact with transport pathways and vectors of expansion, greatly increasing the possibility for accidental – or willing – introductions outside its current range. The main problem to work with *D. galganoi* in this case would be the logistics: working with populations which are very separated geographically, and being one species – the Iberian Painted Frog – endangered in its easternmost populations. For example, for any competition experiment, in order not to bias results, several pairs of both species need to breed synchronously (see e.g. Chapter 5). In this case, this poses a series of nearly unsolvable complications that would eventually lead to the use of hormonal treatments to create a planned calendar of breeding in lab, meaning the extraction of native individuals from its native populations and several extra doses of artificiality due to previous housing and "forced breeding". The distinction of the similar tadpoles of both species during experimentation would also need to be solved, adding an extra layer of complexity in lab trials. On the other hand, and finally, a serious study of the possible hybridization between both species would need of a complementary part to test fitness of the possible offspring in several situations, which would incur similar logistic problems.

CONCLUSIONS

GENERAL CONCLUSIONS

1. The Mediterranean Painted Frog (*Discoglossus pictus*) is not a uniform unit in its invasive range in Europe, neither from a phenotypic nor a genomic point of view: mean size of adults and life-history traits are found to relate to mean annual precipitation, while there are clearly detectable genetic differences among populations most probably created by the expansion history itself. Therefore, recent divergence of populations does not impede to find potentially important geographic differences among populations.

2. The Mediterranean Painted Frog represents, physiologically, a unique addition to the local tadpole guild. Its growth is very rapid and is based mainly on great consumption rates, representing an extreme tactic in the local tadpole guild. Accordingly, the invasive tadpoles possess the ability to increase the quality and quantity of its food intake when this is possible. This combination of high consumptive needs and abilities to secure them, poses these tadpoles as potential disruptors of the natural dynamics of natives.

3. The competitive relationship present between the Natterjack Toad *Epidalea (Bufo) calamita* and the Mediterranean Painted Frog changes according to the previous evolutionary history of each population. In this sense, populations of the native toad that have had a greater number of generations of contact with the invasive frog are able to inflict a greater competitive distress on the invasive tadpoles. Therefore, results globally point at an optimistic scenario for the native species in the long-term.

4. The Mediterranean Painted Frog and the Natterjack Toad differ mildly in their breeding preferences, choosing the Natterjack Toad highly sunlit and shallow ponds and the Mediterranean Painted Frog ponds with abundant surrounding vegetation. Direct competitor avoidance seems also plausible according to results here presented. Anyhow, both species seem forced to end up competing very often.

5. The trophic position of both species within ponds can respond either to evolutionary or to ecological patterns with the data gathered hitherto. Although the most conservative option with the present data would be to discard evolutionary patterns, data from the most differentiated population genetically – La Jonquera – will probably disentangle which is the dominant pattern.

6. The Mediterranean Painted Frog expresses its inducible defences as if it were a native anuran, responding to all native predators effectively, while lacking responses in front of introduced or invasive predators. We should discard predation costs on the invasive frog due to lack of previous knowledge of the native predators.

7. The complex evolutionary history of the local anuran assemblages poses the Mediterranean Painted Frog as a very particular case of invasive species, in which effects of previous shared evolutionary history with native taxa – and viceversa – cannot be discarded. The species has proved to be a good model for studies reaching beyond the invasion process itself to provide new arguments on the future ecological roles of introduced species. A temporal, spatial and – in the case of tadpoles – plastic point of view, in other words, an evolutionary focus, is probably essential for realistic long-term predictions of the impacts and ecological role of any invasive species.

CONCLUSIONS GENERALS

1. La granota pintada – *Discoglossus pictus* – és un anur que no representa un conjunt uniforme al llarg de la seva distribució invasora, ni des d'un punt de vista fenotípic ni genòmic: la mida mitjana dels adults i els trets relacionats amb la seva història vital es troben relacionats amb la precipitació mitjana anual de cada zona, mentre que hi ha algunes diferències genètiques importants entre poblacions probablement creades pel mateix procés d'expansió de l'espècie. Per tant, la recent divergència evolutiva entre les diverses poblacions invasores no ens impedeix trobar diferències geogràfiques entre elles.

2. La granota pintada representa, fisiològicament, una addició única al gremi local de capgrossos. El seu creixement és molt ràpid i es basa principalment en un gran consum alimentari, representant l'espècie una tàctica extrema dins de la comunitat local. En conseqüència, els capgrossos de granota pintada són capaços d'augmentar la qualitat i quantitat de la seva ingesta d'aliments quan això és possible. La combinació d'altes necessitats alimentàries amb aquestes habilitats per aconseguir-ne l'ingesta, presenten els capgrossos d'aquesta espècie invasora com a potencials disruptors de les dinàmiques naturals dels capgrossos autòctons.

3. La competència present entre el gripau corredor – *Epidalea (Bufo) calamita* – i la granota pintada canvia d'acord amb la història evolutiva de cada població. En aquest sentit, les poblacions del gripau autòcton que han tingut un nombre més gran de generacions en contacte amb la granota invasora són capaços d'infligir una major pressió competitiva en els capgrossos invasors. Per tant, els resultats globals apunten a un escenari optimista a llarg termini per l'espècie competidora autòctona.

4. Aquests mateixos dos competidors difereixen lleugerament en les seves preferències reproductives. Les basses efímeres i temporals són triades de manera lleugerament diferent pel gripau corredor – que tria sobretot basses someres i sense ombra – i la granota pintada – per qui les basses amb un mínim de vegetació circumdant són les més preferides. La possibilitat que els dos competidors directament evitin coincidir entre ells sembla també plausible segons les nostres dades, però en qualsevol cas, pel tipus d'hàbitat general ocupat semblen estar obligades a acabar competint amb freqüència.

5. La posició tròfica dels capgrossos dels dos competidors dins de les basses pot respondre tant a patrons evolutius com ecològics segons les dades recollides fins ara. Tot i que la opció més conservadora amb les dades actuals seria decantar-se pels ecològics, les dades pendents de la població més diferenciada genèticament – La Jonquera – probablement seran les que marcaran clarament quin és el patró dominant.

6. La granota invasora presenta un patró d'expressió de defenses induïbles – plasticitat fenotípica – contra depredadors molt semblant al que s'esperaria per a un anur autòcton, responent a tots els depredadors nadius amb eficàcia, i mancant les respostes davant de depredadors introduïts. Hem de descartar totalment possibles costos addicionals en termes de depredació per la granota invasora relacionats amb una hipotètica manca de coneixement dels depredadors nadius.

7. La complexa història evolutiva de les comunitats locals d'anurs planteja el de la granota pintada com un cas molt particular d'espècie invasora, en el qual no es poden descartar els efectes de la seva història evolutiva prèviament compartida amb tàxons natius i viceversa. En aquest sentit, la granota pintada s'ha mostrat com un bon model d'estudi per anar més enllà del mateix procés d'invasió i proveir-nos amb noves aproximacions i reflexions, ajudant a millorar el processos de valoració i discussió del paper ecològic de les espècies introduïdes. Un punt de vista temporal, espacial i - en el cas dels capgrossos - plàstic, és a dir, un enfoc evolutiu, és probablement essencial per realitzar prediccions realistes a mig o llarg termini dels impactes i el futur paper ecològic de les espècies invasores.

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ANNEX

Predation of *Bufo calamita* eggs by *Discoglossus pictus* tadpoles

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Key words: spawn predation, tadpole aggregation, tadpole competition, intraguild predation.

Resumen: El sapillo pintojo (*Discoglossus pictus*) es una especie introducida desde el norte de África que presenta una expansión continua en el noreste de Cataluña y el sur de Francia. En las charcas usadas como punto de reproducción coincide sobre todo con el sapo corredor (*Bufo calamita*), comúnmente considerado su principal competidor nativo. En esta nota informamos de una observación nocturna de un grupo de renacuajos de *D. pictus* alimentándose de una puesta de *B. calamita* en una charca natural cerca del límite sur de expansión de la especie invasora. Posteriores observaciones en charcas cercanas en las que se podría repetir la depredación por la presencia tanto de renacuajos de *D. pictus* como de puestas de *B. calamita* no han dado lugar a observaciones similares, lo que hace pensar a los autores que no se trata de un hecho muy común.

The Mediterranean Painted Frog (*Discoglossus pictus*) is a species native of North Africa and lives in Eastern Morocco, Algeria and Tunis and in the islands of Sicily, Malta and Gozo (Lanza *et al.*, 1986; Pabijan *et al.*, 2012). It was introduced in Banyuls de la Marenda, Southern France, at the beginning of the 20th century (Wintrebert, 1908) and since then, it has expanded north and southwards, with similar rates of spread (Montori *et al.*, 2007; Llorente *et al.*, 2016). It is still expanding (SIARE, 2016), being the southern limit of the continuous expansion found nowadays near St. Celoni (Barcelona Province, NE Spain).

In its invasive range, *D. pictus* usually reproduces in temporary or ephemeral ponds commonly shared with native species with similar preferences, like *Bufo calamita* or *Pelodytes punctatus* (Montori *et al.*, 2007; Llorente *et al.*, 2016). Concretely, Richter-Boix *et al.* (2013) estimated a niche overlap in breeding areas between *Discoglossus pictus* and *Bufo calamita* of 0.99 over 1, meaning that is it very common to see them as larval competitors in the same ponds. When they share ponds, previous studies suggest a displacement of *B. calamita* to non-preferred food resources and greater competitive abilities of *D. pictus* (San Sebastián *et al.*, 2015a).

On 9th March 2016, approximately at 19:00 h, during a nocturnal survey to check the southern expansion front of *D. pictus*, we found in a pond near La Batllòria (UTM: 31T 4619000m N , 463802m E), a group of tadpoles of *D. pictus* feeding on eggs of *B. calamita*. As it is common in the species (authors, unpublished data), a great proportion of tadpoles were feeding together on the same part of the foraging resource, in this case, the *B. calamita* spawn (Figura 1, video: https://www.youtube.com/watch?v=qCB8zfr69hg). This clustering behavior is also common in lab experiments (authors, unpulished data; O. San Sebastián, personal communication), where a large proportion of tadpoles compete among them but at the same time probably

Figure 1: A group of tadpoles of *D. pictus* feeding together on a stretch of a spawn of *B. calamita*.
Figura 1: Un grupo de renacuajos de *D. pictus* se alimentan juntos de un trozo de puesta de *B. calamita*.

use the other individuals as reliable indicators of the presence of food.

Tadpoles of *D. pictus* have already been mentioned as predators of their own eggs (Licata *et al.*, 2015) and of *Hyla intermedia* eggs as well (Escoriza, 2014). On the other hand, *B. calamita* eggs are commonly eaten, being prey of *Pelodytes punctatus* and *Pelobates cultripes* (Tejedo, 1991), *Bufo bufo* and *Rana temporaria* (Banks & Beebee, 1987) and *Procambarus clarkii* (Cruz & Rebelo, 2005; Cruz *et al.*, 2006; Portheault *et al.*, 2007), among other species.

In this case, the pond was starting to dry out due to the lack of rain: two weeks later a re-visit to the same reproduction point confirmed it was already empty of water. According to lab studies (San Sebastián *et al.*, 2015b), tadpoles of *D. pictus* prefer resources with high protein contents if they are available. Moreover, these tadpoles are able to grow and develop faster if they forage on highly proteinic resources. Therefore, the simple preference for proteinic resources, plus the need to increase their rates of growth and development before the pond dried out (Kupferberg, 1997; San Sebastián *et al.*, 2015b), could have led these tadpoles to exploit an unusual resource. Posterior surveys of ponds with the same combination did not yield the same kind of observations. This, and the fact that, after several years of studies on competition among these species, it is the first time that this behavior has been described, makes the authors think that it is not common.

However, the high ecological similarity between these species (Richter-Boix *et al.*, 2013), makes this behavior very interesting in terms of competition: when eggs are laid the same rainy night, *D. pictus* tapdoles hatch and develop faster than *B. calamita* ones (authors, unpublished data), potentially making *D. pictus* a more powerful competitor than thought if this predation events were not to be as uncommon as it seems.

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El sapillo pintojo mediterráneo (*Discoglossus pictus*) en la península ibérica

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El sapillo pintojo mediterráneo, *Discoglossus pictus* (Figura 1), se distribuye por el norte de África (Marruecos, Argelia y Túnez tanto en la parte continental como en las islas adyacentes) así como en las islas de Sicilia, Malta y Gozo (Lanza *et al.*, 1986). Su distribución en Europa continental se circunscribe a una pequeña área en el noreste de la península ibérica y el sur de Francia (Geniez & Cheylan, 1987; Llorente *et al.*, 1995, 1997; Fradet & Geniez, 2004).

El origen de las poblaciones que se distribuyen por Cataluña y el sur de Francia no tiene ningún soporte paleobiogeográfico y únicamente se puede interpretar como fruto de una introducción. Los datos moleculares también apuntan a esta interpretación para explicar su distribución actual en la península ibérica (Martínez-Solano, 2004). Todos los datos disponibles indican que fue introducido a finales del siglo XIX o, más probablemente, a principios del XX, en la zona francesa de Banyuls de la Marenda (Knoepffer, 1962; Lanza, 1989). También fue introducido a finales del siglo XIX en Saint-Hilaire (Val-de-Marne), en Amboise (Indre-et-Loire), en el Indre y en el Jardin des Plantes en París (Frétey *et al.*, 2003), donde parece ser que es el único lugar donde sobrevive además de las poblaciones del Languedoc-Roussillon y del nordeste ibérico.

La primera cita corresponde a Wintrebert y está datada en el año 1906 (Wintrebert, 1908). Desde su introducción, se ha expandido hacia el norte por el departamento de Pirineos Orientales, sobrepasando Narbona, así como a otras localidades de los departamentos del Aude y del Hérault. Su expansión hacia el nordeste ha alcanzado más de 70 km (Fradet & Geniez, 2004) y actualmente ya se encuentra en las proximidades de Montpellier. Hacia el sur ha penetrado en la península ibérica unos 110 km. No obstante, la excesiva distancia (51 años) entre la primera cita en territorio francés (1906) y la primera en territorio español (1957) sugiere que muy probablemente esta especie debió colonizar el nordeste ibérico bastantes años antes. Es muy probable que la ausencia de observaciones sea debida a la ausencia de estudios herpetológicos entre las décadas 1920-1970 (Montori *et al.*, 1985). Por otra parte, la presencia en Perpiñán en 1948, sólo a unos 35 km al norte de Banyuls (Yakowleff, 1948) apoya esta hipótesis, ya que la frontera española se sitúa a tan sólo unos 6 km de esta localidad y no existe ninguna razón aparente que dificulte o limite una expansión hacia el Sur de esta especie de carácter mediterráneo.

En la península ibérica, hasta la década de 1980, su distribución se centraba en las comarcas del Alt y Baix Empordà, y en el Pla de l'Estany, penetrando sólo levemente en el Gironés. En la década de 1990 colonizó la citada comarca y La Selva, alcanzando en la década de 2000 la cuenca del río La Tordera hacia el Sur, por donde colonizó por primera vez la provincia de Barcelona, y La Garrotxa hacia el Oeste (Montori *et al.*, 2007). Actualmente su límite meridional de distribución está llegando a la población de Sant Celoni (Vallés Oriental), y entre Malgrat de Mar y Pineda de Mar (comarca del Maresme).

Recientemente se ha encontrado una población reproductora en el delta del río Llobregat (Franch *et al.*, 2007), en la provincia de Barcelona, a unos 60 km al sur oeste del límite del área de distribución conocida de la especie. En esta área presenta una expansión constante y rápida, colonizando gran parte de la llanura deltaica y aledaños, y mostrando poblaciones con un buen número de efectivos.

Discoglossus pictus en Cataluña ha mantenido una expansión constante y paulatina desde su introducción, inicialmente hacia el Sur y posteriormente también hacia el Oeste. La expansión hacia el Sur es mayor que hacia el interior, habiéndose estimado una tasa de dispersión media de 1,53 km/año ± 0,8 km (1,14 km/año ± 0,4 km hacia el Oeste y 2,70 km/año ± 0,34 km hacia el Sur; Montori *et al.*, 2007).

Las poblaciones europeas continentales están asignadas a la subespecie *Discoglossus pictus auritus* y con toda probabilidad corresponden a poblaciones argelinas (Busack, 1986; Glaw & Vences, 1991; Veith & Martens, 1992; García-París & Jockusch, 1999; Fromhage *et al.*, 2003; Martínez-Solano, 2004; Martínez-Solano *et al.*, 2004; San Mauro *et al.*, 2004; Real *et al.*, 2005; Zangari *et al.*, 2006). Todos estos autores sugieren o demuestran que las poblaciones del nordeste ibérico y sureste de Francia proceden de Argelia y pertenecen a dicha subespecie, aunque el estudio realizado a partir de datos moleculares por Zangari *et al.* (2006) duda de la validez subespecífica de *D. p. pictus* y *D. p. auritus*.


Foto Gustavo A. Llorente

Figura 1: Morfos rayado y moteado de *D. pictus*, encontrados en la misma charca en las proximidades de Riudarenes (Girona).

En lo que se refiere a las vías de penetración, la más lógica es por la franja litoral, por Port Bou. Sin embargo, la colonización del Alt Empordà debe haberse producido por diversos puntos de forma casi simultánea, ya que el Macizo de l'Albera posee en promedio una altitud insuficiente como para constituirse en una barrera geográfica insalvable. Sin embargo, Martens & Veith (1987) no tuvieron en cuenta esta última posibilidad, y únicamente consideraron la zona costera y el puerto de Le Perthus (290 msnm) como las probables zonas de penetración hacia el Sur. En apoyo de la hipótesis de una colonización más continua Norte-Sur se cuentan los datos de presencia de la especie en L'Albera publicados por Knoepffler (1962). Este autor indicó que *D. pictus* es abundante en Les Albères hasta los 900 msnm, aunque escasea a partir de los 600 msnm, y que en el bosque de La Massana, limítrofe con el Alt Empordà y con zonas de contacto con altitudes inferiores a los 600 msnm, era abundante. Ante esta situación, es difícil aceptar que la colonización se produjo únicamente por dos vías y parece más lógico aceptar una penetración por diversos puntos, siempre por zonas de altitud no muy elevada como podría ser el Coll de Banyuls (356 msnm).

Se han localizado poblaciones en la península ibérica presentes desde el nivel del mar hasta unos 500 msnm, generalmente en áreas de escaso relieve. De manera excepcional existen poblaciones a mayor altitud, a 980 msnm en Girona (Escoriza *et al.*, 2007) y hasta 1.250 msnm en Francia (Duguet & Melki, 2003). Esta situación difiere de la que se encuentra en las poblaciones autóctonas (1.800 msnm en Argelia o 1.500 msnm en Sicilia, en el Etna; Lanza, 1983).

En la península ibérica la especie es fundamentalmente termofílica (Escoriza & Boix, 2012a), y se distribuye por zonas con un rango de pluviosidad de entre 600 y 900 mm y temperatura media anual de 14-15ºC (Llorente *et al.*, 1995) en biotopos típicamente mediterráneos. Los individuos casi siempre pueden encontrarse próximos al agua, preferentemente en zonas de poca profundidad y con abundante substrato herbáceo en las proximidades. Toleran bien la salinidad (Llorente *et al.*, 1997).

Como especie introducida y en un proceso de expansión continuada, podría sospecharse de una interferencia con otras especies de anfibios. Los pocos datos que se poseen de la fase adulta indican que *Bufo calamita* y *D. pictus* presentan dietas similares (Montori *et al.*, 2007). Estos resultados no coinciden con los aportados por Veith & Martens (1987), quienes obtienen una semejanza de dietas mucho mayor entre *D. pictus* e *Hyla meridionalis*. Sin embargo, la excesiva simplificación en el número de categorías de presas usado por estos autores (siete frente a 25), es probable que enmascare la realidad. La escasez de estudios sobre este tema impide extraer conclusiones fehacientes sobre la posible competencia trófica de los adultos.

Sin embargo existe abundante información sobre la fase larvaria. *Discoglossus pictus* se reproduce en acequias, canales de riego, riachuelos, charcos naturales e incluso en charcos de agua de mar con alta salinidad. Prefiere charcas de ciclo hidrológico temporal o efímero, y se han observado puestas desde finales de invierno hasta otoño, aunque éstas se concentran fundamentalmente en primavera (Llorente *et al.*, 1997).

Discoglossus pictus puede solapar su periodo larvario con todas las especies presentes en su área de distribución. Sin embargo, y debido a que esta especie utiliza preferentemente charcas temporales y/o secundariamente efímeras, las especies con las que solapa mayoritariamente son *B. calamita* y *Pelodytes punctatus* siendo la primera de ellas la especie con la que más competiría en estado larvario.

El desarrollo larvario en charcas de carácter efímero puede conllevar riesgos de desecación, a los cuales *D. pictus* responde acortando el período larvario, acelerando la metamorfosis y modificando su morfología gracias a su gran plasticidad fenotípica. La escasez de alimento es un factor determinante que puede modificar esta respuesta, pues en estas condiciones disminuye la tasa de crecimiento, se retrasa la metamorfosis, se generan metamórficos de menor tamaño y masa corporal, y cambia la longitud de las patas, lo que modifica la mecánica del salto (Enríquez-Urzelai *et al.*, 2013). La plasticidad fenotípica también se manifiesta cuando las larvas de *D. pictus* son expuestas a diferentes depredadores, generando una cola más alta y una talla mayor (Pujol-Buxó *et al.*, 2013).

En experimentos realizados en el laboratorio en presencia de larvas de *D. pictus*, las larvas de *B. calamita* reducen la actividad y la talla corporal, aumentan la duración del periodo larvario y reducen su supervivencia. Estudios con isótopos estables de carbono y nitrógeno realizados con larvas de *D. pictus* y *B. calamita* procedentes de charcas en las que convivían y de charcas en las que se encontraba una sola de ellas sugieren un desplazamiento del nicho de *B. calamita* hacia recursos no preferidos y muestran una mayor capacidad competitiva de *D. pictus*. Esta última especie muestra un nicho más amplio en las dos condiciones (convivencia y no convivencia), lo que indica una mayor capacidad para explotar la diversidad de los recursos. Ello, en definitiva, puede favorecer indirectamente a su capacidad invasora, mostrando un cierto nivel de competencia con la especie nativa (San Sebastián *et al.*, 2015a).

Por otra parte, *D. pictus* muestra capacidad de escoger alimento de alta calidad. En condiciones de posible competencia con larvas de *B. calamita* cuando las dos especies se encuentran en alta densidad y comparten los recursos alimentarios, *D. pictus* mostró valores de actividad y alimentación más altos que los de las larvas de *B. calamita* (desplazado al alimento de menor calidad). Así, *D. pictus* presenta rasgos tróficos que son favorables para la invasión y que podrían limitar la aptitud de *B. calamita* cuando los recursos son limitados o se corre el riesgo de desecación del estanque (San Sebastián *et al.*, 2015b).

Aunque estos estudios indican el posible carácter de especie invasora de *D. pictus*, no se ha detectado un descenso de las poblaciones de los competidores nativos en su área de distribución aunque algunos autores indican que la capacidad invasora de *D. pictus* en Cataluña depende más de las características abióticas favorables, que de una ventaja adaptativa sobre las especies nativas (Escoriza *et al.*, 2014).

Es probable que, en definitiva, la invasión por *D. pictus* pudiera estar dando lugar a comunidades de anfibios menos estructuradas (Richter-Boix *et al.*, 2013). Además, *D. pictus* muestra en Cataluña una correlación positiva entre su presencia y la riqueza de especies, lo que, según Escoriza & Boix, (2012b), sugiere una pérdida en la estructura de la comunidad. El seguimiento de la expansión de *D. pictus* se está llevando a cabo desde hace más de 10 años, lo que permite calcular la tasa de expansión de esta especie y comprobar la colonización de nuevos territorios en tiempo real. Numerosos estudios se han realizado (y se están realizando) sobre las interacciones entre esta especie y las poblaciones autóctonas, mayormente en su fase larvaria, por lo que se puede afirmar que ya se posee un conocimiento importante sobre la magnitud del impacto que está causando *D. pictus* en la comunidad de anfibios. Aunque no hay datos fiables de densidad de población se puede aseverar que éstas son abundantes. Aunque en condiciones de estrés es probable que *D. pictus* presente una ventaja sobre *B. calamita*, ello no es suficiente para recomendar que se deban realizar medidas conducentes a la limitación de la expansión de *D. pictus*, ni a especiales medidas de conservación.

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El sapo balear (*Bufotes balearicus*) en las islas Baleares

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Estudios recientes han revisado las relaciones filogenéticas y la sistemática del complejo de especies de sapos verdes (*Bufotes viridis*) de la región Paleártica, incluidas las poblaciones de Europa Occidental, tradicionalmente adscritas a *B. viridis* y en la actualidad consideradas tres especies bien diferenciadas: *Bufotes balearicus*, *Bufotes siculus*, y *Bufotes viridis sensu stricto* (Stock *et al.*, 2006, 2008). Aunque aún existe cierta confusión (véase Sillero *et al.*, 2014, donde se presenta la distribución del complejo de especies en su conjunto, en lugar de cada especie por separado), los estudios moleculares existentes permiten una delimitación general de las áreas de distribución de cada especie, así como de sus zonas de contacto secundario. Así, *B. balearicus* (Figura 1), la especie presente en las islas Baleares y cuya localidad tipo es de hecho Palma de Mallorca, tiene como área de distribución natural la mayor parte de la península itálica, así como las islas de Córcega y Cerdeña. Existen además poblaciones en Sicilia, donde podrían haber llegado en un proceso de colonización natural desde Calabria en el Pleistoceno, formando una zona de contacto con *B. siculus*, la especie endémica de esta isla, en su extremo noreste (Stock *et al.*, 2008; Colliard *et al.*, 2010). En la península itálica, *B. balearicus* contacta con *B. viridis* en el noreste, en la llanura aluvial de la desembocadura del río Po, mientras que en el Norte los Alpes actúan como barrera natural entre ambas especies (Stock *et al.*, 2008; Dufresnes *et al.*, 2014). En las islas Baleares, *B. balearicus* se encuentra presente en Mallorca y Menorca, donde es relativamente común, y en Ibiza, donde es mucho menos abundante. Se han registrado observaciones puntuales de ejemplares aislados en Formentera, donde no obstante no hay constancia de la existencia actual de poblaciones reproductoras (Muntaner-Yangüela, 2002).

Hemmer *et al.* (1981) analizaron la distribución de *B. balearicus* (refiriéndose a esta especie como *B. viridis balearicus*) en el Mediterráneo Occidental y, basándose en evidencias moleculares, morfológicas, bioacústicas, y en restos arqueológicos atribuidos a la Edad de Bronce, infirieron que las poblaciones baleares proceden muy probablemente de Córcega y Cerdeña. Si bien existen

Síntesis de las introducciones de anfibios y reptiles en España

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La diversidad de especies introducidas es tan importante en la renovación de las biotas de países del Mediterráneo durante el Holoceno, que suele superar a la diversidad de especies extinguidas en el mismo período de tiempo (Planhol, 2004; Pascal *et al.*, 2006). En España se conocen 52 especies de anfibios y reptiles introducidas desde otros países, o desde zonas de la geografía española donde las especies son nativas a zonas donde no lo son, 17 casos de anfibios y 35 de reptiles. Este trabajo es una síntesis de las 28 especies tratadas en este volumen especial del Boletín de la Asociación Herpetológica Española, seis de anfibios y 22 de reptiles. A excepción de algunas introducciones en las islas Canarias, todas las especies introducidas en España y consideradas en esta revisión tienen su origen en el Mediterráneo Occidental y Central (Tabla 1), pues son aquéllas que, por proximidad filogeográfica, podrían plantear dudas sobre su carácter nativo o introducido en España. Recuérdese que las especies alóctonas que forman parte del comercio de mascotas, procedentes de zonas geográficas más alejadas, incluso otros continentes (e.g., *Trachemys scripta* y *Lampropeltis getula*), no han sido incluidas en este estudio. Igualmente, no se han incluido otras introducciones debido a su carácter puntual y reciente, como *Mesotriton alpestris* y *Ommatotriton ophryticus* en el prepirineo catalán (Fibla *et al.*, 2015 y Fontelles *et al.*, 2011, respectivamente), *Lissotriton boscai* en el macizo del Montseny (Amat & Carranza, 2011), y *Triturus pygmaeus* en Crevillente, Alicante (Sancho *et al.*, 2015), así como apariciones de ejemplares del género *Tarentola* en puertos y estaciones de tren, debidas a transporte pasivo con mercancías (e.g., *Tarentola mauritanica* cerca de estaciones de tren en localidades de Galicia; Cabana, 2008). Tampoco se han tratado especies canarias del género *Tarentola* que, siendo endémicas de alguna isla, han aparecido puntualmente en puertos y zonas próximas de otras islas del mismo archipiélago debido posiblemente al transporte marítimo; en ningún caso han conformado poblaciones viables (Pether *et al.*, 2009). La Tabla 1 resume el período de la introducción y el modo de entrada de cada especie tratada. Para cada especie se ha considerado por separado las distintas zonas de introducción en su caso, pues en general las evidencias genéticas indican que se trata de procesos independientes. De esta manera, esta síntesis incluye datos de 52 introducciones, 33 ya estudiadas mediante marcadores moleculares (el 62,3% de las introducciones documentadas), especialmente mitocondriales (entre los que destacan fragmentos del citocromo *b*) pero también nucleares (sobre todo microsatélites).

Las islas Baleares constituyen la zona receptora del mayor número de especies alóctonas (18 especies y 31 introducciones independientes), mientras que las islas Canarias han recibido cinco especies introducidas, además de seis translocaciones entre islas (no se contabilizan otras translocaciones de gecos endémicos canarios del género *Tarentola* entre islas debido a su carácter muy puntual). Además, nueve especies en al menos 12 eventos independientes han sido introducidas en la península ibérica, principalmente en zonas costeras y el sur peninsular (Tabla 1).

Los estudios moleculares, junto a otras evidencias históricas, han permitido precisar la procedencia de 43 poblaciones alóctonas (Figura 1): 12 introducciones proceden de diferentes puntos de la península ibérica, 10 de otras partes de Europa (incluidas islas del Mediterráneo), 14 del norte de África y en siete casos se desconoce el origen (Tabla 1).

Gran parte de las introducciones ha ocurrido de manera pasiva según nuestras estimas (n = 29; 54,7% de los casos), aunque el número de introducciones activas no es desdeñable (n = 16; 30,2% de los casos), y aún hay dudas sobre la intencionalidad en al menos siete casos (Tabla 1). Las introducciones pasivas se deben a menudo al tráfico marítimo de mercancías, tanto en tiempos históricos como actuales, aunque recientemente el comercio de olivos centenarios ha desencadenado un alud de introducciones, tanto de saurios como de ofidios (Valdeón *et al.*, 2010; Silva-Rocha *et al.*, 2015). Muchas de las introducciones activas se han producido en épocas históricas, aunque se han constatado también en los últimos 25 años (Pinya & Carretero, 2011).

La Figura 2 muestra los períodos con mayor número de introducciones. Destacan especialmente la Edad Antigua (siglos VIII a.e.c.-V; según la clasificación de Celarius, 1688; véase Blondel *et al.*, 2010), para la que se constata que hubo al menos 13 introducciones independientes, casi todas en las islas Baleares. Es la época del periodo romano clásico, en la que se desarrollan las ciudades, especialmente las redes de comunicación, hechos que favorecieron los movimientos de especies antropófilas (Lepetz & Yvinec, 2002; Pascal *et al.*, 2006). En el siglo XXI se registra un repunte de las introducciones, 12 casos, tanto en la península ibérica

Tabla 1: Procesos de introducción de anfibios y reptiles en España.

Nombre científico	Introducida en España	Origen
Mesotriton alpestris	Macizo de Peñalara	Cornisa cantábrica
Discoglossus pictus	Península ibérica (NE)	Argelia
Bufotes balearicus	Mallorca	Península itálica, Córcega, Cerdeña
Bufotes balearicus	Menorca	Península itálica, Córcega, Cerdeña
Bufotes balearicus	Ibiza	Península itálica, Córcega, Cerdeña
Hyla meridionalis	Menorca	Desconocido
Hyla meridionalis	Islas Canarias	Marruecos
Hyla meridionalis	Península ibérica (Norte)	Norte de Marruecos
Pelophylax perezi	Mallorca y Pitiusas	Desconocido
Pelophylax perezi	Islas Canarias	Desconocido
Pelophylax saharicus	Gran Canaria	Seghiat el Hamra (Sáhara Occidental)
Mauremys leprosa	Mallorca	Desconocido
Emys orbicularis	Mallorca y Menorca	Desconocido (*)
Testudo graeca graeca	Doñana	Sureste ibérico
Testudo graeca marokkensis	Doñana	Marruecos
Testudo graeca	Mallorca	Norte de África
Testudo hermanni (linaje continental)	Mallorca	NE península ibérica, Sur Francia
Testudo hermanni (linaje Menorca)	Menorca	Posible Tirreno (actualmente linaje exclusivo de Menorca)
Chamaeleo chamaeleon	Península ibérica	Norte de África
Hemidactylus turcicus	Península ibérica	Medit. Oriental (clado Europeo y Africano)
Hemidactylus turcicus	Mallorca	Medit. Oriental (clado Africano)
Hemidactylus turcicus	Menorca	Medit. Oriental (clado Africano)
Hemidactylus turcicus	Pitiusas	Medit. Oriental (clado Africano)
Hemidactylus turcicus	Gran Canaria y Tenerife	Medit. Oriental (clado Africano)
Tarentola mauritanica	Península ibérica (costa)	Marruecos
Tarentola mauritanica	Islas Baleares	Norte de África
Chalcides viridanus	La Palma	Tenerife
Chalcides sexlineauts	La Palma	Gran Canaria
Gallotia atlantica	Gran Canaria	Lanzarote
Gallotia galloti	Fuerteventura	Tenerife
Gallotia stehlini	Fuerteventura	Gran Canaria
Teira dugesii	Gran Canaria	Madeira
Podarcis pityusensis	Bermeo y San Sebastián	Ibiza o Murada (Mallorca)
Podarcis pityusensis	Mallorca	Ibiza
Podarcis sicula	Menorca	Sicilia (probablemente vía Cerdeña)
Podarcis sicula	Cantabria	Toscana
Podarcis sicula	Almería	Sicilia (probablemente via Cerdeña o Menorca)
Podarcis sicula	Rioja	S península italiana
Podarcis sicula	Cataluña	S península italiana
Psammodromus algirus	Mallorca	Desconocido
Scelarcis perspicillata	Menorca	Probablemente Orán, Argelia
Macroprotodon cucullatus	Mallorca y Menorca	Túnez
Rhinechis scalaris	Menorca	Península ibérica
Rhinechis scalaris	Mallorca	Península ibérica
Rhinechis scalaris	Ibiza	Península ibérica
Rhinechis scalaris	Formentera	Península ibérica
Hemorrhois hippocrepis	Mallorca	Península ibérica
Hemorrhois hippocrepis	Ibiza	Península ibérica
Hemorrhois hippocrepis	Formentera	Ibiza (probablemente)
Malpolon monspessulanus	Mallorca	Península ibérica
Malpolon monspessulanus	Ibiza	Península ibérica
Natrix maura	Mallorca	Sur Francia
Natrix maura	Menorca	Desconocido

Modo de introducción	**Fecha de introducción**	**Marcadores moleculares**
Activa	1984	ADNmt y microsatélites
Activa	1906	ADNmt
Desconocido	Desconocido	ADNmt y ADNn
Desconocido	Desconocido	ADNmt y ADNn
Desconocido	Desconocido	No disponibles
Probable pasiva, con mercancías	Aprox. S. II a.e.c.	No disponibles
Activa	Aprox. S. XV	ADNmt
Probable pasiva, con mercancías	Desconocido, anterior S. XX	ADNmt
Probable pasiva, con mercancías	Aprox. S. I	No disponibles
Probable pasiva, con mercancías	Aprox. S. XV	No disponibles
Activa	Aprox. 1989	No disponibles
Probablemente activa	Siglo XXI	No disponibles
Probablemente activa	Aprox. S. I	ADNmt y microsatélites
Probable introducción activa	Desconocido	ADNmt y microsatélites
Activa	Siglo XX	ADNmt y microsatélites
Activa	Desconocido	ADNmt
Activa	Probable en Edad Antigua	ADNmt
Desconocido	Desconocido	Microsatélites
Desconocido	Neolítico - Bronce	ADNmt
Probable pasiva, con mercancías	Probable en Edad Antigua	ADNmt y ADNn
Probable pasiva, con mercancías	Probable en Edad Antigua	No disponibles
Probable pasiva, con mercancías	Probable en Edad Antigua	ADNmt y ADNn
Probable pasiva, con mercancías	Probable en Edad Antigua	No disponibles
Probable pasiva, con mercancías	SXV-XVIII	ADNmt y ADNn
Probable pasiva, con mercancías	Desconocido	ADNmt y ADNn
Probable pasiva, con mercancías	Aprox. S. IV a.e.c.	ADNmt y ADNn
Probable pasiva	Final s. XX	No disponibles
Activa	1960’s	No disponibles
Desconocido	1980’s	ADNmt
Activa	1980’s	No disponibles
Desconocido	Final s. XIX	No disponibles
Probable activa	2000’s	ADNmt
Activa	Aprox. 1992	ADNmt
Activa	1980’s	No disponibles
Probable pasiva, con mercancías	Aprox. S. XIV	ADNmt
Probable pasiva, con mercancías	1930’s	ADNmt
Probable pasiva, con mercancías	1930’s	ADNmt
Pasiva, en olivos	2010’s	ADNmt
Pasiva, en olivos	2010’s	ADNmt
Probable pasiva, en olivos	1980’s	No disponibles
Probable pasiva, con mercancías	Aprox. S. XVIII	ADNmt
Probable pasiva, con mercancías	Aprox. S. II a.e.c.	ADNmt
Activa, Culto religioso?	S. II a.e.c. o anterior	ADNmt
Pasiva, en olivos	2000’s	ADNmt
Pasiva, en olivos	2000’s	ADNmt
Pasiva, en olivos	2000’s	No disponibles
Pasiva, en olivos	2006	ADNmt
Pasiva, en olivos	2003	ADNmt
Pasiva	2010	No disponibles
Pasiva, en olivos	2000’s	ADNmt
Pasiva, en olivos	2000’s	No disponibles
Activa, Culto religioso?	Aprox. S. I a.e.c.	ADNmt y ADNn
Activa, Culto religioso?	Aprox. S. I a.e.c.. (?)	No disponibles

(*) Desconocido en el rango de distribución de *E. o. galloitalica* y *E. o. orbicularis*.

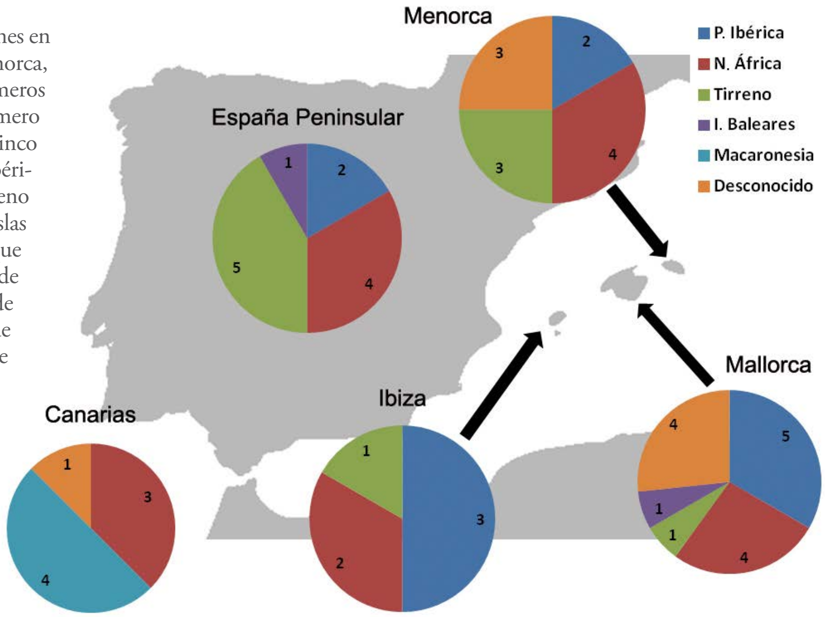

Figura 1: Origen de las introducciones en la península ibérica, Mallorca, Menorca, Ibiza y las islas Canarias. Los números en cada figura corresponden al número de introducciones procedentes de cinco regiones geográficas (península ibérica, norte de África, región del Tirreno incluyendo Italia, islas Baleares e islas Canarias) y una última categoría que corresponde a las introducciones de origen desconocido. El tamaño de cada porción corresponde al % de introducciones respecto al total de cada zona.

como en las islas Baleares, fundamentalmente ligadas al comercio de plantas de jardín, como los olivos centenarios. En cambio, no se detecta un incremento del número de especies de anfibios y reptiles introducidas en la Edad Moderna (siglo XVI- s. XVIII), relacionado con el inicio de los viajes transoceánicos de los europeos, como es común para el resto de los vertebrados introducidos globalmente (Simberloff, 2006). Se desconoce el período aproximado de la introducción en siete casos (Figura 2). La tasa de especies introducidas por siglo en los diferentes períodos muestra valores parecidos desde la Edad Antigua hasta 1950, momento a partir del cual se dispara (Figura 2). Además, esta elevada cifra en la época más reciente está infravalorada, pues en esta revisión no se incluyen las especies introducidas procedentes del mercado de animales de compañía.

La tendencia poblacional y corológica en las poblaciones introducidas es muy variable (Tabla 2). Parece estable en 20 casos, en expansión en siete, en regresión en otros nueve (considerando al mismo nivel los cambios de las áreas de distribución y del tamaño de las población) y solamente en cinco casos se ha constatado la extinción de

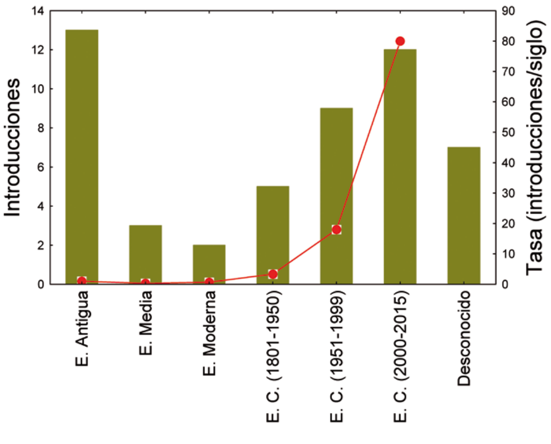

Figura 2: Cronograma del número de las 51 introducciones independientes en España (histogramas verdes; *C. chamaeleon* no ha sido considerado en este gráfico por estimarse una posible introducción anterior a la Edad Antigua) y la tasa de introducciones por siglo (puntos y líneas rojas). Los períodos basales están basados en la clasificación de Celarius (1688): Edad Antigua (s. VIII a.e.c. - s. V e.c.), Edad Media (s. VI – s. XV), Edad Moderna (s. XVI – s. XVIII) y Edad Contemporánea (E.C.; s. XIX – Actualidad), aunque el último período se ha subdividido, por el elevado número de introducciones recientes.

las poblaciones introducidas (Silva-Rocha *et al.*, 2015). En las introducciones acaecidas en la península ibérica, el patrón general muestra que la distribución es mayor en las especies de introducción antigua, e.g., *Hemidactylus turcicus* y *T. mauritanica*. Este último caso es complejo pues conviven, incluso en sintopía y sin aparente hibridación, dos linajes, uno autóctono y otro de introducción reciente, que podrían corresponder a dos especies distintas de salamanquesas (Rato *et al.*, 2016).

Se han constatado impactos negativos de las especies de anfibios y reptiles introducidas sobre la biota nativa en 19 casos, el 35,8% de los considerados en la Tabla 2, un porcentaje mayor que el que se encuentra en Francia para la comunidad de vertebrados introducidos (16%; Pascal *et al.*, 2006). Mayoritariamente están relacionados con la competencia entre especies de nicho ecológico similar (e.g., *Discoglossus pictus* con otros anuros nativos; Richter-Boix *et al.*, 2013) o con la depredación sobre fauna local (ofidios introducidos que depredan sobre saurios y aves autóctonos y endémicos; Ayllón *et al.*, 2014). En el caso de *Mesotriton alpestris*, la población introducida en el Sistema Central puede ser un reservorio de quitridiomicosis (Bosch *et al.*, 2015, en este volumen) y en la recientemente localizada en el prepirineo catalán se ha detectado la presencia de *Ranavirus* spp., agente infeccioso que ya ha provocado mortalidad masiva de anfibios en España (Price *et al.*, 2014). Los casos documentados de impacto sobre la fauna nativa corresponden tanto a introducciones históricas (e.g., *Natrix maura*; Moore *et al.*, 2004) como a recientes (e.g., *Hemorrhois hippocrepis*, Ayllón *et al.*, 2014). Se ha constatado un posible impacto beneficioso de una especie introducida, *Testudo graeca*, que parece ser buena dispersora de semillas (Cobo & Andreu, 1988), y también de culebras introducidas en las islas Baleares como *Macroprotodon cucullatus*, a través del consumo de micromamíferos alóctonos. Sorprendentemente, el impacto de las poblaciones introducidas sobre la fauna local es desconocido en 26 casos, es decir, en el 49,1% de los casos considerados. Esto representa un campo abierto y prioritario para la investigación, pues la información sobre los impactos de las especies introducidas es de vital importancia para tomar decisiones en su gestión (Simberloff *et al.*, 2005), y estos procesos son excelentes modelos de investigación en ciencia básica (Simberloff, 2003; Pascal *et al.*, 2006).

Además de los recientes programas de erradicación de los galápagos procedentes del comercio de mascotas, solamente en 10 de las especies incluidas en este estudio (18,9%) se está realizando la gestión de las poblaciones introducidas (o se ha planificado a corto plazo), como control y/o erradicación, o sencillamente un seguimiento de los cambios en su distribución y demografía poblacional. A efectos comparativos, esta cifra es mucho menor que en Francia, donde el 70% de las poblaciones introducidas de vertebrados ha sido objeto de alguna medida de gestión (Pascal *et al.*, 2006). Los casos en los que se realiza gestión corresponden a poblaciones de pequeña distribución (e.g., *Podarcis sicula*; Valdeón *et al.*, 2010) en diferentes puntos de la península ibérica, y a poblaciones de diversos ofidios (e.g., *Rhinechis scalaris*, *Malpolon monspessulanus* y *Hemorrhois hippocrepis*) en las islas Baleares (Montes *et al.*, 2015). Tan sólo en dos de los casos en los que se ha gestionado una población con el fin de la erradicación ésta ha sido exitosa (las poblaciones de *Podarcis sicula* en Cataluña y La Rioja; Valdeón *et al.*, 2010). No se realizan acciones de gestión en 34 casos de poblaciones introducidas, es decir, en el 64,2% de las introducciones documentadas en España (Tabla 2).

Se han realizado o se realizan acciones para la conservación de poblaciones introducidas de cinco especies, las poblaciones de las islas Baleares

Tabla 2: Impactos de la introducción de anfibios y reptiles en España.

Nombre científico	Introducida en España	Tendencia Rango	Tendencia Población
Mesotriton alpestris	Macizo de Peñalara	Expansión	Aumento fuerte
Discoglossus pictus	Península ibérica (NE)	Expansión	Aumento
Bufotes balearicus	Mallorca	Regresión	Declive
Bufotes balearicus	Menorca	Regresión	Declive
Bufotes balearicus	Ibiza	Regresión	Declive
Hyla meridionalis	Menorca	Estable	Desconocido
Hyla meridionalis	Islas Canarias	Estable	Desconocido
Hyla meridionalis	Península ibérica	Estable	Desconocido
Pelophylax perezi	Mallorca y Pitiusas	Desconocido	Declive (?)
Pelophylax perezi	Islas Canarias	Expansión	Desconocido
Pelophylax saharicus	Gran Canaria	Extinta	Extinta
Mauremys leprosa	Mallorca	Estable	Estable
Emys orbicularis	Mallorca y Menorca	Estable	Desconocido
Testudo graeca	Doñana	Regresión	Estable
Testudo graeca	Mallorca	Regresión	Declive
Testudo hermanni	Mallorca	Regresión (?)	Declive (?)
Testudo hermanni	Menorca	Estable	Estable
Chamaeleo chamaeleon	Península ibérica	Expansión	Estable (?)
Hemidactylus turcicus	Península ibérica	Expansión	Aumento
Hemidactylus turcicus	Mallorca	Estable	Estable
Hemidactylus turcicus	Menorca	Estable	Estable
Hemidactylus turcicus	Pitiusas	Estable	Estable
Hemidactylus turcicus	Gran Canaria y Tenerife	Estable	Estable
Tarentola mauritanica	Península ibérica (costa)	Desconocido	Desconocido
Tarentola mauritanica	Islas Baleares	Desconocido	Desconocido
Chalcides viridanus	La Palma	Desconocido	Desconocido
Chalcides sexlineatus	La Palma	Estable	Estable
Gallotia atlantica	Gran Canaria	Estable	Incremento
Gallotia galloti	Fuerteventura	Estable	Estable
Gallotia stehlini	Fuerteventura	Estable	Desconocido
Teira dugesii	Gran Canaria	Desconocido	Desconocido
Podarcis pityusensis	Euskadi	Estable	Estable
Podarcis pityusensis	Mallorca	Estable	Desconocido
Podarcis sicula	Menorca	Estable	Desconocido
Podarcis sicula	Cantabria	Regresión	Desconocido
Podarcis sicula	Almería	Expansión	Aumento
Podarcis sicula	Rioja	Erradicada	Erradicada
Podarcis sicula	Cataluña	Erradicada	Erradicada
Psammodromus algirus	Mallorca	Regresión (?)	Declive (?)
Scelarcis perspicillata	Menorca	Desconocido	Desconocido
Macroprotodon cucullatus	Mallorca y Menorca	Desconocido	Desconocido
Rhinechis scalaris	Menorca	Estable	Desconocido
Rhinechis scalaris	Mallorca	Expansión	Aumento
Rhinechis scalaris	Ibiza	Estable	Estable
Rhinechis scalaris	Formentera	Extinta (?)	Extinta (?)
Hemorrhois hippocrepis	Mallorca	Desconocido	Desconocido
Hemorrhois hippocrepis	Ibiza	Expansión	Aumento
Hemorrhois hippocrepis	Formentera	Desconocido	Desconocido
Malpolon monspessulanus	Mallorca	Desconocido	Desconocido
Malpolon monspessulanus	Ibiza	Extinta	Extinta
Natrix maura	Mallorca	Regresión	Regresión
Natrix maura	Menorca	Estable	Desconocido

Impactos de la población alóctona	Gestión actual población alóctona
Competencia y depredación (anfibios); reservorio quitridiomicosis	Propuesta de Erradicación
Competencia (anfibios)	Seguimiento
Desconocidos	Conservación
Desconocidos	Conservación
Desconocidos	Conservación
Desconocidos	No
Desconocidos	No
Desconocidos	No
Competencia y depredación (anfibio)	No
Desconocidos	No
Desconocidos	No
Desconocidos	No
Positivos (dispersor de semillas); Negativos (introgresión genética entre spp.)	Conservación
Desconocidos	Conservación
Desconocidos	Conservación
Desconocidos	Conservación
No parecen existir	Conservación
Desconocidos	No
Desconocidos	No
Desconocidos	No
Desconocidos	No
Desconocidos	No
Desconocidos	No
Competencia (*Podarcis lilfordi*)	No
Desconocidos	No
Desconocidos	No
Competencia (*Gallotia stehlini*)	No
Desconocidos	No
Desconocidos	No
Desconocidos	No
Competencia (*Podarcis muralis*)	Seguimiento y control
Desconocidos	No
Competencia (*Podarcis lilfordi* en Ses Mones?)	Seguimiento
Competencia (*Podarcis muralis*)	No
Competencia (*Podarcis hispanica*)	No
Desconocidos	No
Desconocidos	No
Desconocidos sobre especies nativas, positivo sobre micromamíferos introducidos	No
Depredación (aves)	No
Depredación (aves)	No
Depredación (aves)	Erradicación
Depredación (aves y *Eliomys quercinus*)	No
Desconocidos	No
Depredación (*Podarcis pityusensis*)	Erradicación
Depredación (*Podarcis pityusensis*)	No
Depredación (aves)	No
Depredación (*Alytes muletensis*)	Erradicación
Depredación (*Bufotes balearicus*)	No

de *Bufotes balearicus*, *Testudo graeca* y *Testudo hermanni*, las poblaciones continentales de *T. graeca* en Doñana, y las de *Chamaeleo chamaeleon* en el sur de la península ibérica. Se trata en la mayoría de los casos de especies con presencia en España desde tiempos históricos o prehistóricos (o que incluso presentan dudas sobre su carácter alóctono; e.g., *C. chamaeleon*), especies emblemáticas, sin aparente impacto sobre la fauna nativa, los ecosistemas, y que en algunos casos presentan problemas de conservación en la zona nativa (e.g., *T. hermanni*; Bertolero, 2014).

Atendiendo a los criterios señalados por Pleguezuelos & Mateo (2015) en este volumen, con pocas excepciones, las especies estudiadas en los artículos anteriores de este volumen deben ser consideradas alóctonas al tratarse de especies que han sido introducidas en áreas del territorio español donde no estaban de forma natural. Para las introducciones en las islas Baleares e islas Canarias no hay dudas. Sin embargo, el panorama es más complejo en la península ibérica, y algunas especies o poblaciones deben ser consideradas como autóctonas, aunque sea por el principio de precaución. Tal es el caso de *T. graeca* en el sur ibérico, y podría serlo para *C. chamaeleon* y las poblaciones del suroeste ibérico de *Hyla meridionalis*. En los dos últimos casos hay dudas sobre una posible dispersión natural desde la orilla africana del Estrecho de Gibraltar, un hecho frecuente en muchos grupos taxonómicos desde el Mioceno (Carranza *et al.*, 2006), o una introducción muy antigua. En otros casos, también en el ámbito de la península ibérica, los marcadores moleculares han permitido constatar la cercanía de haplogrupos de origen diferente que pertenecerían a colonizaciones/introducciones diferentes desde la orilla marroquí del Estrecho de Gibraltar (e.g., *Emys orbicularis*, Stuckas *et al.*, 2014; Velo-Antón *et al.*, 2015) y hasta la coexistencia en simpatría de linajes de origen tanto autóctono como alóctono; éste es el caso de las poblaciones del centro peninsular de *T. mauritanica* (Rato *et al.*, 2016). Para estas especies, nuestra opinión es que deben ser consideradas como especies/poblaciones autóctonas, sin perjuicio de futuros estudios que puedan reinterpretar su estatus.

En resumen, el conjunto de las introducciones de anfibios y reptiles en España muestra un complejo panorama del que se desprenden escasos patrones comunes. Los reptiles han sido más frecuentemente introducidos que los anfibios, debido a su mayor resistencia a condiciones ambientales desfavorables (e.g., transporte marítimo; Lever, 2003). El transporte marítimo por el Mediterráneo ha favorecido desde la Edad Antigua el intercambio comercial y cultural, y ello ha acarreado la introducción voluntaria o accidental de numerosas especies (Lepetz & Yvinec, 2002; Blondel *et al.*, 2010), sobre todo de reptiles que podemos llamar antropófilos (*T. mauritanica*, *H. turcicus*, *H. hippocrepis*), y especialmente en las islas Baleares (Pinya & Carretero, 2011).

Detectamos la necesidad de nuevos estudios en diferentes campos del conocimiento: 1) el uso de marcadores moleculares variables que permitan identificar el origen de las poblaciones alóctonas y, en algunos casos, los límites entre poblaciones alóctonas y autóctonas, y los casos de hibridación; 2) trabajo de campo y experimental para estudiar la interacción de las especies introducidas con la biota nativa; 3) modelos de nicho ecológico proyectando hacia el futuro la tendencia del rango de distribución de las poblaciones introducidas; 4) divulgación y concienciación de la sociedad en el problema que genera la introducción de especies; 5) control de las vías de entrada de especies exóticas, por una parte restringiendo la importación de especies para el comercio de mascotas, y por otro, con un control riguroso de las especies vegetales que transportan anfibios y reptiles alóctonos; y 6) fomento de una clara y decidida participación de las administraciones en la gestión de las poblaciones introducidas bajo la supervisión de expertos. En este sentido, y para tener un respaldo administrativo y legal en este proceso, es necesario replantear las listas de especies protegidas e introducidas en los catálogos nacionales y autonómicos (véase Ayllón *et al.*, 2015, en este volumen).

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Propuesta de revisión de los listados y catálogos nacionales y autonómicos de especies amenazadas o protegidas, y del Catálogo Nacional de Especies Invasoras

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Key words: herps, conservation, Spain, list, threatened species, exotic species.

En España, si de algo adolece la legislación comunitaria, nacional y autonómica en materia de protección de especies, es de la falta de actualizaciones periódicas de sus catálogos, un ejercicio necesario por el mejor conocimiento de la taxonomía y del estado de conservación de nuestra biota. Esta falta de actualización implica la utilización de unidades de gestión erróneas en la toma de decisiones que afecten a nuestra biodiversidad. Por ejemplo, en casos de translocación de especies autóctonas hacia áreas de nuestro territorio sin presencia previa, los catálogos de especies amenazadas en ocasiones incluyen estas especies no diferenciándolas de las poblaciones autóctonas. De modo parecido, algunas especies introducidas que se han llegado a reconocer como especies invasoras debido al impacto sobre la biota nativa, pueden seguir en los catálogos de especies protegidas y a menudo no están recogidas en los catálogos de especies invasoras.

Se ha realizado una revisión de la presencia en el Catálogo Español de Especies Amenazadas (CEEA) y en el Listado de Especies Silvestres en Régimen de Protección Especial (LESRPE) de las especies alóctonas tratadas en este volumen. Igualmente, se han consultado 15 catálogos y listados regionales de especies amenazadas, y la Ley de Protección de Animales de Cataluña (este último no es un catálogo propiamente dicho). No se ha podido valorar el estatus de estas poblaciones y especies en Castilla y León, por la inexistencia del catálogo regional correspondiente (véase Anexo I).

A partir de la presencia / ausencia de las poblaciones / especies alóctonas en los catálogos anteriormente mencionados, se ha procedido a realizar una propuesta de modificación de estos. Dicha propuesta se basa en evidencias científicas aportadas por los expertos sobre el carácter alóctono y/o invasor de las especies. El criterio general ha sido que las poblaciones / especies alóctonas no deberían aparecer en ningún catálogo nacional de especies amenazadas ni en los catálogos autonómicos de las zonas de introducción, y por tanto, con contadas excepciones, proponemos que dichas poblaciones / especies sean eliminadas de estos catálogos.

Igualmente, se ha procedido a consultar el Catálogo Español de Especies Exóticas Invasoras (CEEEI). En este caso se propone incluir en dicho listado a aquellas poblaciones / especies que, conocido su carácter alóctono e impacto en las regiones de introducción, no figuran actualmente en el CEEEI.

La Tabla 1 recoge el listado de poblaciones / especies introducidas en los catálogos nacionales y autonómicos. Las propuestas de cambio se marcan en amarillo y rojo. Las propuestas concretas de modificación son:

- En el CEEA, proponemos retirar el estatus de Vulnerable para las poblaciones de *Mesotriton alpestris* en Peñalara y de *Testudo graeca* en las islas Baleares, dado su carácter invasor en el primer caso y la constatación de ser una introducción histórica procedente del norte de África en el segundo.

- En el LESRPE, proponemos excluir del listado las poblaciones alóctonas de *M. alpestris* (Macizo de Peñalara), *Discoglossus pictus* (toda la distribución en España), *Bufotes balearicus* (islas Baleares), *Hyla meridionalis* (poblaciones de islas Baleares, País Vasco y Cataluña), *Emys orbicularis* (poblaciones de islas Baleares), *Mauremys leprosa* (poblaciones de Islas Baleares),*Tarentola mauritanica* (poblaciones de islas Baleares), *Hemidactylus turcicus* (toda la distribución en España), *Gallotia atlantica* (población de Gran Canaria), *Gallotia stehlini* (población de Fuerteventura), *Podarcis pityusensis* (poblaciones de País Vasco, Barcelona y Mallorca), *Podarcis sicula* (toda la distribución en España), *Psammodromus algirus* (poblaciones en Mallorca), *Macroprotodon cucullatus* (toda la distribución en España), *Rhinechis scalaris* (poblaciones en islas Baleares), y *Hemorrhois hippocrepis* (poblaciones en islas Baleares). Excepcionalmente, proponemos mantener en el LESRPE aquellas especies o poblaciones alóctonas resultado de introducciones históricas que tengan un destacado valor científico o para la conservación de poblaciones autóctonas; en ningún caso podrán ser poblaciones que hayan presentado un comportamiento invasivo o que condicionen negativamente la dinámica de las especies nativas o en general el funcionamiento de los ecosistemas. En particular proponemos mantener en el listado las poblaciones de islas Baleares de *Testudo hermanni* (stock reproductivo del linaje ibérico de esta tortuga y poblaciones con características genéticas únicas en toda su distribución) y la población de *Testudo graeca* de Doñana y Mallorca. En el caso de *T. graeca*, proponemos mantener a la especie en el listado sin hacer distinciones entre poblaciones, para proporcionar herramientas administrativas que limiten la tenencia en cautividad de tortugas y prácticas como la cría en cautividad o la liberación de tortugas en el medio. Estas prácticas suponen una seria amenaza para la población autóctona de *T. graeca* en el sureste ibérico, pudiendo conllevar erosión poblacional, exogamia genética e introducción de patógenos y parásitos. La inclusión de una especie en el listado supone la prohibición expresa de su captura, tenencia e intercambio (Art. 57, Ley 42/2007), prácticas que deben quedar limitadas en todo el territorio nacional con independencia del origen de la población. En cualquier caso la inclusión de las poblaciones de *Testudo* en dicho listado no debe justificar la creación de "poblaciones" satélite con tortugas procedentes de centros de recuperación y/o cautividad. Las introducciones y refuerzos poblacionales deben siempre obedecer a las necesidades de conservación de las poblaciones autóctonas y, en cualquier caso, han de quedar recogidos en sus documentos de gestión (como planes de conservación o de recuperación). La consideración de la especie en el listado no implica el desarrollo de acciones proactivas de conservación y, por tanto, no ha de suponer un detrimento de recursos paras las poblaciones autóctonas.

Tabla 1: Propuesta de cambios para las especies de anfibios y reptiles con poblaciones introducidas en territorio español en el Catálogo Español de Especies Amenazadas (CEEA), Listado de Especies Silvestres en Régimen de Protección Especial (LESRPE), Catálogo Español de Especies Exóticas Invasoras (CEEEI), y →

Especie	Introducción	CEEA*1	LESRPE*2
Mesotriton alpestris	Peñalara	Vu (1)	In (1)
Discoglossus pictus	PI	NI	In→NI
Bufotes balearicus	IB	NI	In→NI
Hyla meridionalis	PI, IB, IC	NI	In (2)(3)
Pelophylax perezi	IB, IC	NI	NI
Mauremys leprosa	PI, IB	NI	In (2)
Emys orbicularis	PI, IB	NI	In (2)
Testudo graeca	PI, IB	Vu (2)	In (2)
Testudo hermanni	IB	NI	In
Chamaeleo chamaeleon	PI	NI	In
Hemidactylus turcicus	PI, IB, IC	NI	In →NI
Tarentola mauritanica	PI, IB	NI	In (2)
Chalcides viridanus	La Palma	NI	In
Chalcides sexlineatus	La Palma	NI	In (4)
Gallotia atlantica	Gran Canaria	NI	In (5)
Gallotia galloti	Fuerteventura	NI	NI
Gallotia stehlini	Fuerteventura	NI	In (6)
Podarcis pityusensis	PI, Mallorca	NI	In (7)
Podarcis sicula	PI, IB	NI	In →NI
Psammodromus algirus	Mallorca		In (2)
Scelarcis perspicillata	IB	NI	NI
Macroprotodon cucullatus	IB	NI	In→NI
Rhinechis scalaris	IB	NI	In (2)
Hemorrhois hippocrepis	IB	NI	In (2)
Malpolon monspessulanus	IB	NI	NI
Natrix maura	IB	NI	NI

→ en catálogos autonómicos de especies amenazadas (los símbolos indican las iniciales de las comunidades autónomas). Las propuestas de cambios se indican en cuadros amarillos y rojos: los cuadros amarillos indican que la modificación no afecta a la catalogación actual de toda la especie sino solamente a alguna de sus poblaciones (indicadas entre paréntesis). Los cuadros rojos son propuestas de modificación de una categoría, y por ello se indica la catalogación actual y la propuesta de cambio. Cuando dichos cambios son parciales (afectan solo a algunas poblaciones o áreas geográficas), se especifica a continuación de la catalogación con un número entre paréntesis.

CEEEI*3	CAM	Cat	IB	IC	PV	An	Mu	CV	Ar	Ga	Ca	LR	Na	CIM	Ex
NI→In (8)	EN→NI														
NI		NI													
NI			NI												
NI	NI	In	NI	NI	EN	NI	NI							DIE	DIE
NI→In (2)			NI	NI											
NI	DIE	In	NI		VU	NI	NI	NI	DIE	NI	NI	NI	DIE	DIE	DIE
NI	EN	In	EP→NI		VU	NI		VU	VU	EN		NI	SAH	VU	SAH
NI			Vu→NI			EN	VU								
NI		In	NI					EN→NI							
NI						NI	NI	NI							
NI		In	NI	NI		NI	NI	NI	NI					DIE	DIE
NI	NI	In	NI			NI	NI	NI	NI	NI	NI	NI		DIE	DIE
NI				NI											
NI				NI											
NI→In (9)				NI											
NI				NI											
NI				In (6)											
NI		NI	NI		NI										
NI→In		NI	EP→NI		NI	NI									
NI			EP→NI												
In			NI												
In			NI												
In			NI												
In			NI												
In			NI												

Siglas: PI (península ibérica),
IB (islas Baleares),
IC (islas Canarias),
EN (En Peligro de Extinción),
VU (Vulnerable),
SAH (Sensible a la Alteración de su Hábitat),
DIE (De Interés Especial),
EP (Especial Protección),
In (Incluida),
NI (No incluida).

(1) Excluir la población del Macizo de Peñalara.
(2) Excluir poblaciones de islas Baleares.
(3) Excluir las poblaciones de País Vasco y Cataluña.
(4) Excluir la población de La Palma.
(5) Excluir la población de Gran Canaria.
(6) Excluir la población de Fuerteventura.
(7) Excluir las poblaciones del País Vasco, Barcelona y Mallorca.
(8) Incluir la población del Macizo de Peñalara.
(9) Incluir la población de Gran Canaria.

- En el CEEEI, se propone incluir las poblaciones de *M. alpestris* en Peñalara, de *Pelophylax perezi* en Mallorca, de *G. atlantica* en Gran Canaria, y de *P. sicula* en toda la geografía española. En todos los casos se ha constadado el carácter dañino de estas introducciones para la biota nativa.

- En los catálogos autonómicos de protección se propone excluir aquellas poblaciones cuya naturaleza alóctona ha sido probada. En concreto se propone excluir: *M. alpestris* del catálogo de especies protegidas de la Comunidad de Madrid; *E. orbicularis*, *T. graeca*, *P. sicula* y *Scelarcis perspicillata* del catálogo de las islas Baleares; *T. graeca* en Doñana del catálogo de Andalucía y *T. hermanni* del catálogo de la Comunidad Valenciana. En este último caso, aunque hay restos fósiles que atestiguan la presencia de la especie en la comunidad, actualmente solamente se conocen dos poblaciones reintroducidas a partir de ejemplares de linajes mezclados, algunos de ellos procedentes de Menorca, hecho que no justificaría catalogar esas poblaciones como En Peligro.

Esta propuesta actualizaría el panorama vigente de introducciones en España en los catálogos nacional y autonómicos. No se incluyen en este listado introducciones procedentes del mercado de mascotas que en muchos casos ya aparecen en el CEEEI. Finalmente, dado que la dinámica de las introducciones en España es un proceso que no se detendrá, proponemos revisiones periódicas avaladas por grupos de expertos para la actualización de catálogos y listados.

ANEXO 1. RELACIÓN DE CATÁLOGOS ANALIZADOS:

Nacionales:

1. Real Decreto 139/2011, de 4 de febrero, para el desarrollo del Listado de Especies Silvestres en Régimen de Protección Especial y del Catálogo Español de Especies Amenazadas. (LESRPE y CEEA)
2. Real Decreto 630/2013, de 2 de agosto, por el que se regula el Catálogo Español de Especies Exóticas Invasoras. (CEEEI)

Regionales:

1. Andalucía: DECRETO 23/2012, de 14 de febrero, por el que se regula la conservación y el uso sostenible de la flora y la fauna silvestres y sus hábitats.
2. Aragón: DECRETO 49/1995, de 28 de marzo, de la Diputación General de Aragón, por el que se regula el Catálogo de Especies Amenazadas de Aragón. ORDEN de 4 de marzo de 2004, del Departamento de Medio Ambiente, por la que se incluyen en el Catálogo de Especies Amenazadas de Aragón determinadas especies, subespecies y poblaciones de flora y fauna y cambian de categoría y se excluyen otras especies ya incluidas en el mismo.
3. Asturias: Decreto 32/90, de 8 de marzo, por el que se crea el Catálogo Regional de Especies Amenazadas de la Fauna Vertebrada del Principado de Asturias y se dictan normas para su protección.
4. Islas Baleares: Decreto 75/2005, de 8 de julio, por el cual se crea el Catálogo Balear de Especies Amenazadas y de Especial Protección, las Áreas Biológicas Críticas y el Consejo Asesor de Fauna y Flora de les Illes Balears.
5. Islas Canarias: LEY 4/2010, de 4 de junio, del Catálogo Canario de Especies Protegidas.
6. Cantabria: Decreto 120/2008, de 4 de diciembre por el que se regula el Catálogo Regional de Especies Amenazadas de Cantabria.
7. Castilla La Mancha: Decreto 33/1998, de 05-05-98, por el que se crea el Catálogo Regional de Especies Amenazadas de Castilla-La Mancha. Decreto 200/2001, de 06-11-2001 por el que se modifica el Catálogo Regional de Especies Amenazadas.
8. Cataluña: DECRET LEGISLATIU 2/2008, de 15 d'abril, pel qual s'aprova el Text refós de la Llei de Protecció dels Animals.
9. Comunidad Valenciana: ORDEN 6/2013, de 25 de marzo, de la Conselleria de Infraestructuras, Territorio y Medio Ambiente, por la que se modifican los listados valencianos de especies protegidas de flora y fauna.
10. Extremadura: DECRETO 37/2001, de 6 de marzo, por el que se regula el Catálogo Regional de Especies Amenazadas de Extremadura.
11. Galicia: Decreto 88/2007 do 19 de abril, polo que se regula o Catálogo Galego de Especies Ameazadas.
12. La Rioja: Decreto 59/1998, de 9 de octubre, por el que se crea y regula el Catálogo Regional de Especies Amenazadas de la Flora y Fauna Silvestre de La Rioja.
13. Madrid: Decreto 18/1992, de 26 de marzo, por el que se aprueba el Catálogo Regional de Especies Amenazadas de Fauna y Flora Silvestres y se crea la categoría de árboles singulares.
14. Murcia: LEY 7/1995, de 21 de abril, de la Fauna Silvestre, Caza y Pesca Fluvial.
15. Navarra: DECRETO FORAL 563/1995, de 27 de noviembre, del Gobierno de Navarra, por el que se aprueba la inclusión en el Catálogo de Especies Amenazadas de Navarra de determinadas especies y subespecies de vertebrados de la fauna silvestre.
16. País Vasco: ORDEN de 10 de enero de 2011, de la Consejera de Medio Ambiente, Planificación Territorial, Agricultura y Pesca, por la que se modifica el Catálogo Vasco de Especies Amenazadas de la Fauna y Flora Silvestre y Marina, y se aprueba el texto único. ORDEN de 18 de junio de 2013, de la Consejera de Medio Ambiente y Política Territorial, por la que se modifica el Catálogo Vasco de Especies Amenazadas de Fauna y Flora Silvestre y Marina.

Conclusiones y propuesta de gestión de las poblaciones y especies de anfibios y reptiles alóctonos en España

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Key words: herps, Spain, exotic species, management.

El criterio fundamental para considerar que una especie es alóctona en el territorio español se basa en la constatación de que la colonización no ha sido natural, sino de origen antrópico. Según este criterio, debemos considerar como poblaciones alóctonas la mayoría de las tratadas en este volumen, con algunas excepciones como *Testudo graeca* en el SE Ibérico. Existen dudas razonables sobre las poblaciones de *Hyla meridionalis*, *Emys orbicularis* y *Chamaeleo chamaeleon* del suroeste ibérico, especies / poblaciones que temporalmente consideramos criptogénicas, es decir, de origen desconocido en el área de estudio. Planteamos en los tres casos la necesidad de nuevos estudios que aporten evidencias más concluyentes sobre su carácter autóctono o alóctono. Igualmente alentamos la realización de nuevos estudios en la población de *T. graeca* en Doñana, enclave donde sería interesante estudiar los efectos en la población de la hibridación entre la subespecie *Testudo graeca marokkensis*, introducida a mediados del siglo pasado, y la subespecie *Testudo graeca graeca*, con presencia anterior en este espacio.

Entre los anfibios y reptiles considerados alóctonos, se ha constatado el impacto que algunos de ellos producen sobre la biota nativa, en forma de competencia con especies de parecido nicho ecológico, depredación, hibridación o transmisión de enfermedades, entre otros procesos. Dichas especies / poblaciones deben ser consideradas invasoras en el territorio donde han sido introducidas, y por tanto, se recomienda su exclusión de los catálogos de especies protegidas, tanto a nivel nacional como autonómico (según cada caso). Sería aconsejable que las diferentes administraciones fomentaran acciones de erradicación de dichas poblaciones cuando fuera razonablemente posible en base a criterios de expertos.

En determinadas especies alóctonas para las cuales no se ha detectado un impacto negativo en las especies nativas, además de su exclusión de los catálogos de especies protegidas, alentamos la realización de estudios de seguimiento en dos frentes: 1) anotar posibles cambios en su rango de distribución o su tamaño poblacional mediante trabajo de campo y modelos de distribución proyectados para el futuro; y 2) examinar la interacción con especies nativas (en la forma de competencia, depredación, transmisión de enfermedades, hibridación y alteración del medio).

Finalmente, se propone la protección de algunas poblaciones alóctonas o con dudas sobre su origen autóctono / alóctono. Tal es el caso de *Testudo hermanni* en Mallorca y Menorca, donde la especie se mantiene en buenas condiciones en contraste con la muy amenazada población de l'Albera, única localidad donde es nativa en toda la península ibérica. De esta manera, la protección de esta población introducida quedaría justificada al tratarse de un stock reproductivo del linaje ibérico de esta tortuga. En el caso de *T. graeca*, los estudios moleculares han sugerido el carácter alóctono de la población de Mallorca, por lo que no creemos necesaria su consideración en el CEEA ni en el catálogo balear. Sin embargo proponemos mantener en el catálogo andaluz a la población de *T. graeca* en Doñana, donde goza de un hábitat natural continuo y muy protegido, muy en contraste con el resto de poblaciones de tortugas de tierra en la península ibérica. Finalmente, sí consideramos que debiera mantenerse a la especie a nivel nacional en el LESRPE con el fin de limitar las actividades relativas a la tenencia en cautividad de tortugas, y como medida de conservación expresa de la población autóctona de *T. graeca* del sureste ibérico.

En resumen, las propuestas de gestión para cada una de las poblaciones y especies tratadas en esta revisión (Tabla 1), bajo el criterio colegiado de la Asociación Herpetológica Española, se concretan en cinco tipos:

- Erradicación: el impacto de la especie introducida sobre la biota nativa es importante.
- Seguimiento: hay sospechas de que la especie introducida puede alterar algún componente de los sistemas nativos.
- Nuevos estudios: son necesarios estudios que conduzcan a un mejor conocimiento del carácter autóctono o alóctono de la especie.
- No acción: hay evidencias de que se trata de una especie introducida, pero no hay aparentemente impacto negativo sobre la biota nativa.
- Protección: a pesar de ser una especie introducida, por diversas razones biológicas y culturales, se propone su protección.

No proponemos acciones de control (mantenimiento de las poblaciones dentro de un límite geográfico o poblacional), pues implica una actuación permanente, más costosa a largo plazo que la erradicación, y con el riesgo de perder todo el esfuerzo en los casos de cese temporal de esta gestión. Concluimos esta revisión con una propuesta muchas veces repetida, pero nosotros lo haremos aquí una vez más, por su importancia: en la gestión de las introducciones biológicas, la mejor política es evitar que se produzcan.

Tabla 1: Lista de especies alóctonas en España y propuestas de gestión.

Nombre científico	Introducida en	Propuesta
Mesotriton alpestris	Macizo de Peñalara	Erradicación
Discoglossus pictus	Península ibérica (NE)	Seguimiento
Bufotes balearicus	Mallorca	No acción
Bufotes balearicus	Menorca	No acción
Bufotes balearicus	Ibiza	No acción
Hyla meridionalis	Menorca	Nuevos estudios
Hyla meridionalis	Islas Canarias	No acción
Hyla meridionalis	Península ibérica	Nuevos estudios
Pelophylax perezi	Mallorca	Erradicación
Pelophylax perezi	Menorca	No acción
Pelophylax perezi	Ibiza	No acción
Pelophylax perezi	Islas Canarias	No acción
Pelophylax saharicus	Gran Canaria	No acción / Extinta
Mauremys leprosa	Islas Baleares	No acción
Emys orbicularis	Islas Baleares	No acción
Testudo graeca	Doñana	Nuevos estudios
Testudo graeca	Mallorca	No acción
Testudo hermanni	Mallorca	Protección
Testudo hermanni	Menorca	Protección
Chamaeleo chamaeleon	Península ibérica	Nuevos estudios
Hemidactylus turcicus	Península ibérica	No acción
Hemidactylus turcicus	Islas Baleares	Seguimiento
Hemidactylus turcicus	Islas Canarias	Erradicación
Tarentola mauritanica	Península ibérica (costa)	No acción
Tarentola mauritanica	Islas Baleares	Seguimiento
Chalcides viridanus	La Palma	Erradicación
Chalcides sexlineatus	La Palma	Erradicación
Gallotia atlantica	Gran Canaria	Erradicación
Gallotia galloti	Fuerteventura	Erradicación
Gallotia stehlini	Fuerteventura	Erradicación
Teira dugesii	Gran Canaria	Erradicación
Podarcis pityusensis	Euskadi	Erradicación
Podarcis pityusensis	Mallorca	Seguimiento
Podarcis sicula	Menorca	Seguimiento
Podarcis sicula	Cantabria	Erradicación
Podarcis sicula	Almería	Erradicación
Podarcis sicula	Rioja	Erradicada
Podarcis sicula	Cataluña	Erradicada
Psammodromus algirus	Mallorca	Seguimiento
Scelarcis perspicillata	Menorca	Seguimiento
Macroprotodon cucullatus	Mallorca y Menorca	No acción
Rhinechis scalaris	Menorca	No acción
Rhinechis scalaris	Mallorca	Erradicación
Rhinechis scalaris	Ibiza	Erradicación
Rhinechis scalaris	Formentera	Erradicación
Hemorrhois hippocrepis	Mallorca	Erradicación
Hemorrhois hippocrepis	Ibiza	Erradicación
Hemorrhois hippocrepis	Formentera	Erradicación
Malpolon monspessulanus	Mallorca	Erradicación
Malpolon monspessulanus	Ibiza	No acción / Extinta
Natrix maura	Mallorca	Erradicación
Natrix maura	Menorca	No acción