UNIVERSITAT DE BARCELONA

La invasió de *Gambusia holbrooki* en ecosistemes eutrofitzats.

Dels mecanismes ecològics a la seva gestió

Oriol Cano Rocabayera

Aquesta tesi doctoral està subjecta a la llicència ***Reconeixement- NoComercial 3.0. Espanya de Creative Commons***.

Esta tesis doctoral está sujeta a la licencia ***Reconocimiento - NoComercial 3.0. España de Creative Commons.***

This doctoral thesis is licensed under the ***Creative Commons Attribution-NonCommercial 3.0. Spain License.***

Les invasions biològiques d'espècies exòtiques es troben íntimament lligades a l'activitat humana, des de l'etapa inicial d'introducció en el nou hàbitat fins a l'establiment i expansió. La cerca dels factors ecològics i socioeconòmics que fan que una espècie introduïda esdevingui invasora i provoqui un impacte és l'objectiu fonamental de la ciència de les invasions biològiques. En aquesta tesi s'explora la invasió en ecosistemes eutrofitzats d'un petit peix nord-americà introduït a Europa el 1921 per al control biològic de mosquits, la gambúsia (*Gambusia holbrooki*). Mitjançant estudis de laboratori i de camp, s'intenta esbrinar si l'eutrofització per l'excés de nitrats estaria entre les causes que promouen l'èxit invasor de *G. holbrooki*. Aquesta informació permetria estimar la importància relativa de l'excés de nutrients entre el conjunt de factors ambientals que afavoreixen l'espècie, per tal d'ajudar els gestors del medi natural a contenir els seus efectes negatius sobre la biodiversitat nativa mitjançant una aproximació que tingui en compte el conjunt de l'ecosistema.

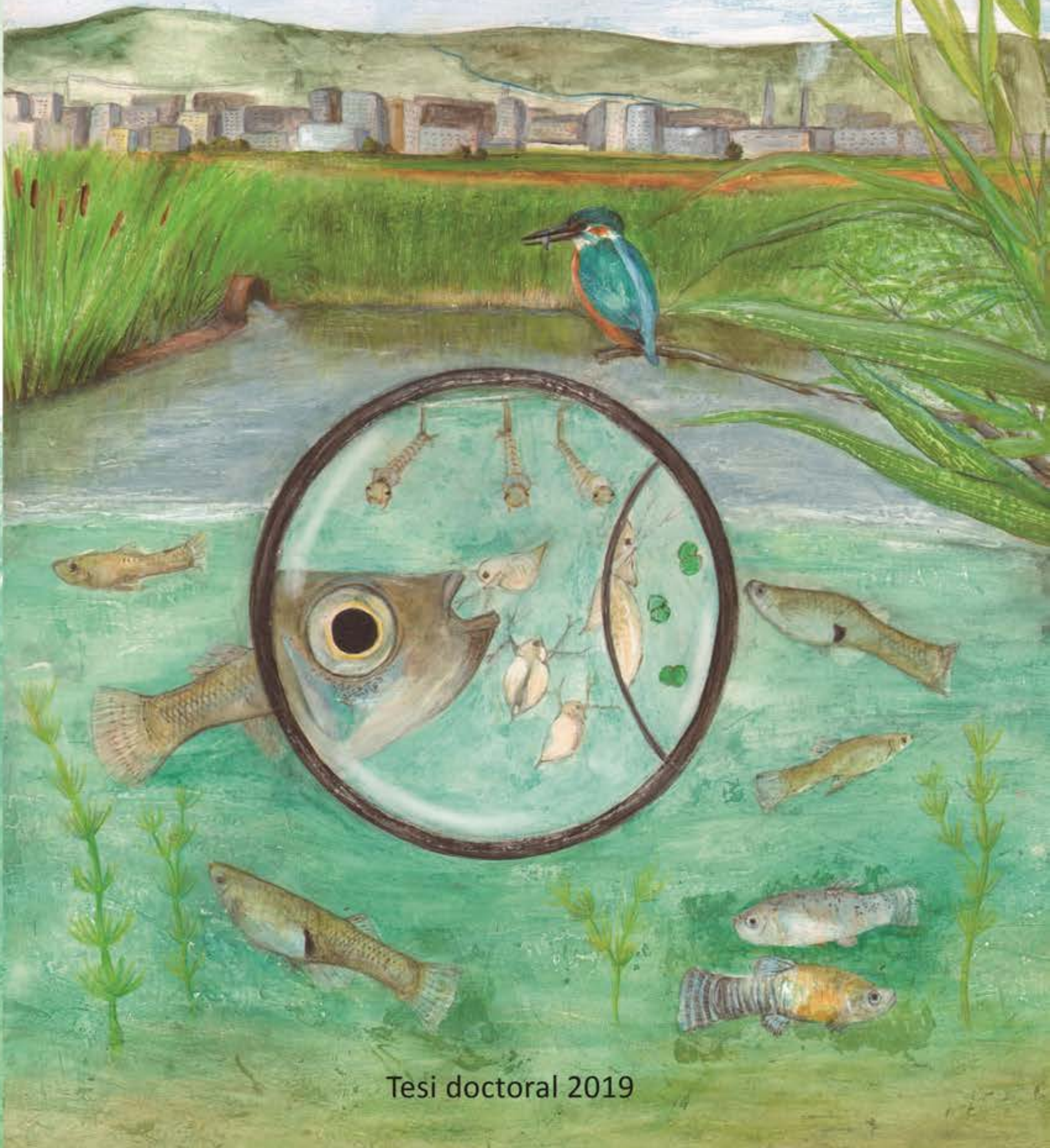
La invasió de *Gambusia holbrooki* en ecosistemes eutrofitzats

Oriol Cano Rocabayera

La invasió de *Gambusia holbrooki* en ecosistemes eutrofitzats

Dels mecanismes ecològics a la seva gestió

Oriol Cano Rocabayera



Tesi doctoral 2019

Departament de Biologia Evolutiva, Ecologia i Ciències Ambientals

Programa de Doctorat en Biodiversitat HDK04

La invasió de *Gambusia holbrooki* en ecosistemes eutrofitzats.

Dels mecanismes ecològics a la seva gestió

Memòria presentada per Oriol Cano Rocabayera per optar al grau de doctor per la Universitat de Barcelona

Barcelona, juliol de 2019

El doctorand

Oriol Cano Rocabayera

El director i tutor de tesi

Dr. Adolfo de Sostoa Fernández

El director de tesi

Dr. Alberto Maceda Veiga

Oriol Cano Rocabayera (2019). La invasió de *Gambusia holbrooki* en ecosistemes eutrofitzats. Dels mecanismes ecològics a la seva gestió. Tesi doctoral, Universitat de Barcelona, 218 pp.

Il·lustració de la portada i la contraportada: Irene Jiménez Blasco
Il·lustracions interiors: Adolfo de Sostoa Fernández

A la meva família i amics propers

Agraïments

Qui podria imaginar, quan vaig picar la porta del Departament de Biologia animal aquell agost de 2010 per preguntar si hi havia la possibilitat de fer alguna col·laboració, que estaria escrivint finalment aquest text. Ha anat passant i entrant molta gent, fins i tot ha canviat el nom del Dept, però aquestes línies faran palès el meu sincer agraïment durant l'aprenentatge de tot aquest temps. En primer lloc al meu director, l'Adolf de Sostoa, volia agrair la confiança i suport dipositats en aquest llarg camí, pels bons records que m'emporto del treball de camp i per l'estima a la ictiologia, el dibuix i la docència. Si no hi ha sorpreses seré dels últims alumnes així que molta sort en aquesta nova etapa. A l'altre director i amic, l'Alberto Maceda, que ha estat el far i part fonamental en l'arquitectura d'aquest treball, en la tria dels ingredients dipositats i en la cocció a foc lent però segur, i sempre amb bon ambient. Espero poder seguir el vincle i que trobis aviat l'estabilitat necessària. Sense sortir del grup, un agraïment sincer als companys i amics amb els quals he compartit laboratori, camp i moltes més coses, en especial per a en Mario Monroy, "profe" en molts aspectes excepte en anàlisi futbolística i amb qui he compartit vàries aventures als dos costats del *charco*, la Nicole Colin amb el seu esforç, dedicació i predisposició a ajudar i en Sergi Vargas, "bitxòleg" per excel·lència i company i copilot de fatigues en l'atrotinat Suzuki. Finalment, no puc oblidar els diferents estudiants de grau i màster que han donat un cop de mà, imprescindibles per tirar endavant aquesta tesi i que massa sovint queda diluïda la seva empremta. En Lluís Coll "Purito", les incansables Clàudia Pastor, Daniela Díaz i Marina Vergotti, l'Alessandro di Peso, l'entusiasta Joan Vilumara, el flamant nou doctorand Gustavo Ceballos i la Lorena Cárdenas, als qui desitjo molta sort. També per descomptat al David Ansón, que probablement ostenta el rècord d'hores visualitzant vídeos de gambúsies.

Sense sortir de l'àmbit acadèmic vull agrair també als professors que han participat d'alguna manera en aquest treball, la Isabel Muñoz, per la seva ajuda, qualitat personal i coneixement d'ecologia, l'Humbert Salvadó, per fer gran el que sembla petit, i al Francesc Padrós, per la seva tutoria i acollida en la Facultat de Veterinària de la UAB. Finalment agrair a en Xavier Remesar i la Mercè Durfort, per l'acollida i ensenyaments en calorimetria i histologia, en Jordi Guinea i l'equip de l'estabulari humit, imprescindibles per a realitzar l'experimentació animal, i als tècnics de laboratori, Jordi Correas, Jessica Borrego, Cèlia Arcarons, Pilar Rubio, Regina Roca, i el personal de la Unitat de Medi Ambient dels CCiTUB. Menció apart es mereix el grup del Servei de Control de Mosquits, en concret en Carles Aranda, i el Francesc X. Santaeufèmia del Consorci per a la protecció dels espais naturals del delta del Llobregat per les seves aportacions i coneixement sobre el terreny necessari per realitzar en conjunt més de la meitat de capítols d'aquesta tesi.

Sortint de la tesi, perquè no tot és treballar, volia tenir un record per als companys de departament, tant la "vella guàrdia" que vaig agafar de resquitllada, és a dir, els supercracks Manolo García, Laura Stefan i Eloy Gálvez, la Blanca Figuerola (visca Reus!), en Raül Ramos i en Fran Ramírez, el racional Edu Galea, la Vero Cortés i la Teresa Militão, l'Olatz San Sebastián i en Marc Franch (en especial també per l'acollida a Porto), en Marc López "Batmarc" i la Noëlle Fabre; com els més "contemporanis", Marta Cruz, Fernanda Pereira, Morghana Vighi, Francesc "kazakh" Cuscó, José M. de los Reyes, Carolina Quirós, Laura Zango, Virginia Morera, Eli Cortés, Virginia Morera, Irene Jiménez (realitzadora de la portada), Patricia Campos, tocayo Oriol Canals, Felipe "tranquilo", Pol Pintanel i Sofía Salinas, Javi de Salamanca, Iván "merengue" i Zuzana Zajkova. I també els que vénen darrere, però malauradament cada cop menys: el comando EPIF Paula de Castro i Raquel Vernet, Marc Domènech "Mr. Bugs", Sarah Saldanha, Júlia Alcaraz, els inquiets Álvaro i Mariona Sardà i en Moisès Sánchez.

Persones que han fet més agradable l'estada en aquests nou anys a la facultat, en especial pel seu interès en el meu progrés, destacaria la Dolors Vinyoles, amb qui he compartit pesques en mars i rius, les històries curioses i l'afabilitat del Xavier Ferrer, d'en Domingo Rodríguez i d'en Jacint Nadal, els acudits de l'Eduardo Mateos, d'en Joan Real i en Toni Hernández, i en Lluís Cardona. A en Lluís Jover, que malauradament ja no està aquí. També els de l'entresol, el museu de zoologia, les bones fotògrafes Helena Basas i la Mònica Utjés, amb qui he gaudit d'unes excel·lents excursions naturalistes pel territori, i en Toni Serra; i els del pis de dalt, la Marta Goula, les històries de navegants d'en Manuel Ballesteros i la Creu Palacín; i els de l'àtic, en Quico Sabaté i la Núria Cid, i l'Eli Ballester a les llargues hores de dinar. Una salutació també per tota la gent de Silwood Park, especialment en Dan Perkins, en Guy Woodward, l'Éoin O'Gorman i la colla d’italians, i en Michael Ahrens i companyia de la Universitat J. Tadeo Lozano de Bogotà que van ajudar a fer més fàcils les estades a l’estranger.

Finalment, però no menys important, agraeixo a la gent de sempre, que han recolzat abans i durant aquesta tesi, és a dir, tots els amics de Sant Adrià, els *tronners* de ciències ambientals i sobretot a la meva família, tant els que veig cada dia com els que veig no tan sovint, els quals m’han inculcat aquesta curiositat que han permès acabar aquesta feina.

Ah, me n’oblidava! Els pobres peixos que van haver d’aguantar els llargs experiments.

Sinopsi

Invasive species, together with other perturbations like nutrient enrichment, threaten native biodiversity worldwide. This thesis is framed within the context of the invasion of a small fish, the eastern mosquitofish *Gambusia holbrooki* (Girard, 1859) in ecosystems subject to environmental degradation due to excessive fertilisation with nitrates. By means of experimental and field observational studies, the hypothesis of whether eutrophication mediates the invasive success of *G. holbrooki* is explored. Specifically, the initial aim is to understand the ecological mechanisms exerted by fish introduction and nitrate pollution in altering the native communities, the ecological processes and their interaction. Nutrient enrichment causing eutrophication, but also physical habitat quality, abundance of predators and parasites prevalence could influence the life history and the invasive success of *G. holbrooki*. Should the invader fitness be mediated by any factor, managers could carry out an ecosystem approach by favouring such desired conditions against the invader success. Either by direct elimination or through an ecosystemic approach, the loss of *G. holbrooki* populations should be substituted by other native species, such as *Aphanius iberus* and provide a similar mosquito control efficiency in clean and eutrophic waters. Results indicate a negligible interaction between the impacts exerted by both fish introduction and nitrate pollution on the recipient ecosystem. The loss of fish biomass in high nitrate concentration would be attributed to indirect effects such as a decline in prey abundance, rather than a direct alteration on fish physiology. This low susceptibility of *G. holbrooki* to nitrates, especially in females, confirms its high tolerance to a wide gradient of environmental conditions reported by previous authors. Moreover, the low prevalence of parasites and the high variability of life history traits, mainly attributed to *G. holbrooki* density in the pond over other environmental factors leave managers with low chances of controlling the invader apart from its eradication. Given the similar but slower efficiency of the native community both in eutrophic and clean waters in reducing mosquito abundances and the difficulty of eradicating *G. holbrooki* once established, favouring the presence of native competitors and preventing further introductions of *G. holbrooki* for biological control seems a priority.

Índex

INTRODUCCIÓ GENERAL

"Pare, digueu-me què li han fet al riu, que ja no canta"
Joan Manuel Serrat, Pare

La globalització, definida com la integració del conjunt d'economies locals en un únic mercat global amb una mínima regulació, ha multiplicat per deu l'intercanvi de béns i serveis els últims 50 anys (Guttal, 2007; Díaz et al., 2019). Una de les conseqüències d'aquest moviment és la introducció d'espècies exòtiques, entesa com el trasllat per via humana d'una espècie des de la seva regió d'origen cap a una altra fora del seu rang natiu (Kolar i Lodge, 2001). Algunes introduccions poden ser de forma accidental, com el mosquit tigre *Aedes albopictus* (Skuse, 1894), que vingué en el transport de pneumàtics reciclats (Aranda et al., 2006), o deliberada, com la perca del Nil *Lates niloticus* (Linnaeus, 1758) alliberada per contrarestar la pèrdua d'estocs de pesca al llac Victòria (Lowe, 2000).

Una espècie exòtica es considera invasora quan s'estableix fora del seu rang de distribució històric i causa impactes sobre la biodiversitat nativa fruit de la manca d'un context de coevolució (Fig. 1; Kolar i Lodge, 2001). La majoria d'introduccions fracassen durant l'establiment i expansió en l'ecosistema receptor, però, en alguns casos, tenen èxit i completen les quatre etapes, i és l'anàlisi dels factors que el determinen l'objecte d'estudi de la ciència de les invasions biològiques (Richardson i Ricciardi, 2013). Així mateix, donat que no és l'espècie la que esdevé invasora, sinó una població, hom considera més adient emprar el terme població invasora que no el d'espècie invasora. Per exemple, la llamprea de mar *Petromyzon marinus* (Linnaeus, 1758) és una espècie amb poblacions natives en regressió a la península ibèrica i invasora a la regió dels Grans Llacs nord-americans (Hansen et al., 2016). La taxa global d'introduccions amb èxit ha incrementat des de 1950, arribant a valors que multipliquen per 60 la taxa històrica preindustrial (Seebens et al., 2017). D'aquestes introduccions, el petit percentatge que esdevenen invasores, juntament amb factors com la degradació dels hàbitats i el canvi climàtic, representa una amenaça per a la conservació de la biodiversitat (la taxa d'extinció actual és fins a 100 vegades superior a la taxa dels últims 10 milions d'anys), però també per la salut i l'economia (Díaz et al., 2019).

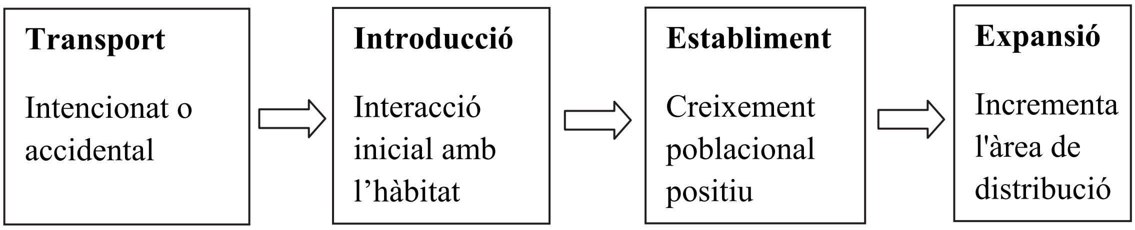


Figura 1. Etapes d'invasió d'una espècie exòtica en un nou hàbitat receptor.

La ciència de les invasions biològiques és relativament recent i es parla de "ciència" perquè inclou factors sociològics i econòmics, a més dels ecològics, que en determinen l'èxit invasor d'una espècie exòtica i els seus impactes. Ara bé, el concepte d'invasió biològica és molt antic; Charles Darwin (1089 – 1882) i Alfred R. Wallace (1823 – 1913) ja parlaven de la naturalització d'espècies exòtiques, tot i que s'atribueixen els fonaments de la disciplina al llibre 'The Ecology of Invasions by Animals and Plants' de Charles S. Elton (1900 – 1991):

> *Un sistema ecològic, com qualsevol comunitat humana organitzada, té els seus propis centres d'acció – com el sòl, el cobricel arbori, l'aiguamoll i el rierol, el tronc caigut i el niu d'ocell – però sempre en algun punt hi pots trobar connexions entre tots ells, i això podria afectar l'equilibri entre poblacions. L'invasor es troba per tant obrint-se pas com pot dins un sistema complex, tal com un emigrant intenta trobar una feina, una casa i començar una família en un nou país o ciutat. La manera més curta de descriure aquesta situació [...] seria que l'invasor es troba amb una 'resistència ecològica'.*
>
> *C.S. Elton, 1958*

Aquest concepte seminal d'Elton fou la base de conceptes clau en biologia d'invasions formulats posteriorment, que giren al seu voltant: i) hipòtesi de la comunitat simple, que postula que és més fàcil envair comunitats pobres que riques en espècies (Elton, 1958; Johnstone, 1986). Això va molt relacionat amb la hipòtesi de nínxols[1] buits, els quals augmenten quan es perden espècies o se'n generen de nous per l'activitat humana, i que aprofiten les espècies introduïdes per a colonitzar. ii) hipòtesi de la mancança d'enemics, que lliga l'èxit invasor al fet que una espècie exòtica es troba "lliure" dels depredadors i

---

[1] C.S. Elton ho defineix com l'espai que ocupa un individu dins l'entorn biòtic, la seva relació amb l'aliment disponible i els depredadors. Per tant ho limita a un concepte tròfic. L'ecòleg G.E. Hutchinson (1903 – 1991) ho amplia a una sèrie de condicions ambientals i de recursos que defineixen els requeriments d'un individu per tal que la població persisteixi.

patògens que tenia a l'hàbitat d'origen (Keane i Crawley, 2002). A partir de les idees sorgides en l'obra d'Elton s'han proposat tres mecanismes diferents per explicar els patrons d'invasions, que no s'exclouen entre ells. En primer lloc, l'activitat humana, que concerneix a les quatre etapes de la invasió. Una pressió de propàguls [2] incrementada tant en nombre com en freqüència i l'efecte de les pertorbacions d'origen antropogènic facilitaran l'establiment d'una espècie no nativa (Leprieur et al., 2008; Chiron et al., 2009). En segon lloc, la resistència biòtica, que concerneix a l'establiment i l'expansió únicament. Les comunitats pobres en espècies albergaran un major nombre d'espècies exòtiques, ja que l'espècie no nativa es trobarà una competència menor en comparació amb una comunitat rica (Levine et al., 2004). Per últim, l'acceptació biòtica, que concerneix a l'establiment i l'expansió únicament, apareix en contraposició a les hipòtesis d'Elton. Se suggereix que l'establiment d'una espècie no nativa serà més probable en àrees riques en espècies natives perquè són zones riques en recursos i, per tant, hi ha més energia i espai físic disponibles (Stohlgren et al., 2006).

No hi ha cap metanàlisi o evidència experimental clara que donin un suport inequívoc per a cap de les tres hipòtesis. En el cas dels peixos continentals, Leprieur et al. (2008) trobaren que hi havia una estreta relació entre la biogeografia d'invasions i la intensitat de l'activitat humana exclusivament. Altres estudis han trobat el mateix patró per a altres grups d'animals i zones geogràfiques (Chiron et al., 2009; Jeschke i Genovesi, 2011). Però per altra banda, les altres dues hipòtesis han tingut recolzament a escales més petites o en altres grups taxonòmics (vegeu Levine et al., 2004; Fridley et al., 2007; Chen, 2012).

Aquesta tesi s'emmarca en el context d'una invasió biològica en ecosistemes sotmesos a forta degradació ambiental, en concret les zones humides[3] dels agroecosistemes del delta del Llobregat. Les zones humides han estat tradicionalment lligades al desenvolupament humà, però són també zones de gran valor ecològic amenaçades per l'activitat agrícola (Joosten, 2009). En alguns casos, el canvi d'usos de sòl tal com la dessecació de terres per a ús agrícola i com a mesura de control dels mosquits, vectors de malalties endèmiques com el paludisme, ha transformat substancialment l'ecosistema

---

[2] Nombre de vegades que s'allibera un nombre absolut d'individus d'una espècie en una regió no nativa.

[3] Zona humida o *wetland* en un sentit ampli. La definició del Conveni de Ramsar inclou llacs i rius, aqüífers subterranis, maresmes, torberes, estuaris i masses d'aigua modificades com embassaments i basses.

original. La modernització de l'agricultura ha suposat un increment en l'ús de fertilitzants, tot sovint sintètics (ex. sals de nitrogen), que arriben a les masses d'aigua per escorrentia superficial o percolació als aqüífers (Joosten, 2009). En conseqüència, els ecosistemes aquàtics experimenten eutrofització antròpica. La principal diferència amb l'eutrofització natural rau en la intensitat dels efectes i els canvis que experimenten les masses d'aigua, incloent un augment de la terbolesa i una reducció de la concentració d'oxigen durant la nit que, en casos extrems, suposa la mort de tota la comunitat biòtica. Així doncs, si ha aparegut una degradació ambiental, hom podria dir que la població invasora actua com a mer "passatger amb el bitllet comprat" en aquesta transició ambiental (Didham et al., 2005; MacDougall i Turkington, 2005). Però la població invasora pot, segons el context, exercir una pertorbació addicional en aquests ecosistemes prèviament degradats, alterant les interaccions entre les espècies natives i els serveis ecosistèmics que d'aquestes se'n deriven, per exemple el reciclatge de nutrients o el control de plagues (Didham et al., 2005). La identificació dels canvis en l'estructura de la comunitat i el funcionament de l'ecosistema per la presència de múltiples factors de degradació i com aquests indicadors es relacionen entre sí està molt poc estudiat en ecosistemes aquàtics.

La gestió d'invasions biològiques en ecosistemes "novells"

Un ecosistema novell és un terme de nou encuny, caracteritzat per ser un ecosistema on les condicions ambientals i la composició d'espècies han sofert un canvi tan marcat que no s'assemblen en res a les característiques que hi havia en un període històric recent (Seastedt et al., 2008; Moyle, 2014). Sempre ha existit una continuïtat en la modificació de les condicions dels ecosistemes, inclús la seva degradació, però actualment el gran canvi és que aquesta taxa d'alteració s'ha accelerat enormement, impulsada sovint per diverses pertorbacions actuant simultàniament (Díaz et al., 2019; Fig. 2). Per exemple, amb la construcció d'una represa en un riu es canvia d'un hàbitat lòtic a un altre lèntic, sovint acompanyat de l'alliberament d'espècies exòtiques relacionades amb la pesca (Moyle, 2014). Per tant es fa difícil comparar les noves condicions en quant a la seva biodiversitat i el seu funcionament amb l'estat de referència, és a dir, la variabilitat natural en un període i zona geogràfica concrets que no es troben afectats per l'activitat humana (Landres et al., 1999). A més, en zones altament desenvolupades és complicat trobar zones de referència (Moss et al., 2003; Bonada et al., 2006). Amb això no es vol donar la idea que la restauració per acostar-se al màxim a la situació de referència sigui impossible, o que es doni per bo un increment en la tolerància a la degradació (Soga i Gaston, 2017).

La gestió d'espais naturals comprèn des de polítiques de *laissez-faire*, és a dir, de mínima intervenció independentment de l'impacte, fins a les polítiques molt intervencionistes. Aquestes últimes tradicionalment s'han centrat en el manteniment o restauració dels ecosistemes a l'estat pre-impacte com podria ser la revegetació d'una pedrera abandonada o l'eradicació d'una espècie exòtica (Seastedt et al., 2008). En ecosistemes degradats, però, la semblança amb el seu estat natural històric és tan baix que les mesures de restauració desitjades tenen més possibilitats de fructificar si l'objectiu fixat és realista i es té un coneixement profund de l'ecologia del medi i les seves característiques socioeconòmiques (Moyle, 2014). En aquests hàbitats enormement alterats, el fet d'eradicar una espècie invasora o millorar la qualitat de l'aigua, però mantenint un règim hidrològic alterat, pot suposar que l'ecosistema segueixi sense recuperar-se (Fig. 2). Concretament, en determinades situacions com en el cas de vertebrats aquàtics de mida petita en coexistència amb múltiples fonts de degradació, la probabilitat d'èxit en l'eradicació i la restauració de l'hàbitat és baixa (Prior et al., 2018).

Així doncs, malgrat les invasions biològiques estan entre les causes de pèrdua de biodiversitat del planeta (MEA, 2005; Díaz et al., 2019), l'eradicació d'espècies exòtiques d'àmplia distribució no és factible i només resta aplicar estratègies de control o mitigació dels seus impactes a nivell local (Norton, 2009). A més, quan tenim una població exòtica establerta des de fa molts anys, aquesta pot estar fortament integrada en la comunitat nativa, pot haver-hi establert interaccions de competència o depredació, i tenir un paper funcional rellevant fins i tot per les espècies natives (Zavaleta et al., 2001; Carroll, 2011). En aquests casos l'òptima gestió hauria d'actuar no només en el control de l'expansió de la població invasora, sinó analitzant el context ecològic per evitar que apareguin "sorpreses ecològiques" derivades d'una cascada tròfica (Caut et al., 2009). L'eliminació de l'espècie exòtica tindria un efecte negatiu sobre depredadors natius si aquests depenen en gran mesura d'aquest recurs, o sobre altres espècies natives del mateix nivell tròfic si els depredadors canvien de presa (Zavaleta et al., 2001). Aquesta aproximació ecosistèmica no només pot preveure efectes inesperats, sinó que es pot utilitzar com a pròpia eina de control de la població invasora si es coneixen les condicions ambientals que redueixin el seu èxit invasor. Ara bé, no s'ha d'oblidar que la millor estratègia de maneig de les espècies invasores és la prevenció, possiblement a través de llistats que prohibeixin la importació d'espècies amb elevat risc d'invasió i amb programes d'educació ambiental (Hulme et al., 2006).

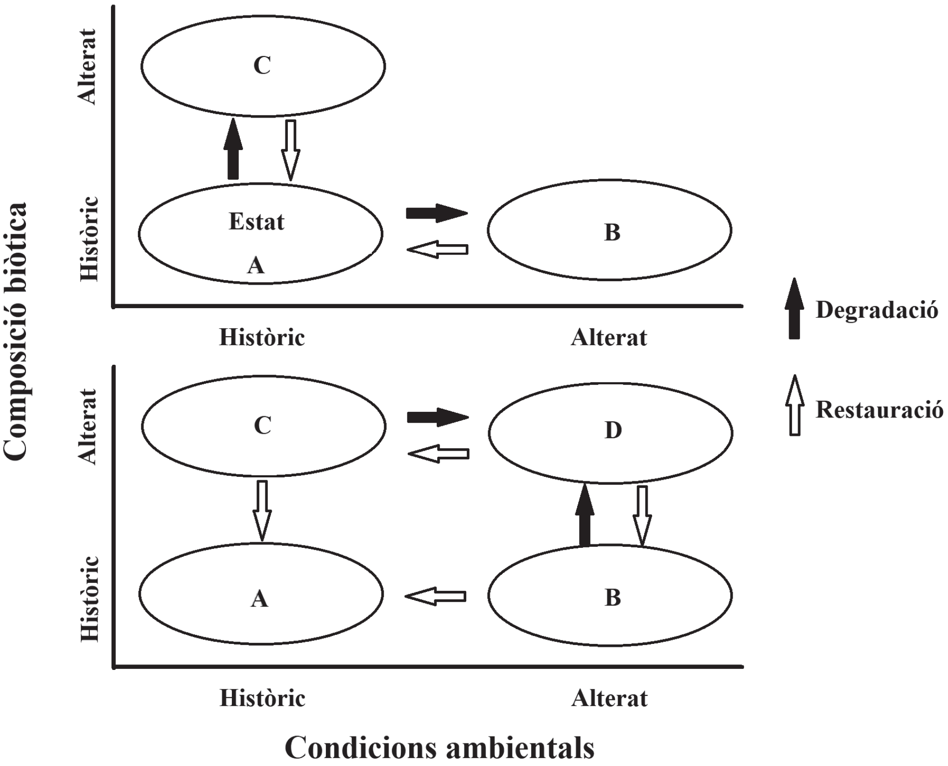


Figura 2. Alteracions en les condicions ambientals i la composició biòtica. Un ecosistema, amb la seva variabilitat natural (encaixada dins l'el·lipse A), que per l'acció d'un factor ambiental (A → B), o per l'addició o pèrdua d'una espècie (A → C) pateix una degradació. Un cop estem en el nou estat (B o C), l'addició de noves alteracions biòtiques o abiòtiques, o la mateixa reestructuració interna (pèrdua d'espècies perquè no poden tolerar les noves condicions ambientals, o bé la degradació de l'hàbitat per la pèrdua d'una espècie clau en el funcionament de l'ecosistema), poden portar cap a un estat encara més degradat (D). Per tornar a l'estat inicial (A), és probable que s'hagin d'adreçar mesures de restauració que tinguin en compte tant la composició d'espècies com les condicions ambientals. El fet de dur a terme una mesura que no tingui en compte el conjunt de l'ecosistema, per exemple eradicar una espècie invasora sense més, pot portar a un estat (B) que no era l'objectiu inicial de la restauració (A). Adaptat de Seastedt et al. (2008).

La invasió de la gambúsia *Gambusia holbrooki* (Girard, 1859)

Aquesta tesi utilitza la invasió per *G. holbrooki* com a exemple d'invasió difícil de gestionar un cop l'espècie s'ha establert i expandit pel territori. Originària de Nord-amèrica, *G. holbrooki* és un peix d'aigua dolça de la família dels pecílids, de petita talla, com a màxim 35 mm en mascles i 60 – 70 mm en femelles, de color platejat sobretot en la zona ventral i una tonalitat gris – verdosa la resta del cos (Vargas i de Sostoa, 1996;

Pyke, 2005). Té una sola aleta dorsal, i una aleta caudal arrodonida. El dimorfisme sexual, a part de la talla, és molt evident (Fig. 3). Els mascles tenen gonopodi, que és l'aleta anal modificada en forma d'òrgan copulador (Meffe i Snelson, 1989). És una espècie ovovivípara, o sigui, els ous fecundats romanen a l'interior de la femella fins a descloure. La mida de camada típica d'una femella és de 5 – 100 juvenils, la qual depèn en gran part de la mida de la femella i la disponibilitat d'aliment (Meffe, 1986; Vargas i de Sostoa, 1996). Es tracta d'una espècie molt prolífica, amb camades cada tres o quatre setmanes i juvenils que maduren sexualment a l'edat d'entre tres i deu setmanes (Meffe, 1992). La longevitat és curta, típicament menys de 3 anys en femelles i menys de 2 anys en mascles (Vargas i de Sostoa, 1996). Les poblacions tenen una proporció de sexes, tot i que amb excepcions temporals, esbiaixada cap a les femelles amb una mitjana d'un mascle per cada quatre femelles (Hildebrand, 1927; Vargas, 1993).

És una espècie ecològicament generalista, però amb una preferència per aigües someres i càlides (31 – 35 ºC), de corrent nul·la o molt baixa, amb una bona presència de vegetació submergida (Pyke, 2005) i moderada salinitat (< 20 ‰, Chervinski, 1983; Alcaraz i García-Berthou, 2007). A més, han esdevingut molt abundants en masses d'aigua properes a nuclis urbans, per exemple en estanys urbans, basses i canals de zones agrícoles, els quals solen tenir problemes de pol·lució (Lloyd et al., 1986; Arthington i Lloyd, 1989).

La seva dieta inclou artròpodes terrestres que reposen o cauen en la superfície aquàtica, crustacis, oligoquets, nematodes, mol·luscs, larves i pupes d'invertebrats aquàtics, algues i detritus, diatomees i petits peixos, incloent-hi juvenils de la mateixa espècie (Vargas, 1993; García-Berthou, 1999). Experimentalment s'ha demostrat que es poden alimentar també d'ous i larves d'amfibi o causar serioses lesions a aquests o d'altres espècies natives de petita mida (Pyke, 2005; Preston et al., 2018), tot i que els seus impactes sobre el funcionament dels ecosistemes encara són força desconeguts (vegeu Hinchliffe et al., 2017).

Figura 3. Femella i mascle adults de *Gambusia holbrooki*. Fotografies cortesia de Chris Appleby, Fishbase.org.

El rang de distribució natural de *G. holbrooki* és la costa est dels Estats Units, des de la costa de Nova Jersey fins a Florida (Rosen i Bailey, 1963). No obstant, tant l'espècie d'estudi *G. holbrooki* com la seva espècie germana, *Gambusia affinis* (Baird & Girard, 1853), han estat àmpliament distribuïdes per tots els continents excepte l'Antàrtida. La invasió de *G. holbrooki* té el seu origen en el control biològic de mosquits en zones de presència endèmica del paludisme (Hildebrand, 1919; De Buén i De Buén, 1922). No obstant, gairebé no hi ha dades quantitatives sobre l'eficàcia de *G. holbrooki* en el control de mosquits en condicions ambientals variables, així que la seva introducció arreu del món podria haver estat innecessària, tal i com albiraren ictiòlegs de l'època (De Buén, 1929). Malgrat ser extensa la literatura sobre com afecten els canvis ambientals a la biologia de *G. holbrooki* (Taula A2 a l'annex), és força desconegut com li afecta l'eutrofització, força comú a les masses d'aigua on prolifera una de les espècies de mosquit de més àmplia distribució: el mosquit comú *Culex pipiens* (Linnaeus, 1758).

El nitrogen: de nutrient a contaminant

La producció primària depèn dels compostos de nitrogen, entre d'altres nutrients, que estan al medi natural com a sals inorgàniques. Les formes principals de nitrogen inorgànic són l'amoni, tant ionitzat (NH_4^+) com no ionitzat (NH_3), el nitrit (NO_2^-) i el nitrat (NO_3^-), tots ells presents a molt baixa concentració als ecosistemes aquàtics tret que hi hagi fertilització antròpica, superi la seva capacitat d'assimilació natural i causi eutrofització (Taula 1). En condicions naturals la concentració dels diferents compostos de nitrogen ve determinada pel cicle del nitrogen (Fig. 4). Els microbis heteròtrofs (ex. bacteris, fongs) degraden la matèria orgànica com femtes i restes vegetals i generen NH_3 que s'oxida a NO_2^- i NO_3^- per acció dels bacteris nitrificants. Els dos primers compostos

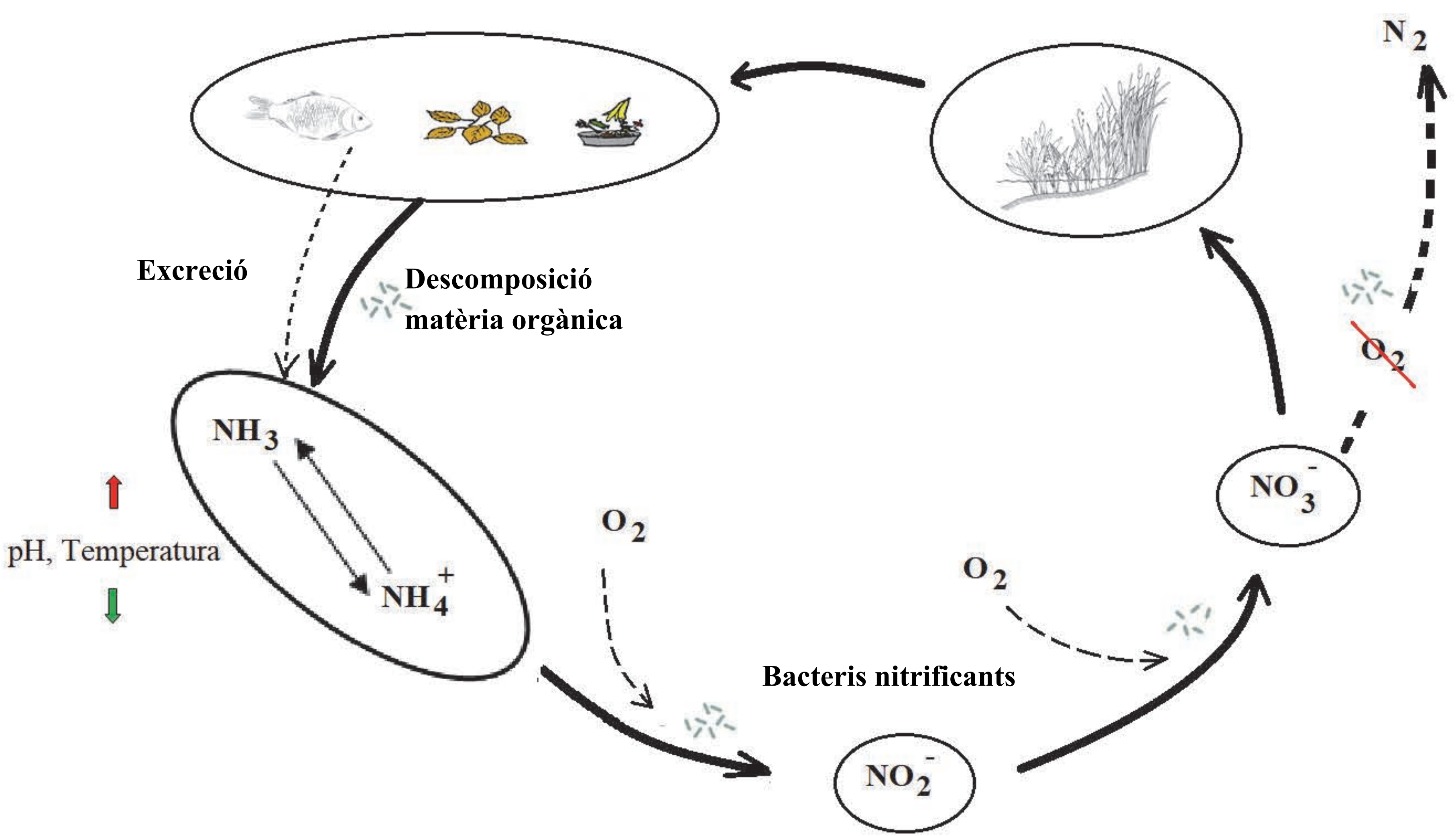


Figura 4. Fonts principals d'entrada i sortida de nitrogen en un ecosistema aquàtic.

són molt tòxics per a la fauna aquàtica a baixes concentracions [4] (ex. 1 mg/l) i, a més, donat que la seva oxidació és ràpida, fins i tot a baixa concentracions d'oxigen, la seva presència indica un vessament recent d'aigües amb elevat contingut de matèria orgànica (Jensen, 1995). Per contra, el nitrat és més estable en el medi i s'acumula, tret que sigui assimilat per plantes i algues, o hi hagi desnitrificació, és a dir, una reducció a nitrogen molecular (N_2) en condicions anòxiques (Jensen, 1995; Camargo et al., 2005). De fet, la seva concentració màxima permesa és de 50 mg/l, dos ordres de magnitud més alt que el recomanat per al nitrit (Directiva 91/676/CE; Noga, 2010). Per tant, els organismes aquàtics han desenvolupat més tolerància a la seva presència, que pot arribar a ser excepcionalment superior a 300 mg/l al medi natural o de 2000 mg/l en aigües de piscifactoria (Honda et al., 1993; ACA, 2014; Grup de Defensa del Ter, 2019).

Taula 1. Principals fonts de nitrogen d'origen antròpic, que pot arribar als ecosistemes principalment per dues vies. Adaptat de Camargo i Alonso (2006).

Fonts puntuals

Aigües residuals procedents de l'activitat ramadera.

Emissions de la indústria d'aqüicultura.

Efluents d'aigües residuals, procedent tant de les estacions depuradores d'aigües residuals (EDAR) com de zones industrials.

Desbordament de clavegueres en episodis de pluja torrencial.

Fonts difuses

Cultiu de plantes fixadores de nitrogen.

Ús de fems i fertilitzants de nitrogen inorgànics (ex. salnitre) en agricultura, i la posterior escorrentia.

Escorrentia procedent de zones urbanes, zones d'abocaments de residus, lixiviats sèptics i de zones d'activitat minera.

Nitrogen emmagatzemat en aigües subterrànies que alimenten les aigües superficials (rius, llacs, estuaris).

Emissions a l'atmosfera per volatilització dels fertilitzants o la crema de combustibles i la posterior deposició atmosfèrica.

Mobilització de nitrogen emmagatzemat en ecosistemes que funcionen com embornals de nitrogen, com la tala i crema de boscos i dessecació de zones humides.

---

[4] En aquest treball totes les concentracions es donen en mg/l total, per exemple mg NH_4^+/l, en contrast amb la bibliografia nord-americana on s'acostuma a donar en mg N-NH_4^+/l.

Les administracions públiques han desenvolupat diferents mecanismes per controlar els valors de nitrats en aigües superficials, subterrànies i de consum humà per tal de protegir la fauna aquàtica però sobretot per garantir la salut pública. Veient que la problemàtica es posà de manifest especialment durant els anys 70 i 80, la Unió Europea va implementar la Directiva 91/676/CE, de 12 de desembre, relativa a la protecció de les aigües contra la contaminació produïda per nitrats procedents de fonts agràries. A més d'establir un valor màxim per a l'aigua de consum humà, la Directiva Nitrats també obliga els estats membres a elaborar un codi de bones pràctiques agràries, d'aplicació voluntària, i s'introduí el concepte de zona vulnerable als nitrats, delimitació territorial on seria necessari establir un programa d'actuació d'obligat compliment per a prevenir la contaminació per nitrats. La Directiva 91/676/CE no va ser transposada a la legislació espanyola fins al 1996, amb el Reial decret 261/1996, on es traslladava a les comunitats autònomes l'elaboració dels plans d'actuació. Tot i els esforços per controlar els valors de nitrat, la realitat és que en zones d'alta activitat agrícola la normativa es troba repetidament vulnerada (Figs. 5 i 6).

Tot i el seu interès en estudis d'eutrofització, la toxicitat del nitrat està poc estudiada en relació a la de l'amoníac i el nitrit, i menys en condicions d'exposició crònica. Una bona part dels estudis de toxicitat del nitrat han cercat la concentració letal (LC_{50}) en tests de 48 – 120 hores (Camargo et al., 2005; Hickey i Martin, 2009), tot i que s'han realitzat alguns estudis de toxicitat crònica amb paràmetres no letals (ex. Hamlin et al., 2008; Adelman et al., 2009; Pereira et al., 2017). Es creu que el principal mecanisme de toxicitat no és degut al nitrat en sí, ja que té una baixa permeabilitat d'entrada per via branquial, sinó quan aquest es redueix a nitrit de forma endògena, principalment en el tracte digestiu dels vertebrats (Jensen, 1995). El nitrit, tant endogen com el que capten activament els animals aquàtics a través de les cèl·lules del clorur de les seves brànquies, entra en el torrent sanguini i oxida els ions de Fe(II) a Fe(III) de l'hemoglobina (Hb), la proteïna responsable de transportar l'oxigen des dels òrgans respiratoris fins als teixits en vertebrats (Lewis i Morris, 1986). La proteïna resultant, la metahemoglobina (MetHb), que dóna a la sang un característic color marronós, té molt poca afinitat amb l'oxigen, provocant dificultats respiratòries a l'animal (Noga, 2010).

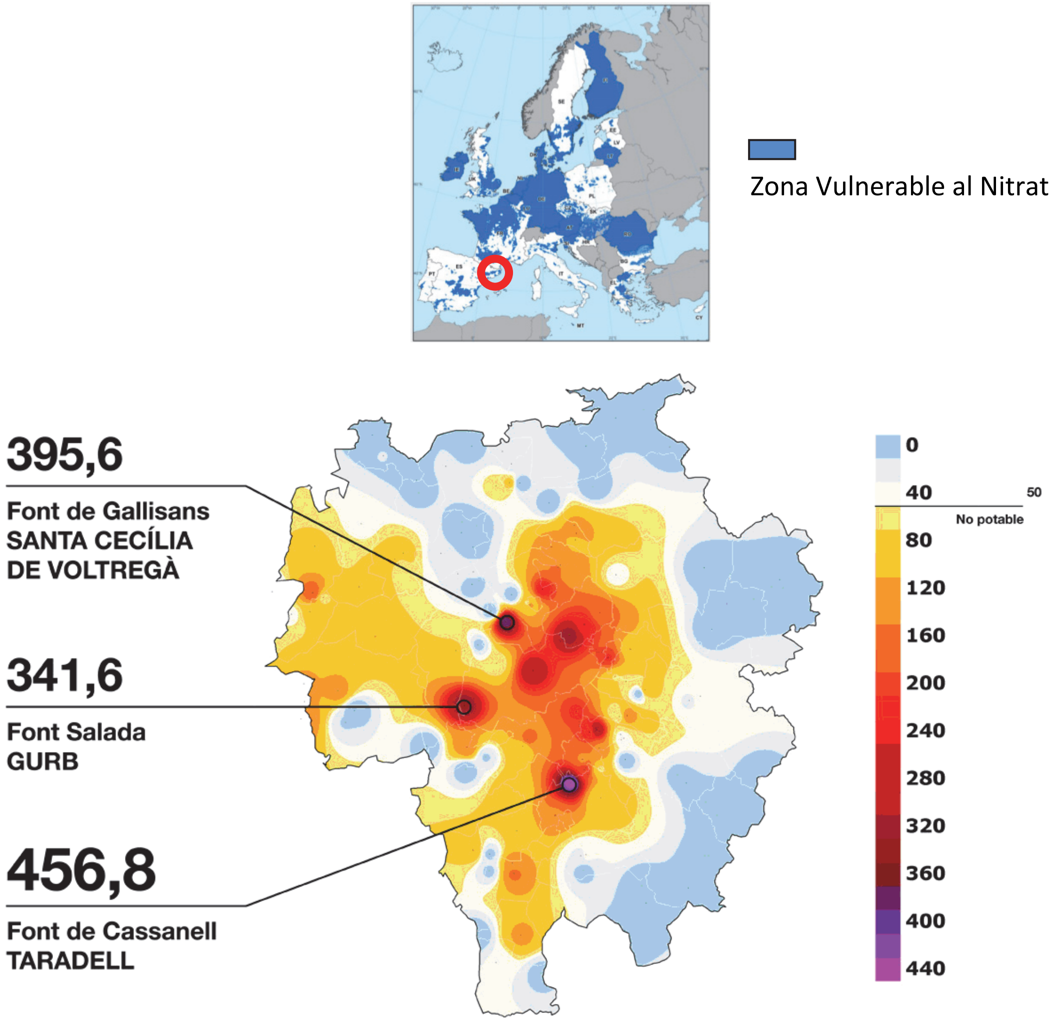
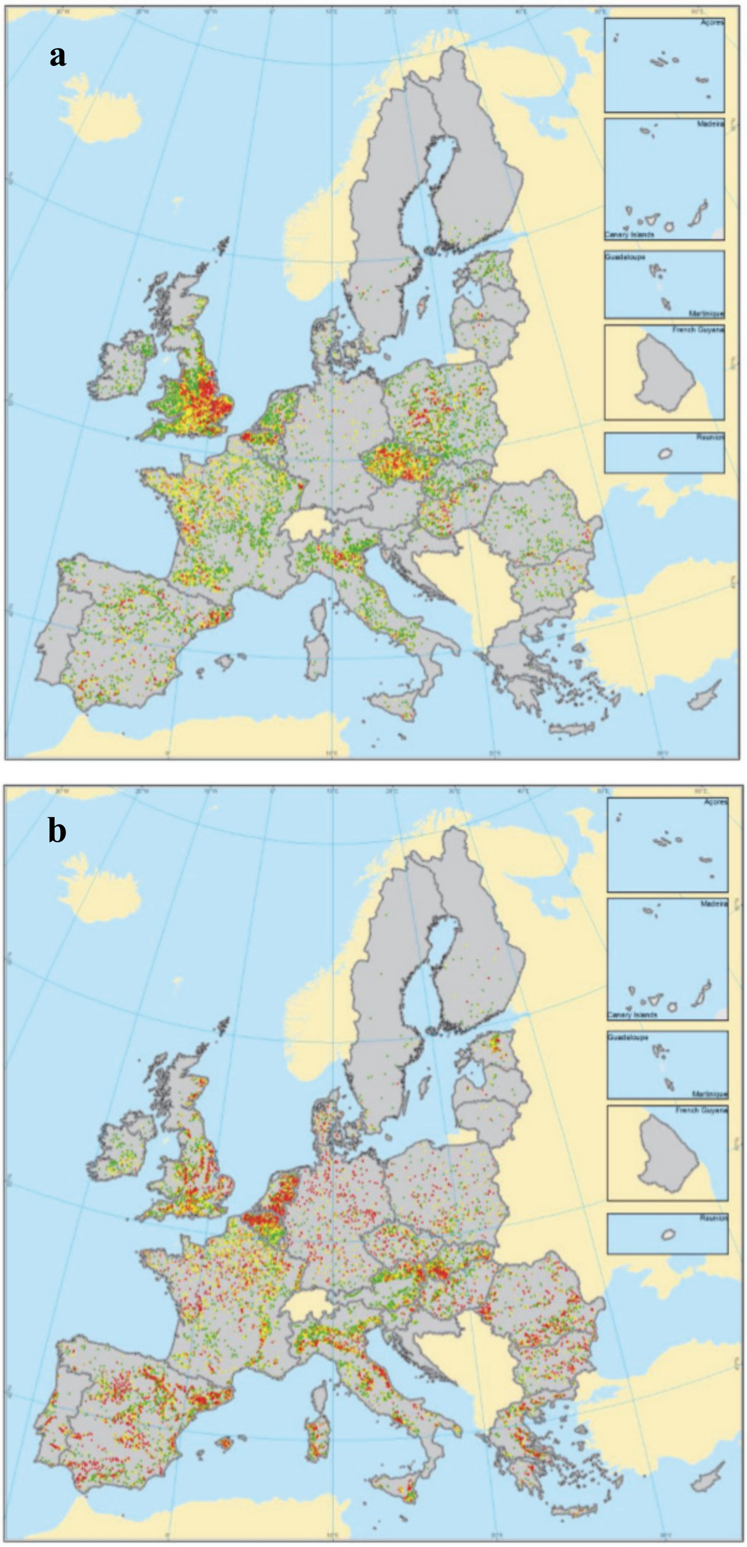


Figura 5. Mapa de les concentracions de nitrats mesurades el 2019 en les fonts de la comarca d'Osona i el Lluçanès, una zona declarada com a vulnerable als nitrats, degut a l'alta activitat ramadera i la gestió ineficient dels purins. Font: Grup de Defensa del Ter, 2019.

Si bé la toxicitat del nitrat en exposició crónica és força desconeguda, hi ha evidències que capgrossos de gripau comú *Bufo bufo* (Linnaeus, 1758), certs gammàrids i juvenils de truita irisada *Oncorhynchus mykiss* (Walbaum, 1792) estarien entre els taxons més afectats, fins i tot a concentracions per sota del llindar màxim establert (Grabda et al., 1974; Baker i Waights, 1993; Camargo et al., 2005). No obstant, aquests estudis també posen de manifest que la toxicitat del nitrat depèn de la salinitat, la duresa de l'aigua, la mida corporal i la pre-exposició prèvia al nitrat, que n'augmenten la tolerància (Johansson et al., 2001; Camargo et al., 2005; Baker et al., 2017). Per contra, s'han descrit efectes sinèrgics amb herbicides (Pandey et al., 2011; Geyer et al., 2016) i la radiació ultraviolada (Hatch i Blaustein, 2003). Per tant, encara és aviat per qualificar el



◄**Figura 6**. a) Concentracions màximes de nitrat (mg/l) durant el període 2012 – 2015 en aigües superficials i subterrànies. De tots els rius monitoritzats en els 28 estats membres, el 12 % i el 7 % es van classificar com a eutròfics i hipereutròfics, respectivament. Els països amb >50 % de rius amb aquesta categorització són Àustria, Luxemburg, Espanya, Lituània, República Txeca, Bèlgica, Croàcia, Malta i Anglaterra. Entre els llacs monitoritzats, el 18 % es van catalogar com a eutròfics i el 8 % com a hipereutròfics. Els països amb pitjors registres són Bulgària, Croàcia i Polònia. En estuaris, només 10 dels estats membres tenen programes de seguiment, amb 6 estats del total de 10 que van reportar el 100% de les seves localitats com a eutròfiques o hipereutròfiques.

b) Concentracions màximes de nitrat (mg/l) durant el període 2012 – 2015 en aigües subterrànies.

Concentració màxima

● 10 – 25 mg/l ● 25 – 40 mg/l

● 40 – 50 mg/l ● >= 50 mg/l

Font: Comissió Europea (2018). Informe per al període 2012 – 2015 de la Comissió al Consell i el Parlament Europeus sobre la implementació de la Directiva 91/676/EEC sobre la protecció d'aigües contra la contaminació per nitrats causada per l'agricultura.

nitrat de relativament innocu. A més de la mencionada metahemoglobinèmia, la resta d'alteracions conegudes descrites en vertebrats aquàtics estan principalment relacionades amb afectacions a la reproducció i el desenvolupament per una disrupció endocrina (Taula A1 a l'annex). Els mecanismes d'acció del nitrat serien els següents: a) afectacions a l'osmoregulació per interferència en la concentració intracel·lular de clorur i iodur, en ser el nitrat un ió monovalent que n'afectaria el transport a través de la membrana cel·lular, b) alteracions neuroendocrines per conversió de NO_3^- a òxid nítric (NO), que és un neurotransmissor (Guillette i Edwards, 2005; Poulsen et al., 2018).

En gran part dels estudis fets amb nitrat, tant de toxicitat aguda o crònica, la variable resposta ha estat o bé mortalitat i creixement (vegeu Hickey i Martin, 2009; Adelman et al., 2009), o alteracions a nivell bioquímic com el balanç iònic i hormonal (Jensen, 1996; Hamlin et al., 2008). Entre les dues aproximacions apareix un compromís entre la rellevància ecològica i la rapidesa de reacció al tòxic. L'ús combinat de diferents biomarcadors permet fer una anàlisi més acurada de l'efecte del tòxic, ja que algunes alteracions no suposen un perill per a la viabilitat de l'individu, però podrien estar impedint un correcte funcionament fisiològic. Tanmateix, estem lluny d'entendre la correspondència entre biomarcadors de naturalesa diversa, ni les conclusions extretes sobre la salut de l'animal (Colin et al., 2016). La incertesa es fa més palesa si només s'estudia els estadis immadurs (ex. Pereira et al., 2017) o algun dels dos sexes (Hamlin et al., 2008), ja que la finestra de toxicitat pot variar entre aquests (Adelman et al., 2009). La integració de diversos biomarcadors de resposta temporal variant en el conjunt d'edats i sexes de la població podria ajudar a dilucidar aquesta incertesa sobre l'abast del nitrat, per exemple en aspectes tan poc estudiats i de gran rellevància ecològica com alteracions en la captura dels nutrients i en l'emmagatzematge de reserves energètiques (Guillette i Edwards, 2005; Poulsen et al., 2018).

Només aquells trets biològics, com els paràmetres fisiològics o la fecunditat, que varien dins una mateixa espècie poden respondre ràpidament a la selecció imposada per l'ambient (Stearns, 1992). Condicionants com la depredació o la qualitat de l'hàbitat influeixen en el desplegament d'aquests trets, que al final determinaran la seva eficàcia biològica o *fitness* (Ricklefs i Wikelski, 2002). Així, la variabilitat interpoblacional resultat de la variabilitat ambiental s'ha utilitzat per estudiar quines condicions afavoreixen en una direcció o una altra determinats trets com el creixement individual, la mida i nombre de la descendència entre altres (Côté et al., 1999; Benejam et al., 2009;

Bassar et al., 2012). L’estudi dels trets d’història de vida està àmpliament utilitzat per tal d’entendre l’èxit invasor d’una espècie (vegeu Taula S2 a l’annex). Ara bé, de la mateixa manera que es pot augmentar la salinitat per limitar l’expansió de *G. holbrooki*, el control del grau d’eutrofització de les aigües, les característiques físiques de l’hàbitat o afavorir la presència de depredadors naturals podria ajudar en el control d’aquesta espècie invasora o bé mitigar els efectes ecològics derivats de la seva introducció (Larson et al., 2011).

OBJECTIUS

Objectiu general

L'objectiu general d'aquesta tesi és l'estudi de la influència que té l'eutrofització en general i l'excés de nitrats en particular sobre la biologia i ecologia del peix invasor *G. holbrooki*. És una tesi multidisciplinar que, a través d'estudis observacionals de camp i experimentals en laboratori i mesocosmos, explora la hipòtesi de si l'eutrofització estaria entre les causes que promouen l'èxit invasor de *G. holbrooki*. En concret, hom dirà que *G. holbrooki* no veu afectat el seu èxit invasor a causa del nitrat si té la capacitat fisiològica de tolerar-lo i si l'impacte de la invasió per *G. holbrooki* sobre espècies i ecosistemes són comparables en presència i absència del mateix.

Objectius específics

1. Avaluar l'impacte de la introducció de *G. holbrooki* sobre l'estructura de les comunitats aquàtiques i els processos ecosistèmics en condicions d'eutrofització per nitrat. Esperem que l'addició de nitrat incrementi la producció primària i que *G. holbrooki* incrementin l'efecte de l'eutrofització en alimentar-se de zooplàncton. No obstant, el seu caràcter omnívor podria fer que en depredar sobre el plàncton, el bentos i els detritus neutralitzi l'impacte net sobre les comunitats.

2. Avaluar l'impacte de l'exposició crònica del nitrat sobre la biologia de *G. holbrooki* utilitzant biomarcadors. La concentració de nutrients és la variable sobre la qual actuen els gestors per evitar l'eutrofització, si bé a l'experiment 1 la toxicitat directa per nitrat queda emmascarada pels efectes ecosistèmics. És per això que l'objectiu 2 busca determinar els mecanismes fisiològics de la toxicitat per nitrat, així com confirmar la hipòtesi que les poblacions invasores de *G. holbrooki* tenen una gran tolerància a canvis ambientals com la fertilització per nitrat.

3. Estudiar dels trets d'història de vida de *G. holbrooki* per explorar mesures de gestió efectives en el control de la seva expansió. Coneguts els efectes directes i indirectes de la contaminació per nutrients sobre *G. holbrooki*, aquest capítol planteja quina és la importància relativa d'aquests en el conjunt de factors ambientals que poden determinar l'èxit invasor de l'espècie, com ara la complexitat de l'hàbitat físic o l'abundància de depredadors com les aus. Els gestors han de desgranar la importància relativa d'aquests factors a l'hora d'establir una aproximació ecosistèmica per a la gestió de les poblacions invasores de *G. holbrooki*.

4. Quantificar la prevalença de macroparàsits en les poblacions invasores de *G. holbrooki*. Junt amb les variables de qualitat de l'aigua i de depredació per vertebrats, un altre dels factors no analitzats en el capítol 3 que podria determinar l'èxit invasor de l'espècie és la manca de paràsits segons la hipòtesi de la manca d'enemics naturals. Un primer recolzament per aquesta hipòtesi seria que la prevalença de paràsits i de lesions als individus de *G. holbrooki* analitzats fos baixa.

5. Comparar l'efectivitat en el control biològic de mosquits en diferents condicions de terbolesa entre *G. holbrooki* i un peix natiu amenaçat. Sigui per eliminació directa o a través d'una aproximació ecosistèmica, *G. holbrooki* és una espècie a eradicar d'acord amb la legislació vigent. Aquest capítol planteja si espècies natives com el fartet *Aphanius iberus* (Valenciennes 1846) podrien substituir *G. holbrooki* en el servei ecosistèmic del control de mosquits en condicions d'eutrofització. D'altra banda, també s'analitza si l'eutrofització donaria avantatge competitiu a l'espècie nativa en relació a la invasora.

INFORME DELS DIRECTORS

El Dr. Adolfo de Sostoa Fernández, com a director, i el Dr. Alberto Maceda Veiga, com a co-director, de la tesi doctoral titulada “La invasió de *Gambusia holbrooki* en ecosistemes eutrofitzats. Dels mecanismes ecològics a la seva gestió” realitzada per Oriol Cano Rocabayera, presenten el següent informe sobre la contribució del doctorand en les publicacions en coautoria que composen la tesi:

Capítol 1. Cano-Rocabayera, O., de Sostoa, A., Muñoz, I., Salvadó, H., Vilumara, J., Cáceres, G., Maceda-Veiga, A. Monitoring additive and interactive effects of a fish invasion and nutrient pollution on experimental ponds: taxonomic vs functional indicators. Ecological indicators (pendent d’enviar).

Contribució del doctorand: Participació en el disseny dels experiments, la presa de dades en camp i laboratori, l’anàlisi dels resultats i la redacció del manuscrit.

Sobre la revista: *Ecological indicators* té un índex d’impacte de 4,490 al Journal Citation Reports (JRC) de 2018. Es troba en el número 45 de 250 a l’àrea de *Environmental Sciences* (1er quartil).

Capítol 2. Cano-Rocabayera, O., de Sostoa, A., Padrós, F., Cárdenas, L., Maceda-Veiga, A. (2019). Ecologically relevant biomarkers reveal that chronic effects of nitrate depend on sex and life stage in the invasive fish *Gambusia holbrooki*. PLOS ONE, 14(1), e0211389. doi:10.1371/journal.pone.0211389

Contribució del doctorand: Participació en el disseny dels experiments, el treball de laboratori, l’anàlisi dels resultats i la redacció del manuscrit.

Sobre la revista: *PLOS ONE* té un índex d’impacte de 2,776 al Journal Citation Reports (JRC) de 2018. Es troba en el número 24 de 69 a l’àrea de *Multidisciplinary sciences* (2n quartil).

Capítol 3. Cano-Rocabayera, O., de Sostoa, A., Coll, L., Maceda-Veiga, A. (2019). Managing small, highly prolific invasive aquatic species: Exploring an ecosystem approach for the eastern mosquitofish (*Gambusia holbrooki*). Science of The Total Environment, 673, 594–604. doi:10.1016/j.scitotenv.2019.02.460

Contribució del doctorand: Participació en el disseny de l'estudi, la presa de dades en camp i laboratori, l'anàlisi dels resultats i la redacció del manuscrit.

Sobre la revista: *Science of the Total Environment* té un índex d'impacte de 5,589 al Journal Citation Reports (JRC) de 2018. Es troba en el número 27 de 250 a l'àrea de *Environmental Sciences* (1er quartil).

Capítol 4. Maceda-Veiga, A., Cano-Rocabayera, O., de Sostoa, A., Cable, J. (2019). Low parasite prevalence in the invasive fish *Gambusia holbrooki* in riverine and stagnant waters in north-eastern Spain. Bulletin of the European Association of Fish Pathologists, 39(2), 70–76.

Contribució del doctorand: Participació en part de la presa de dades.

Sobre la revista: *Bulletin of the European Association of Fish Pathologists* té un índex d'impacte de 0,483 al Journal Citation Reports (JRC) de 2018. Es troba en el número 102 de 108 a l'àrea de *Marine and freshwater biology* (4^{t} quartil) i en el número 48 de 52 a l'àrea de *Fisheries* (4^{t} quartil).

Capítol 5. Cano-Rocabayera, O., Vargas-Amengual, S., Aranda, C., de Sostoa, A., Maceda-Veiga, A. When the introduction of alien fish for mosquito biocontrol unnecessarily threatens the natural ecosystem service. Hydrobiologia (en revisió).

Contribució del doctorand: Participació en el disseny dels experiments, suport en el treball de laboratori i camp, l'anàlisi dels resultats i la redacció del manuscrit.

Sobre la revista: *Hydrobiologia* té un índex d'impacte de 2,325 al Journal Citation Reports (JRC) de 2018. Es troba en el número 32 de 108 a l'àrea de *Marine and freshwater biology* (2^{n} quartil).

Barcelona, a 24 de juliol de 2019

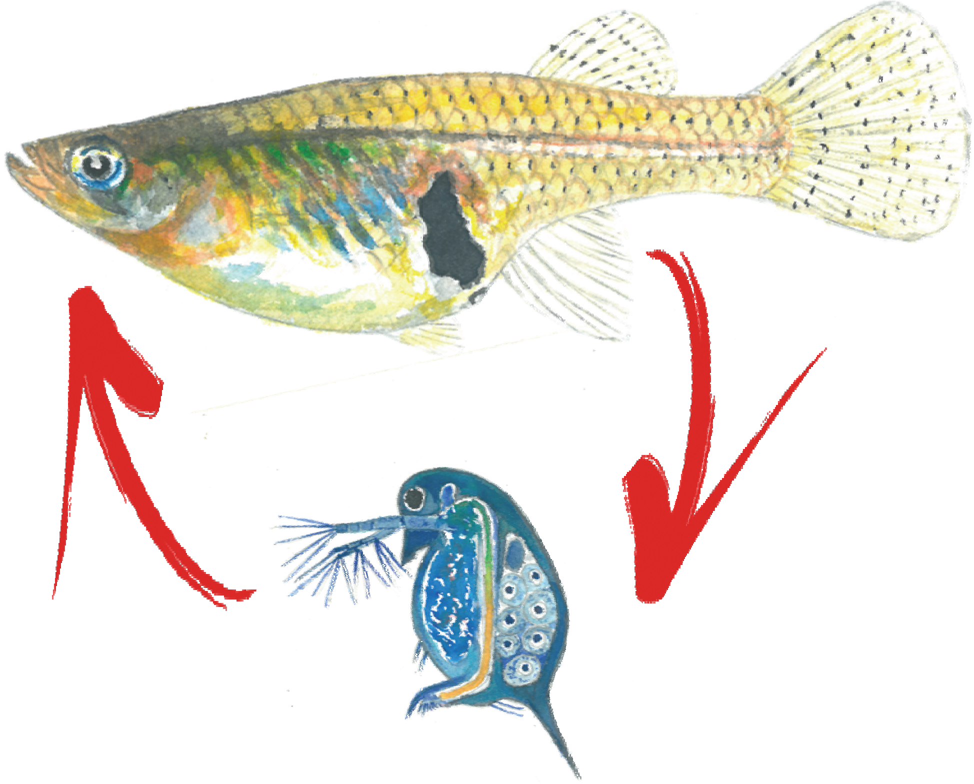
Drs. Adolfo de Sostoa Fernández i Alberto Maceda Veiga (directors de la tesi)

Departament de Biologia Evolutiva, Ecologia i Ciències Ambientals

Facultat de Biologia

CAPÍTOL 1

Monitoratge d'efectes additius i interactius entre una invasió piscícola i la contaminació per nutrients en basses experimentals: indicadors taxonòmics vs. funcionals



Oriol Cano-Rocabayera[a]; Adolfo de Sostoa[a]; Isabel Muñoz[b]; Humbert Salvadó[b]; Joan Vilumara[a]; Gustavo Cáceres[a]; Alberto Maceda-Veiga[a]

[a]Departament de Biologia Evolutiva, Ecologia i Ciències Ambientals – Institut de Recerca de la Biodiversitat (IRBio-UB), Universitat de Barcelona

[b]Departament de Biologia Evolutiva, Ecologia i Ciències Ambientals – Institut de Recerca de l'Aigua (IdRA-UB), Universitat de Barcelona

Monitoring additive and interactive effects of a fish invasion and nutrient pollution on experimental ponds: taxonomic vs functional indicators

ABSTRACT

The number of perturbations affecting aquatic ecosystems is increasing, but there is limited insight into the ecological consequences of their additive and interactive effects. There is also few experimental evidence of how changes in the taxonomic structure of communities affect ecosystem processes, which is important for an accurate monitoring. We set experimental ponds for 11 weeks to explore the additive and interactive effects of nitrate pollution and the invasive fish *Gambusia holbrooki*, two major perturbations threatening aquatic ecosystems around the world. Moreover, we examined congruence among 10 community metrics and 11 ecosystem processes to determine the ecological relevance of the taxonomic approaches often used in official monitoring schemes. There was limited evidence of interactive ecological effects of *G. holbrooki* in eutrophic conditions, whereas nitrate reduced the richness and biomass of planktonic and benthic communities and increased primary production, leaf litter break-down rate and the nitrogen content of the decomposed leaves. *Gambusia holbrooki* only altered the chlorophyll-a concentration and nitrogen immobilisation in litter. The community metrics that best represented variation in ecosystem properties (pH, dissolved organic carbon and decomposition) were zooplankton and phytoplankton biomass, or total richness. Overall, this study shows the ecological effects of eutrophication by nitrate and the context-dependence of the impacts of biological invasions. Furthermore, we provide experimental evidence of the usefulness of eight taxa to monitor the effects of nutrient pollution in freshwaters.

Keywords: nitrate; eutrophication; food web; trophic cascade; biomass; richness; *Gambusia holbrooki*

1. Introduction

Non-native species are altering the composition of biotic assemblages with potential far reaching impacts on ecosystems (Vitousek, 1990; Simberloff et al., 2013). Impacts can be caused by direct interactions to native biota (e.g. competition, predation) and by indirect effects on ecosystems, including turbidity and alterations in primary production, (Gallardo et al., 2015; Sih et al., 2010). The consequences of biological invasions also depend on the characteristics of recipient habitats, including the presence of other perturbations (e.g. habitat degradation) and the diversity of native assemblages (Didham et al., 2007). Nevertheless, the context-dependence of the ecological impacts of invasive species is one of the less studied and more important aspects for a more effective management of biological invasions (Diez et al., 2009).

Nutrient loading from human activities (e.g. agricultural run-off, urban outflows) results in eutrophication, which is contributing to degrading aquatic ecosystems worldwide (Smith et al., 1999). However, nutrients are subsidies or pollutants depending on their concentration (Vitousek et al., 1997). If nutrients increase prey availability, nutrient loading may reduce trophic competition from, or predation by, invasive species on lower trophic levels (Balčiūnas and Fowler, 1995). Conversely, if nutrient pollution reduces prey diversity (Garg and Bhatnagar, 1996; Smith et al., 1999), it may be advantageous to have the generalist feeding strategy of many invasive consumers (García-Berthou, 1999). Nevertheless, invasive species may reduce their fitness (e.g. reduced body condition, offspring) if nutrients are toxic (Edwards et al., 2006; Hickey and Martin, 2009). Moreover, changes in habitat conditions because of eutrophication (e.g. increased macrophyte cover, turbidity) may make prey more difficult to detect and to catch (Scheffer, 2004; Nieman et al., 2018). Therefore, the consequences of fish invasions under eutrophication will be better understood if the fitness of the invader is examined alongside the changes in the structure and function of ecosystems.

Studies addressing relationships between ecosystem processes and the structure of biotic assemblages are central to predict the effects of biological invasions (Simon and Townsend, 2003). Many experimental studies reported changes in the elemental units of aquatic assemblages (taxa identity, abundance and biomass) after invasion (Lancaster and Drenner, 1990; Romo et al., 2004; Cardona, 2006). Less studied is how these structural changes alter ecosystem processes during invasions (Hinchliffe et al., 2017; Hall et al., 2018). The biodiversity-ecosystem functioning hypothesis posits that the higher number of species an ecosystem has, the higher number of functions can secure (Naeem et al., 1994). If a species is extirpated, major changes in ecosystem properties are less likely to occur in species-rich than in species-poor assemblagesdue to potential functional redundancy among some species (Vitousek, 1990). However, the ecosystem impacts of species invasions have been mostly examined between < 2 trophic levels (Shulse and Semlitsch, 2014; Geyer et al., 2016; Hinchliffe et al., 2017). Moreover, few of these studies explored the ecosystem effects of a species invasion and nutrient pollution, in single or combination (Lancaster and Drenner, 1990; Preston et al., 2018), which limits our ability to identify indicators of the impacts of species invasions.

The introduction of omnivorous fish is frequent in freshwater lakes (Eilers et al., 2007). The impacts of fish invasions have been mostly studied in the water column, reporting a decline in macrophyte cover and changes in the abundance, biomass and richness of zooplankton and phytoplankton (Lancaster and Drenner, 1990; Bain, 1993; Romo et al., 2004). The few studies addressing the combined effects of nutrient pollution and introduced omnivorous fish found that the biomass of fish, phytoplankton, periphyton and of snails increased in eutrophic waters (Lancaster and Drenner, 1990; Preston et al., 2018). However, the effects on the microbial loop (e.g. ciliates, decomposition rate), which is important in the organic matter cycle (Elosegui and Sabater, 2009), were not examined. While assessments using multiple taxa and ecosystem functions are desirable (Bonada et al., 2006), logistics often limit the application of these multidisciplinary approaches. Therefore, examining congruence among the taxonomic structure of aquatic communities and ecosystem processes will help to identify metrics with high ecological relevance for the monitoring of invaded or eutrophic aquatic ecosystems.

Our goal was to explore whether the fitness and ecological impacts of eastern mosquitofish *Gambusia holbrooki* (Girard, 1859) was modulated by nitrate pollution within mesocosms. *Gambusia holbrooki* is an omnivorous, invasive fish, which often alters food-webs, including after nitrogen and phosphate additions (Lancaster and Drenner, 1990; Preston et al., 2018). However, we only added nitrate as occurs within EU Nitrate Vulnerable Zones (European Comission, 2018), which should reduce eutrophication effects due to unbalanced nutrient stoichiometry (Vitousek et al., 1997). We expected nitrate and *G. holbrooki* to be important perturbations in aquatic ecosystems (Hickey and Martin, 2009; Hinchliffe et al., 2017), and so, they should alter the taxonomic and functional structure of mesocosms in isolation or combination. In particular, increased ecosystem productivity from nitrate addition should reduce fish predation on invertebrates (but see Preston et al., 2018). Nitrate fertilisation should affect the entire food-web but fish should mostly affect the pelagic food-web because *G. holbrooki* often swims close to the water surface (García-Berthou, 1999). From a biomonitoring perspective, efficiencies might be gained if we determine congruence in responses among multiple taxa and ecosystem functions in invaded or eutrophic waters.

2. Material and methods

2.1. General experimental design

We conducted a two factor fully crossed design within mesocosms to improve mechanistic understanding of how nitrate pollution and *G. holbrooki* invasion alter the functioning of freshwater ecosystems and to develop new monitoring tools. The lowest nitrate concentration was dechlorinated tap water (<10 mg l^{-1}) and the highest level was 400 mg l^{-1}, which is within the range occurring in EU nitrate vulnerable areas (e.g. Grup Defensa del Ter, 2019). Half mesocosms were left fishless and half had *G. holbrooki*. Captive-reared fish (n = 108) were anesthetised using MS-222®, measured (total length), weighed (mg), and nine females and three males were introduced in each tank (total biomass = 3.93 ± 0.08 g). This fish density (10 fish m^{-2}) often occurs in nature (Zulian et al. 1995; Smith and Sargent, 2006). There were five replicates per experimental condition, but two replicates of nitrate addition were lost due to leaking water. Each mesocosm was sampled four times: one week before the start of experiment to check for homogeneous conditions in all ponds (t_0); and at 7 (t_1), 36 (t_2), and 72 (t_3) days after nitrate and fish additions. Fish were only sampled at t_3 but we surveyed on the four sampling occasions the diversity and biomass of several taxa, water quality variables, litter decomposition and primary production (see from section 2.3 onwards).

2.2. Mesocosms set up

The experiment setup started on 19th November 2014. Twenty outdoor mesocosm (117 × 100 × 60 cm, 400 l) were filled with tap water in El Prat de Llobregat (ETR S89/ 31 N/ X:422880, Y:4577115) and left them standing for a week to dechlorinate. Four PVC boxes (42 × 30 × 10 cm) with 10 cm layer of dry granitic sand were added to each mesocosm. After one month, 500 ml of sediment from a nearby freshwater pond (31N X:423328; Y:4575807) was placed on top of each PVC box to settle a homogeneous benthic community. Moreover, 1.5 l of water from two shallow marshes (31N X:425437; Y:4572322 and X:426138; Y:4571734) was introduced to settle a natural planktonic community. A set of four glass slides (26 × 76 mm) was submerged to 10 cm deep in each mesocosm, placed on the facing-south wall, to quantify benthic primary production (see Section 2.4.2).

Fish were introduced on 17th of April and four days later, the experimental nitrate concentration (365.8 ± 72.7 mg l^{-1}) was reached in six hours by means of a drip irrigation system that delivered a concentrated $NaNO_3$ solution (225 g in 12 l of tap water) into each tank. This system was used to refill evaporated water, which mirrors the effects that pumping water from nitrate polluted aquifers may have on surface waters if underground water is used to recharge the surface water table (Martínez-Santos et al., 2008). At the end of the experiment on 2nd July, nitrate concentration was 425.8 ± 63.4 mg l^{-1}. This concentration allows us to identify which taxa and ecological functions are very unlikely to be affected by nitrate, and so, are unsuitable indicators for this compound. All mesocosms were half shaded from the 7th week onwards with a cloth fixed at 20 cm above surface to avoid overheating of the water due to increasing solar irradiance. Furthermore, after nitrate addition five litter bags of 0.5 cm mesh size with eight air-dried *Populus nigra* leaf discs (1.4 cm Ø) in each bag were introduced in each mesocosm to quantify litter decomposition rate (see Section 2.4.2).

2.3. Fitness traits of Gambusia holbrooki

At the end of the experiment fish were collected with a dip net and counted to record breeding success, euthanized with an overdose of anaesthetic MS-222®, and frozen at -28 ºC. Fish were defrosted, sexed and their total length (± 1 mm) and weight (± 0.1 mg) were measured. Fish body condition was calculated using the Scaled Mass Index, $\hat{M}$ (Peig and Green, 2009):

$$\hat{M}_i = M_i \left[\frac{L_0}{L_i} \right]^{b_{SMA}} \qquad 1)$$

where M_i is the weight and L_i is the length of fish i, b_{SMA} is the slope of the Standardised Major Axis regression (M on L) of the population, and L_0 is an arbitrary value of L, e.g. the arithmetic mean length of all fish individuals. $\hat{M}$ is the predicted body mass for fish i when its length is standardised to L_0. Body condition can act as a proxy for predicting future animal growth and reproductive success (i.e. fitness, Wilson and Nussey, 2010; Dempster et al., 2011).

2.3. Community metrics

We surveyed the pelagic and benthic assemblages separately and calculated the biomass and diversity of different taxa as indicators of the structure of biotic assemblages. The pelagic assemblages were surveyed at the centre of each mesocosm with a 20 × 4.2 Ø cm methacrylate corer and 100 ml of the water volume was fixed in 2% Lugol's solution for examination in the laboratory. The sample was gently inverted for 1 minute before pouring it in a 25 ml Utermöhl chamber to count the relative abundance of algae, protists and invertebrates (individuals/ml) under a phase-contrast inverted microscope at 100× and 200×. Phytoplankton and rotifers were identified to species level and biomass for these two groups and ciliates were calculated from previously published mean individual weights (Table S1).

For benthic assemblages, we lidded the PVC box holding sediment and lift it outside the mesocosm. There was one box for each sampling time of the study. We used the same methacrylate tube to take a core of sediment from the centre and two cores from two haphazardly selected corners. The three cores were poured in a plastic bag and were gently mixed with 4% formalin. The sample was sieved in the laboratory through a 250 µm mesh and cleaned with tap water before identifying invertebrates and recording their relative abundances under a stereomicroscope at 10×. All invertebrates were identified to the lowest practical taxonomic level (e.g. crustaceans to family) and their biomasses were estimated by applying the equations published for each taxon to the length measures of 10 – 30 individuals of each taxon and size class (see Table S2).

2.4. Indicators of ecosystem functioning

2.4.1. Water properties

Water quality was altered by the experimental conditions ($NaNO_3$ addition) and by the natural metabolic activity of mesocosms, so that changes in water properties were indicators of ecosystem functioning. On each sampling occasion, we quantified *in situ* dissolved oxygen, temperature, pH, conductivity, and total dissolved solids (TDS) using a portable multiparameter probe (± 0.01 mg O_2 l^{-1}, ± 0.1 ºC, ± 0.01 pH, ± 1 µS cm^{-1} and ± 0.5 mg TDS L^{-1}; YSI Pro Plus, Yellow Springs, USA) and water turbidity using a portable spectrophotometer (± 0.1 Formazin Turbidity Units; Orbeco-Hellige 975-MP). A water volume of 1 l was filtered through pre-ashed Whatman GF/F filters and both were kept at 4 ºC in the darkness. Filter was used to quantify chlorophyll-a (Chl-a, section 2.4.2) and water was used for nutrient analyses. Ammonium (mg l^{-1} NH_4^+; detection limit, d.l. < 0.3 mg l^{-1}), nitrite (mg l^{-1} NO_2^-; d.l. < 0.1 mg l^{-1}), and nitrate (mg l^{-1} NO_3^-; d.l. < 0.1 mg l^{-1}) were quantified using high performance liquid chromatography (HPLC), and dissolved organic carbon (DOC) was quantified using the total organic carbon analyser MULTI N/C 3100 (Analytik Jena, Germany) at the University of Barcelona (CCiT-UB).

2.4.2. Ecosystem processes

Primary production and dead litter decomposition were the ecosystem processes measured. For primary production, we quantified the concentration of Chl-a in the benthos and plankton. For benthic primary production, a glass slide was collected from each mesocosm on each sampling occasion, stored dark at 4 ºC in the field (< 5 h) and frozen at -28 ºC in the laboratory. Each slide was scraped with a brush in a Petri dish with distilled water and the suspension was filtered through a Whatman GF/F filter. For the planktonic primary production, we processed the filters from water quality analyses as follows. Pigments from filters were extracted in 90% acetone overnight and Chl-a concentrations were quantified at 665 and 750 λ-nm using a Perkin-Elmer Lambda2 UV/VIS Spectrophotometer (Elosegui and Sabater, 2009). On the termination of the experiment large carpets of macroalgae covered the tanks (mostly *Cladophora*). Therefore, we took a photograph of each mesocosm at 2 m from the water and calculated the percentage of their surface area covered by macroalgae using ImageJ® to include it in the analyses as an additional indicator of primary production.

2.4.3. Leaf litter decomposition

The litter bag method with a mesh size that allowed the activity of invertebrates and microbes was used to calculate decomposition rate and the carbon and nitrogen content of the decomposed leaves (Elosegui and Sabater, 2009). A single bag was collected from each mesocosm (see Section 2.2) and leaf discs were rinsed in distilled water, oven-dried at 50 ºC, and weighed to the nearest milligram to determine mass loss on each sampling occasion. The mass loss due to leaching and handling was subtracted by weighting five discs before and having them in a bucket with water for 24 h. All leaf discs were soaked in liquid nitrogen, ground to fine powder with a porcelain mortar, and analysed for the % of N and C using the elemental organic analyzer Thermo EA 1108 (Thermo Scientific, Milan, Italy) at CCiT-UB. Breakdown rates were calculated based on a linear regression of log-transformed % of remaining leaf litter biomass at 6, 14, 33, 51, and 71 days following the guidelines of Elosegui and Sabater (2009).

2.5. Data management

Statistical analyses were conducted in R v. 3.4.3 (R Core Team, 2016) using the functions outlined below. The statistical analyses were divided in three steps. Firstly, we examined the ecological impacts of nitrate pollution and *G. holbrooki* invasion using 10 indicators of biotic structure and 11 of ecosystem functioning (Table S3). Indicators of biotic structure (biomass and taxonomic diversity) were grouped by ecosystem compartment (e.g. pelagic food web = zooplankton + phytoplankton; primary producers = phytoplankton). Secondly, we compared the fitness traits of *G. holbrooki* between nitrate and clean waters to better identify the variables that most likely influenced mesocosms responses to *G. holbrooki* invasion. We also examined the ecosystem indicators that best explained variation in the community metrics. Finally, we identified the best sentinel taxa to monitor the effects of nitrate pollution or *G. holbrooki* invasion.

2.5.1. General patterns and correlations among indicators

Spearman's rank correlations (ρ) were used to reduce redundancy by identifying highly correlated pairs of indicators at $\rho > |0.80|$. The removed variables are nitrites, highly correlated with nitrates, and temperature and *Cladophora* biomass, which were highly correlated with conductivity. Ammonium concentration was below the detection limit throughout the experiment and was then omitted. Those highly correlated structure indicators were total tank with benthic macroinvertebrates biomass and richness, phytoplankton with pelagic richness, and zooplankton with pelagic biomass.

We used a partial principal component analysis (PCA, the function *rda* in R, Oksanen et al., 2019) to visualize overall changes in the least correlated indicators as function of nitrate pollution and fish invasion along the 10 weeks of the experiment. Mesocosms ID was the blocking factor in the partial PCA ('Condition' term in the function *rda*) to account for the effects of multiple sampling.

2.5.2. Statistical comparisons among treatments

General lineal mixed models (GLMMs) were used to examine the main and interactive effects of the nitrate and fish treatments on the least correlated indicators of biotic structure (total, zooplankton and pelagic richness; zooplankton, phytoplankton and total biomass) and ecosystem functioning (pH, dissolved O_2, turbidity, macroalgae cover, DOC, planktonic and benthic chlorophyll-a and leaf litter variables, i.e. % remaining mass, carbon and nitrogen content and molar C/N ratio). Visual inspection of the distribution of variables suggested the most appropriate error distribution for GLMMs (e.g. log-normal/identity link). Mesocosm ID was random term in all models and sampling occasion in days was a covariate. The value of the dependent variable at t_0 was introduced in models as covariate to account for any differences in the initial stages of mesocosms. GLMMs were also used to identify the ecosystem indicators that best explained variation in the indicators of biotic structure after retaining only those variables with variance inflation factor < 3 (Zuur et al., 2009). GLMMs with all possible combinations of explanatory variables were built and were ranked by the Akaike Information Criterion corrected for small sample size (AIC_c) to identify the most parsimonious and informative model, i.e. the one with the lowest AIC_c value. GLMMs within 2 units of AIC_c were considered equally plausible, and so, we selected the one with the highest explanatory power (R^2_β) as calculated by Jaeger et al. (2016). The R^2_β compares a model with fixed and random effects to a null model with only the random effects and an intercept.

Gaussian lineal models with identity link were used to examine changes in body length, population size, and body condition of *G. holbrooki* between nitrate polluted and clean waters. The sex of fish (male, female or immature) was added as independent variable. Significance of factors and covariates in all models was assessed using likelihood-ratio χ^2 tests at $P \leq 0.05$ (function *Anova*, Fox and Weisberg, 2011). The statistical assumptions of models (normality, homogeneity of variances and the influence of unduly observations) were checked by visually inspecting the diagnostic plots of residuals following the guidelines of Zuur et al. (2009).

2.5.4. Indicator taxa

The most informative taxa to monitor the effects of nitrate pollution and *G. holbrooki* introduction were identified by means of the R function *multipatt* in the package indicspecies (De Cáceres and Legendre, 2009). An indicator value (*INDVAL*) corrected for unequal group sizes was assigned to each taxon based on their presence and relative abundance in each of the four experimental combinations (nitrate low/high × fish absence/presence, parameter *restcomb*). Taxa with fidelity parameter B < 0.20 (proportion of replicates belonging to a particular experimental condition) were removed from the analysis and the *INDVAL* was tested for significance using a permutation test of 9999 permutations with time as a blocking factor in randomisation (De Cáceres and Legendre, 2009). The t_0 was excluded from the analysis.

2.6. Ethics statement

The experimental procedure was authorised by the Natural Environment and Biodiversity Division at the Catalan Department of Agriculture and Fisheries (Num. DAAM 8288). The experimental design was approved by the Animal Welfare Committee at the University of Barcelona (Num. 192/15). All fish were humanely euthanized on the termination of the experiment in compliance with Spanish legislation for the management of invasive species (Real Decreto 1628/2011).

3. Results

3.1. Ecosystem properties

Many water quality variables and ecosystem processes changed simultaneously in mesocosms and this covariation was summarised in the first two components of a PCA (Fig. 1). The first PCA axis explained 42.4% of total variation and displayed the changes in mesocosms from the beginning (mid-April) to the end of the study (July). The second PCA axis explained 19.9% of total variation and showed the effects of nitrate. There was no clear segregation between fish and fishless mesocosms in the PCA biplot. However, general linear mixed models showed clear univariate statistical effects for all three factors, i.e. nitrate, fish and sampling date (Tables 1 and S4).

3.2. Community metrics and fish fitness

As for other mesocosms measures, the main factors affecting community metrics were sampling date and, to a lesser extent, nitrate addition (Fig. 3; Table S5). Phytoplankton biomass increased from mid-April to July accompanied by a decrease in the total richness and biomass of mesocosms. By guilds, total plankton richness and the richness and biomass of zooplankton reduced throughout the experiment (Table S5). Nitrate addition reduced total richness and biomass but it increased the biomass of phytoplankton (Fig. 3; Table S5). Variation in total richness and biomass was mostly due to changes in benthos (par-wise correlations, Table S6). Changes in plankton richness were mostly due to phytoplankton and those in planktonic biomass were mostly due to zooplankton (Table S6).

Table 1. Statistics for the general linear mixed models examining the response of ecosystem properties indicators along time with nitrate and fish as independent factors. Statistical significance was reached using likelihood-ratio χ^2 tests at $P \leq 0.05$. Chl-a bentos was log-transformed.

Metric	**Factor**	**Effect size**	χ^2	**P-value**		R_β^2
pH	Nitrate	0.65 ± 0.09	51.4	<0.001	*	0.82
	Time	0.03 ± 0.01	35.3	<0.001	*	
	Time^2	-5.1E^{-4} ± 6.4E^{-5}	62.6	<0.001	*	
Dissolved O$_2$	Fish	0.04 ± 0.21	0.03	0.86		0.94
	Nitrate	3.50 ± 0.37	88.9	<0.001	*	
	Time	-0.24 ± 0.02	175.5	<0.001	*	
	Time^2	2.1E^{-3} ± 2.1E^{-4}	99.4	<0.001	*	
	Time:Nitrate	-0.05 ± 0.01	33.0	<0.001	*	
Turbidity	Nitrate	2.03 ± 0.96	4.5	0.03	*	0.40
	Time	0.08 ± 0.02	25.9	<0.001	*	
Cladophora cover	Nitrate	14.02 ± 4.24	10.9	<0.001	*	0.73
	Time	1.52 ± 0.23	45.3	<0.001	*	
	Time^2	-1.2E^{-2}± 2.7E^{-3}	19.4	<0.001	*	
DOC	Nitrate	-1.38 ± 0.39	12.8	<0.001	*	0.39
Chl-a plankton	Fish	1.25 ± 0.59	4.4	0.04	*	0.24
	Time	0.03 ± 0.01	10.1	<0.01	*	
Chl-a benthos	Fish	0.68 ± 0.17	15.7	<0.001	*	0.48
	Nitrate	0.92 ± 0.29	10.4	<0.01	*	
	Time	-5.0E^{-3}± 3.9E^{-3}	1.7	0.19		
	Time:Nitrate	1.0E^{-2}± 5.9E^{-3}	2.9	0.09		

Fish addition did not have discernible effects on community metrics in any treatment, notwithstanding mesocosms had of up to 127 fish/tank in July. Nitrate addition reduced fish body condition, on average, by 6 – 15 % ($P < 0.001$; Fig. S1) and biomass by 18% compared to fish in clean waters. However, no clear statistical effect was found for nitrate on mean fish density (83 ± 32 in controls vs. 108 ± 35 fish/tank; $P > 0.05$).

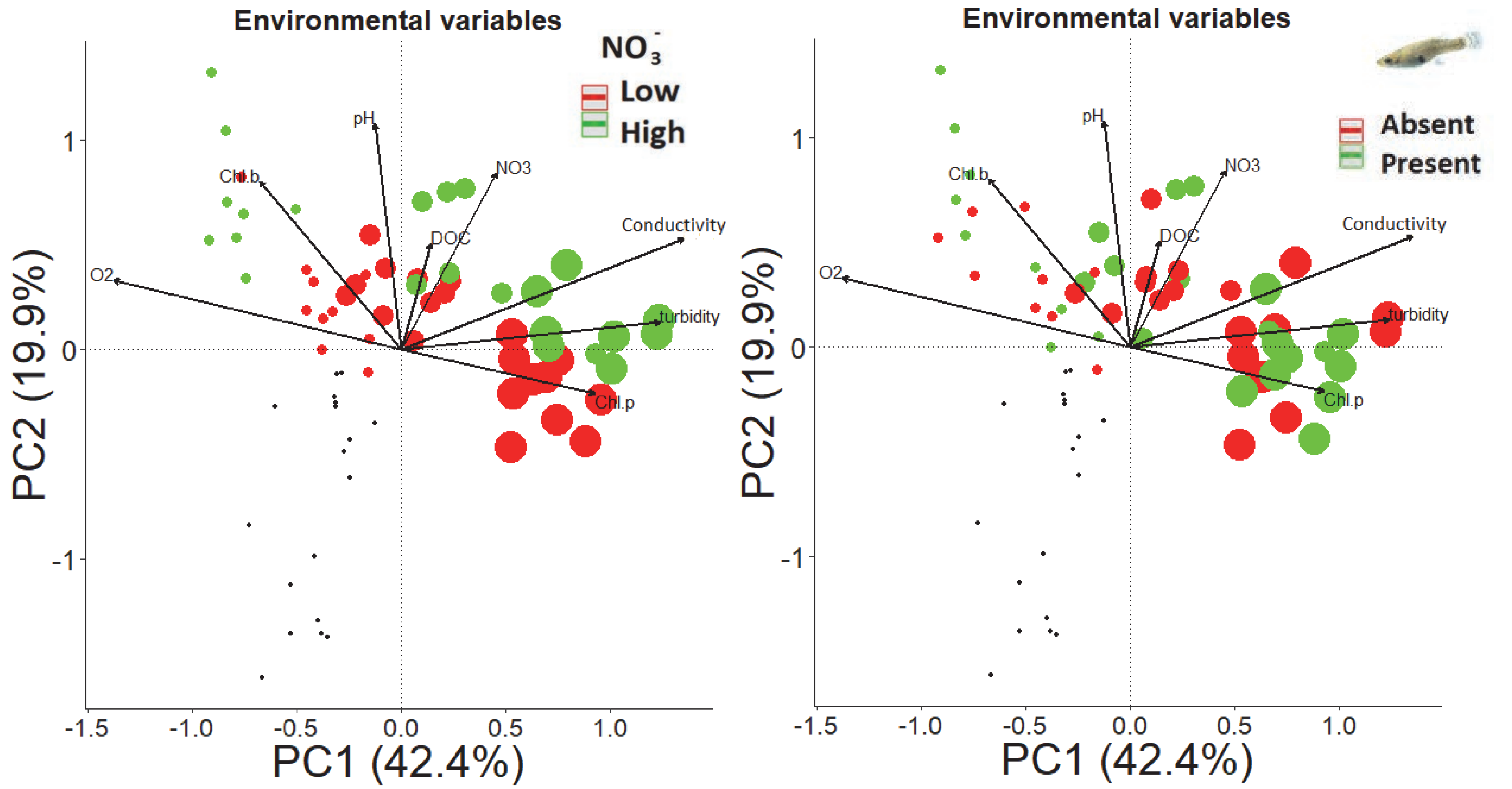


Figure 1. Partial principal component analysis of water quality and ecosystem processes measured over 11 weeks while accounting for the effects of mesocosms identity. Highly correlated variables ($r \geq 0.80$) are omitted to facilitate visualisation. Black dots are mesocosms before adding fish or nitrate (t_0) and bright coloured dots indicate the effects of nitrate addition (left) and of the fish addition (right). Dot size increase with the duration of the experiment: t_1 (1 week), t_2 (5 weeks) and t_3 (10 weeks). DOC: dissolved organic carbon; Chl-b: benthic chlorophyll-a; Chl-p: planktonic chlorophyll-a.

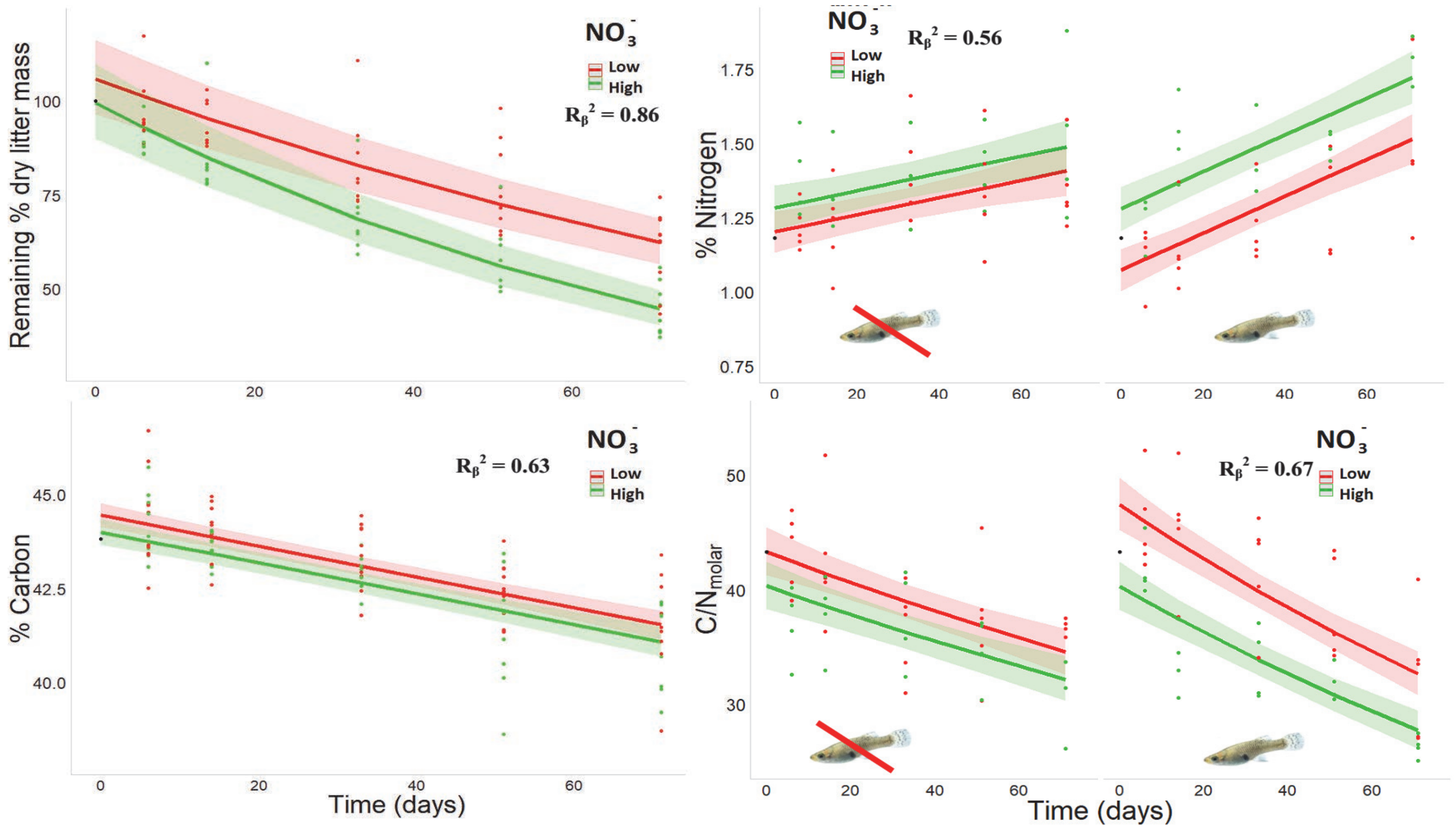


Figure 2. Predicted effects of nitrate and fish on leaf litter decomposition, as estimated by: a) the remaining % dry litter mass, b) the elementary composition of nitrogen (%), c) elementary composition of carbon (%), and d) the carbon to nitrogen molar ratio. The fish effect in % dry litter mass and % C is not significant and thus not splitted in two facets.

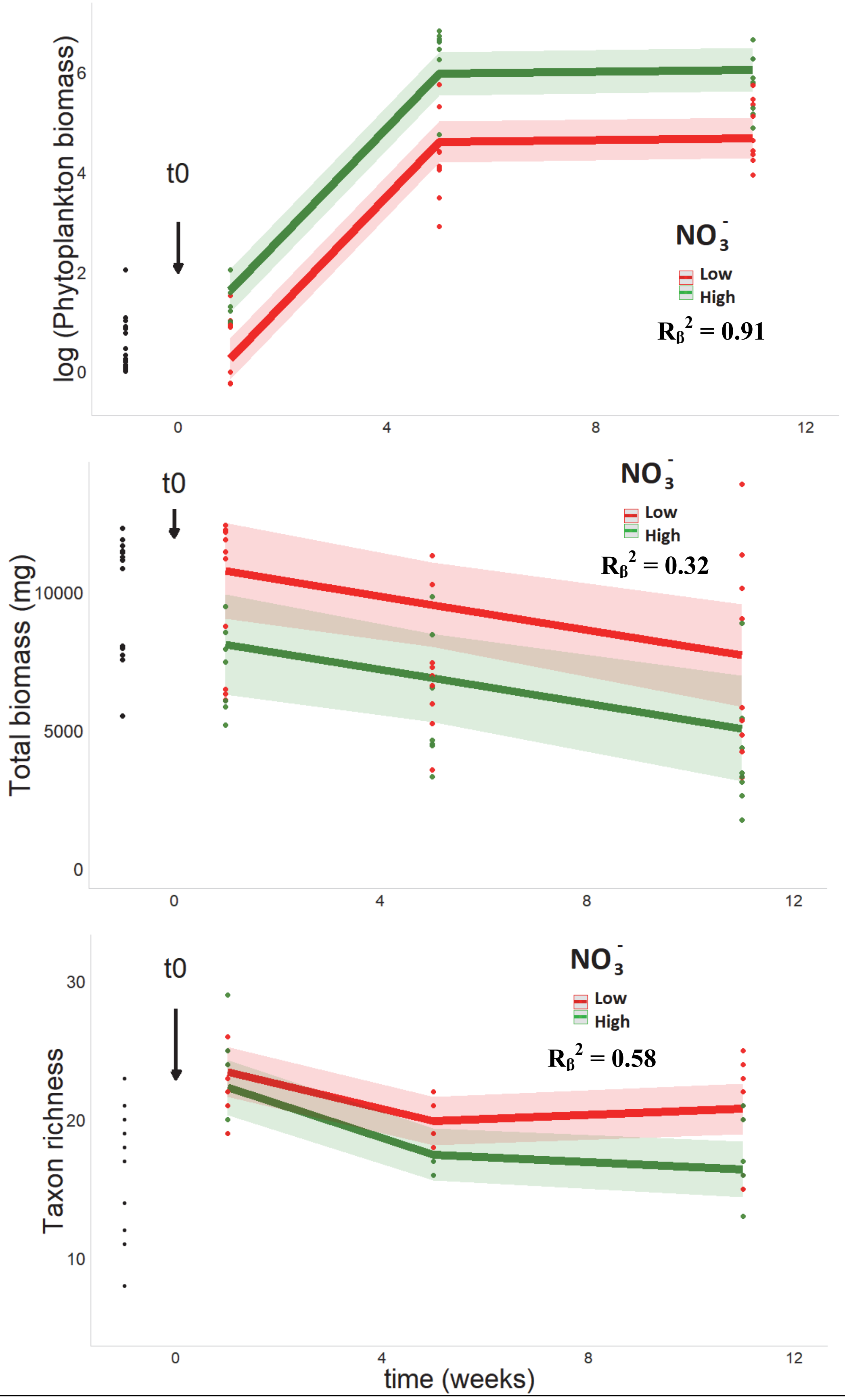


Figure 3. Predicted effects of nitrate on three community metrics from models with the highest explained variance, a) log-transformed phytoplankton biomass, b) total biomass, c) total richness.

3.3. Identification of the best sentinel taxa

Changes in ecosystem properties allowed us to determine which community metrics, or particular taxa, best reflected the ecological effects of nitrate or fish addition (Fig. 4).

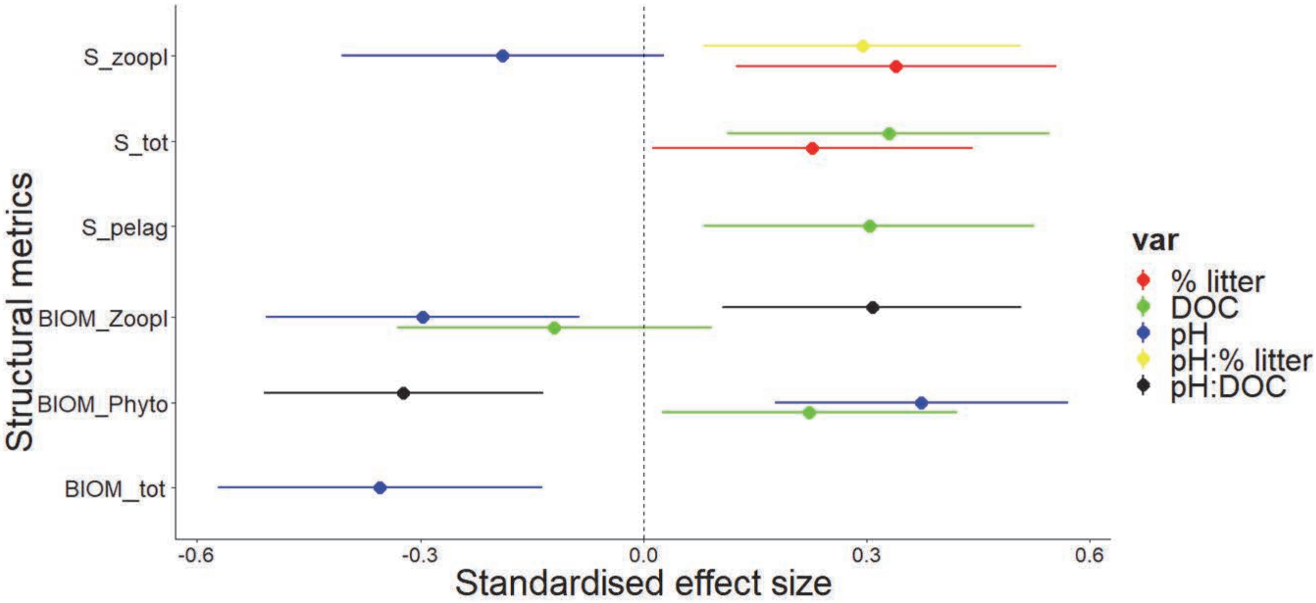


Figure 4. Predicted standardised effect sizes of the functional measured indicators (% remaining litter, dissolved organic carbon, pH and % nitrogen in litter) on the selected community metrics. S_zoopl and BIOM_Zoopl: zooplancton richness and biomass, S_tot and BIOM_tot: total pond richness and biomass, S_pelag: pelagic richness, BIOM_Phyto: phytoplankton biomass. % nitrogen was not selected in any model and is not shown. Interactions between predictors are included when significant.

At the community level, models for zooplankton and phytoplankton biomass had the highest explained variance ($R_\beta^2 = 0.24 – 0.34$) and consistently identified pH and DOC as the most important predictors. Similarly, DOC and % remaining leaf mass were the most important predictors for total richness in mesocosms, but the model had a slightly lower fit ($R_\beta^2 = 0.22$). Models for total biomass, the richness of all pelagic taxa and of zooplankton had the lowest fits (All $R_\beta^2 < 0.20$). Overall, phytoplankton biomass increased with pH, whereas that of zooplankton declined. DOC modulated these associations with greater effects at low levels. Total richness in mesocosms increased with DOC and % remaining leaf mass and total biomass declined at high pH values.

Table 2. Indicator value analysis of all sampled taxa in the pelagic and benthic compartments. Taxa are grouped by size (phytoplankton, microzooplankton and other invertebrates) and assigned to the cluster (nitrate low/high and fish absent/present) with higher indicator value (IndVal.g). IndVal.g is the product of specificity (A) and fidelity (B). P-values of confidence intervals calculated by bootstrapping are also shown and taxa highlighted in bold ($P < 0.05$).

Taxon cluster	Association cluster	A	B	IndVal.g	P
PHYTOPLANKTON					
***Closterium* spp.**	Nitrate low	0.919	0.300	0.525	0.022
Chlorococcum spp.		0.778	0.267	0.455	0.716
***Cosmarium* spp.**	Nitrate high	0.950	1.000	0.974	<0.001
***Tetraedron* spp.**		0.860	1.000	0.927	<0.001
Pediastrum spp.		0.709	0.792	0.749	0.202
Cladophora spp.		0.589	0.625	0.607	0.852
Oocystis spp.	Fish absent	0.582	1.000	0.763	0.909
Scenedesmus spp.		0.533	0.923	0.703	0.882
MICROZOOPLANKTON					
Keratella spp.	Nitrate low	0.653	0.667	0.660	0.171
Ciliates		0.738	0.400	0.543	0.275
Lecane spp.	Nitrate high	0.778	0.625	0.697	0.266
Squatinella spp.	Fish present	0.762	0.370	0.531	0.108
INVERTEBRATES					
Cyclopoida	Nitrate low	0.763	1.000	0.873	<0.001
Chironomini		0.683	0.967	0.813	0.019
Daphniidae		0.794	0.800	0.797	0.011
Oligochaeta		0.611	1.000	0.782	0.002
***Hydra* spp.**		0.793	0.733	0.763	0.002
Ostracoda		0.554	1.000	0.744	0.652
Physa spp.		0.567	0.967	0.741	0.329
Tanytarsini		0.713	0.433	0.556	0.251
Caenidae		0.866	0.300	0.510	0.123
Anisoptera		0.828	0.300	0.498	0.089
Microturbellaria		0.591	0.367	0.466	0.870
Chydoridae	Nitrate high	0.567	0.708	0.633	0.841
Ceratopogonidae		0.726	0.417	0.550	0.154
Copepod nauplii	Fish absent	0.670	0.963	0.803	0.019
Orthocladiinae		0.592	0.963	0.755	0.386
Nematoda		0.606	0.852	0.719	0.432
Juvenile cladocerans		0.945	0.444	0.648	0.001
Macrothricidae	Fish present	0.503	0.889	0.669	0.942
Planorbarius spp.		0.634	0.185	0.343	0.959

Total number of taxa: 31 ($B > 0.2$ in any treatment group). Nº permutations = 10000. Blocked by time.

At the taxon level, the green algae *Tetraedron minimum*, *Cosmarium formosulum* and *C. biretum* were the best indicators of nitrate pollution among the 40 taxa from 15 classes and 20 orders recorded (Table 2). The benthic Cyclopoid copepods were the best indicators of low nitrate pollution. Other taxa showing significant associations with low nitrate levels were Chironomini, benthic daphnids, oligochaetes, the cnidarian *Hydra* spp. and the algae *Closterium moniliferum*. No taxon was clearly related to the presence of fish, but there were more planktonic copepods and cladocerans in fishless tanks. Similarly, *Lecane* spp. was associated with nitrate pollution and *Keratella* spp. was with clean waters, albeit the relationships for rotifers were not statistically clear. Other metazoan (e.g. Nematoda, microturbellaria, Table 2) were neither significantly associated with any treatment combination.

4. Discussion

One of the major challenges of environmental risk assessments is to determine the risk of interactive effects among perturbations and to identify the most sensitive taxa to each perturbation (Friberg et al., 2011). Our study suggests limited risk for interactive effects between nutrient pollution and *G. holbrooki* invasion, two of the major perturbations affecting freshwater ecosystems (Vitousek et al., 1997; Pyke, 2008). We identified ten ecosystem properties, three community metrics and eight taxa that are suitable to monitor nitrate effects. We also proved that *G. holbrooki* increased primary production and nitrogen content in the detrital food-web and reduced the abundance of planktonic copepods and cladocerans. Correlations between community metrics and ecosystem processes are used to discuss the ecological relevance of a total of 21 indicators examined in our 11-week study.

4.1. Additive effects prevail over interactions between nitrate and fish invasion

Nutrients, including nitrate (NO_3^-), were expected to amplify the ecological impacts of *G. holbrooki* by means of increasing fish growth and reproduction (Preston et al., 2018). However, this hypothesis was not supported because *G. holbrooki* biomass in tanks with nitrate was lower than in the controls at the end of the experiment and mesocosms did not differ in fish density. Outcomes are likely to be due to nitrate toxicity because most fish in mesocosms were juveniles and body condition was reduced in juveniles exposed for eight weeks to 250 mg/l NO_3^-/l in aquaria (Cano-Rocabayera et al., 2019a). Reduced fish body condition and growth in mesocosms may also be due to *Cladophora glomerata* outgrowth with nitrate because *Cladophora* increased habitat complexity, and so, prey might have been more difficult to detect and catch (Browder, 1991). Nitrate was expected to alter reproduction because sperm quality was reduced in *G. holbrooki* at 22 mg NO_3^-/l (Edwards and Guillette, 2007). However, fish density in mesocosms probably was not affected because the reproductive plasticity of *G. holbrooki* is high (Cano-Rocabayera et al., 2019b). *Gambusia holbrooki* might have offset any negative effect of nitrate by reducing offspring size but increasing offspring number, as does the also poeciliid guppy (*Poecilia reticulata*) exposed to harsh conditions in Trinidadian streams (Fitzpatrick et al., 2014). Nonetheless, our study cannot discern between females giving birth smaller new-borns when exposed to nitrate or these new-borns growing slower than those in the controls.

The absence of interactive effects between nitrate and *G. holbrooki* neither supported the hypothesis that nutrients can weaken the impacts of *G. holbrooki* through enhancing primary and secondary production (Balčiūnas and Fowler, 1995). Fish biomass was in our study (19 – 31 g/m^3) within the range or 7-fold higher than values (4 – 27 g/m^3) in studies at which impacts of *G. holbrooki* were reported at the community or ecosystem levels (e.g. Lancaster and Drenner, 1990; Cardona, 2006; Preston et al., 2018). However, *G. holbrooki* altered primary production but did not affect consumers. It is possible that nitrate might have altered prey behaviour (Watt and Oldham, 1995) or the increased turbidity might have reduced the ability of *G. holbrooki* to catch prey (Nieman et al., 2018; Cano-rocabayera et al., *under review*), leading to a non-detected effect of fish predation on invertebrates. Potential differences in the ability of prey to deal with *G. holbrooki* are unlikely to be much attributed to changes in taxa composition due to nitrate. Nitrate did not affect the richness or biomass of zooplankton and *G. holbrooki* is mostly zooplanktivorous (García-Berthou, 1999; Pyke, 2008). Functional replacements among species of grazers preferentially captured by *G. holbrooki* (e.g. cladocerans) and other grazers occurring in the community (e.g. Ceratopogonidae, Ostracoda) might also explain the lack of significant interactions between *G. holbrooki* and nitrate.

4.2. A fish invasion may not much affect community metrics but ecosystem properties

Our findings supported the notion that community metrics and measures of ecosystem processes are complementary in biological diagnostics (Friberg et al., 2011). Differences in ecosystem processes (chlorophyll-a concentration and nitrogen content in litter) occurred between invaded and non-invaded tanks irrespective of nitrate concentration. However, exposure to high nitrate levels resulted led to differences in these two ecosystem processes, together with alterations in the rate of litter decomposition and in three community metrics (total biomass and richness, phytoplankton biomass). Greater planktonic chlorophyll-a with *G. holbrooki* probably was due to this species feeding on phytoplankton grazers (cladocerans and copepods, García-Berthou, 1999). Nevertheless, measures of planktonic chlorophyll-a were inconsistent with phytoplankton biomass despite being both often used as proxies for primary production (Elosegui and Sabater, 2009; Longhurst and Harrison, 1989). Light limitation or a nutrient imbalance are often associated with this mismatch (Longhurst and Harrison, 1989; Felip and Catalan, 2000) as was our case because only nitrate was added. Together with planktonic chlorophyll-a, benthic chlorophyll-a increased with *G. holbrooki*, suggesting that fish affected periphyton grazers (e.g. Ostracoda and the snails *Physa acuta* and *Planorbarius* spp.). However, invaded and non-invaded tanks did not differ in the abundance of these taxa, so that fish predation probably was not high. *Gambusia holbrooki* might have bitten grazers reaching the microscope slides at 15 cm from the water surface that we used to quantify benthic chlorophyll-a. This behaviour between *G. holbrooki* and snails is often seen in aquaria (A.M.V. *pers. observ.*) and there are reports of non-lethal injuries caused by *G. holbrooki* to other animals (e.g. amphibians and fish, Alcaraz et al., 2008; Shulse and Semlitsch, 2014). Moreover, primary production might have increased with fish because additional nutrients were released into mesocosms (i.e. excreted ammonia and phosphorous, El-Sabaawi et al., 2015).

Litter-nitrogen content was the only ecosystem process altered by *G. holbrooki* in clean and nitrate-enriched waters with interactive effects. Litter-nitrogen content declined initially with fish invasion but only in clean waters and increased with nitrate pollution regardless of fish addition. However, fish enhanced nitrogen immobilisation rate. Greater litter nitrogen content along the decomposition process is due to microbial colonisation (Pascoal and Cássio, 2004; Elosegui and Sabater, 2009). Our results therefore support the idea that a moderate fertilisation stimulates litter processing (Pascoal and Cássio, 2004; Elosegui and Sabater, 2009). Further evidence of this is that the highest litter break-down rates were in nitrate polluted tanks. Litter breakdown by invertebrates and microbes is faster than by microbes alone (Elosegui and Sabater, 2009), so that effects on litter nitrogen alone might be due to *G. holbrooki* having an impact on litter-processing invertebrates. This contrasts with the fact that effects of *G.*

holbrooki on total mesocosms richness and biomass, which was highly correlated with that of benthic taxa, were not detected. Outcomes are probably attributed to benthic taxa having the coarser classification in the study (e.g. oligochaete, nematode, microturbellaria), which prevents the detection of subtle impacts on diversity measures (Heino and Soininen, 2007). However, total mesocosm biomass, which was highly correlated with that of benthic taxa, was neither affected by *G. holbrooki* and changes in biomass often influence ecosystem processes (Carpenter and Lodge, 1986; Naeem et al., 1994). It is possible that our inferences would have improved with detailed information on the direct impacts of nitrate on the grazing behaviour of detritivores (e.g. Macrothricidae and Ostracoda preferring nitrogen enriched leaves in nitrate ponds). Moreover, our benthic surveys captured dragonfly larvae, which feed on similar prey to *G. holbrooki* (May, 2019) and often replace small fish in the top down control of ponds (May, 2019). Nonetheless, we probably underestimated dragonfly biomass because there were dragonflies in the multifilament aggregations of *C. glomerata*, which we did not survey so as not to disturb the experimental setting using dip-netting.

4.3. Identifying suitable indicators for biological monitoring

Variation in the abundance of 40 taxa from 15 classes and 20 orders across mesocosms allowed us to identify characteristic taxa for each perturbation. For nutrients, we cannot discern between the effects of the ion NO_3^- and other water-quality alterations due to $NaNO_3$ addition, i.e. high conductivity and nitrite. However, all synthetic fertilizers are salts and the concentration of Na^+ was ~1.5-fold lower than that of NO_3^-. Nitrate reduction also occurs in natural ecosystems (An and Gardner, 2002), and the mechanisms of nitrite toxicity are similar to those of nitrate (Edwards and Guillette, 2007). Our indicator values are more ecologically relevant than traditional single-toxicant assays because values reflect changes in water quality and the resultant alterations in species interactions (Colin et al., 2016). The observed decline in total mesocosm biomass and richness due to nitrate is consistent with eutrophication simplifying aquatic communities (Garg and Bhatnagar, 1996; Smith et al., 1999). Besides water quality, the insecticidal and allelopathic properties of the algae *C. glomerata* might have contributed to the extirpation of invertebrate and other algae (Dodds and Gudder, 1992). Mesocosms did not have cyanoprokaryota, which can be toxic (e.g. Camacho, 2008). However, mortality can occur with high concentrations of autotrophs due to oxygen depletion at night (Scheffer, 2004). We only have diurnal measures of oxygen, but *G. holbrooki* can withstand at as low levels as 1 mg O_2/l (Pyke, 2008).

The biomass of phytoplankton was the most informative community metric to detect the effects of nitrate, which is consistent with planktonic algae being the most surveyed taxa in official monitoring programs (e.g. Resh, 2008). However, the second model with the highest explained variance was the one for total-mesocosms richness, supporting the expectation that nitrate reshapes the benthic and pelagic compartments in mesocosms. Although considerably amount of variation in models was attributed to the duration of the experiment, the only significant interaction was between nitrate and the sampling date. The longer the exposure to a contaminant is, the higher are usually the effects on organisms, including nitrate (Cano-Rocabayera et al., 2019a). Water temperature increased from 14 ºC in mid-April to 24 ºC in July and all community metrics were influenced by increasing warming. The exception was total-plankton biomass, possibly because time had opposite effects on phytoplankton (increase) and zooplankton (decline), irrespective of the experimental factors. The absence of strong impacts of *G. holbrooki* on mesocosms may be due to the acclimation of the invertebrate community to the predator when sharing a common evolutionary history (Sih et al., 2010), but this hypothesis needs more replicates and longer experiments than ours to be confirmed.

Regarding the sensitivity of specific taxa to nitrate, the diminished presence of Daphniidae in high nitrate mesocosms is concordant with the lethal effects reported for *Daphnia magna* at 250 mg NO_3^-/l (from KNO_3) in individual assays and microcosms (Maceda-Veiga et al., 2015). Other taxa that were rare in nitrate polluted tanks (oligochaetes, copepods, *Hydra* spp.) are usually not found at salinities $\geq$ 1 g/l (Hart et al., 1991; Tesh et al., 1990), and nitrate polluted tanks had a salinity of c. 1.2 g/l. The lack of definite associations between nitrate and the rotifers *Lecane* sp. or *Keratella* sp. is consistent with these two taxa being water quality generalists (Arora, 1966). Associations of *Cosmarium formosulum*, *C. biretum*, and *Tetraedron minimum* with nitrate pollution confirm the suitability of these algae as bioindicators of eutrophication (Coesel, 1993; Tavera and Castillo, 2000; Allinger and Reavie, 2013). The highest planktonic chlorophyll-a concentration in mesocosms were $\leq$10 mg/m^3, which corresponds to mesotrophic-eutrophic ponds (Nürnberg, 1996). Eutrophication occurred with a highly unbalanced nitrogen and phosphorus ratio (max 0.8 mg PO_4-P/l vs 400 mg NO_3^-/l), so we can say that phosphorus was not a limiting nutrient for primary producers, although it might have been if the experiment would have lasted longer.

5. Conclusions

We provide experimental evidence of the suitability of eight taxa to monitor the effects of eutrophication. Eutrophication was due to high levels of nitrate alone and the ecological effects were lower than those reported for phosphate pollution (Lancaster and Drenner, 1990; Preston et al., 2018). Results show that higher accuracies in assessments will be obtained if biomonitoring accounts for phytoplankton biomass and the total richness and biomass of plankton and benthos. The lack of statistically clear effects for *G. holbrooki* invasion in mesocosms does not diminish its ecological risk, being *G. holbrooki* one of the most pernicious fish invasions around the world (Pyke, 2008). The effects of invasions are often abrupt and hard to predict (Eilers et al., 2007), therefore the prevention of introductions is the only safe management option.

Acknowledgements

We are very grateful to Clàudia Pastor, Marina Vergotti, Alessandro di Peso, Daniela Díaz, Carles Aranda, Sergi Vargas, Nicole Colin and Maria González for laboratory procedures and field assistance. We also thank all the staff from Servei de Control de Mosquits del Baix Llobregat, Parc Agrari and Consorci per a la protecció i la gestió dels espais naturals del delta del Llobregat, who provided the field logistics. Finally, Marisol Felip, Pepita Nolla, Regina Roca, Rosa Mª Marimon and the staff at CCiTUB provided technical help. This study was funded by the Fundació Barcelona Zoo - Ajuntament de Barcelona. OCR was financed by by an APIF grant from the University of Barcelona.

6. References

Alcaraz, C., Bisazza, A., & García-Berthou, E. (2007). Salinity mediates the competitive interactions between invasive mosquitofish and an endangered fish. Oecologia, 155(1), 205–213. doi:10.1007/s00442-007-0899-4

Allinger, L. E., & Reavie, E. D. (2013). The ecological history of Lake Erie as recorded by the phytoplankton community. Journal of Great Lakes Research, 39(3), 365–382. doi:10.1016/j.jglr.2013.06.014

An, S., & Gardner, W. (2002). Dissimilatory nitrate reduction to ammonium (DNRA) as a nitrogen link, versus denitrification as a sink in a shallow estuary (Laguna Madre/Baffin Bay, Texas). Marine Ecology Progress Series, 237, 41–50. doi:10.3354/meps237041

Arora, H. C. (1966). Rotifera as indicators of trophic nature of environments. Hydrobiologia, 27(1-2), 146–159. doi:10.1007/bf00161493

Bain, M. B. (1993). Assessing impacts of introduced aquatic species: Grass carp in large systems. Environmental Management, 17(2), 211–224. doi:10.1007/bf02394691

Balciunas, D., & Lawler, S. P. (1995). Effects of Basal Resources, Predation, and Alternative Prey in Microcosm Food Chains. Ecology, 76(4), 1327–1336. doi:10.2307/1940939

Bonada, N., Prat, N., Resh, V. H., & Statzner, B. (2006). Developments in aquatic insect biomonitoring: A Comparative Analysis of Recent Approaches. Annual Review of Entomology, 51(1), 495–523. doi:10.1146/annurev.ento.51.110104.151124

Browder, J. A. (1991). Periphyton in the Everglades: spatial variation, environmental correlates, and ecological implications. In: Davis, S., Ogden, J. (eds.). Proceedings of Everglades Symposium, Key Largo, Florida.

Cáceres, M. D., & Legendre, P. (2009). Associations between species and groups of sites: indices and statistical inference. Ecology, 90(12), 3566–3574. doi:10.1890/08-1823.1

Camacho, F. A. (2008). Macroalgal and Cyanobacterial Chemical Defenses in Freshwater Communities. Algal Chemical Ecology, 105–119. doi:10.1007/978-3-540-74181-7_5

Cano-Rocabayera, O., de Sostoa, A., Padrós, F., Cárdenas, L., & Maceda-Veiga, A. (2019a). Ecologically relevant biomarkers reveal that chronic effects of nitrate depend on sex and life stage in the invasive fish *Gambusia holbrooki*. PLOS ONE, 14(1), e0211389. doi:10.1371/journal.pone.0211389

Cano-Rocabayera, O., de Sostoa, A., Coll, L., & Maceda-Veiga, A. (2019). Managing small, highly prolific invasive aquatic species: Exploring an ecosystem approach for the eastern mosquitofish (*Gambusia holbrooki*). Science of The Total Environment, 673, 594–604. doi:10.1016/j.scitotenv.2019.02.460

Cardona, L. (2006). Trophic Cascades Uncoupled in a Coastal Marsh Ecosystem. Biological Invasions, 8(4), 835–842. doi:10.1007/s10530-005-0420-0

Carpenter, S. R., & Lodge, D. M. (1986). Effects of submersed macrophytes on ecosystem processes. Aquatic Botany, 26, 341–370. doi:10.1016/0304-3770(86)90031-8

Coesel, P. F. M. (1993). Poor physiological adaptation to alkaline culture conditions inClosterium acutumvar.variabile, a planktonic desmid from eutrophic waters. European Journal of Phycology, 28(1), 53–57. doi:10.1080/09670269300650081

Colin, N., Porte, C., Fernandes, D., Barata, C., Padrós, F., Carrassón, M., … & Maceda-Veiga, A. (2016). Ecological relevance of biomarkers in monitoring studies of macro-invertebrates and fish in Mediterranean rivers. Science of The Total Environment, 540, 307–323. doi:10.1016/j.scitotenv.2015.06.099

Dempster, T., Sanchez-Jerez, P., Fernandez-Jover, D., Bayle-Sempere, J., Nilsen, R., Bjørn, P.-A., & Uglem, I. (2011). Proxy Measures of Fitness Suggest Coastal Fish Farms Can Act as Population Sources and Not Ecological Traps for Wild Gadoid Fish. PLoS ONE, 6(1), e15646. doi:10.1371/journal.pone.0015646

Didham, R., Tylianakis, J., Gemmell, N., Rand, T., & Ewers, R. (2007). Interactive effects of habitat modification and species invasion on native species decline. Trends in Ecology & Evolution, 22(9), 489–496. doi:10.1016/j.tree.2007.07.001

Diez, J. M., Buckley, H. L., Case, B. S., Harsch, M. A., Sciligo, A. R., Wangen, S. R., & Duncan, R. P. (2009). Interacting effects of management and environmental variability at multiple scales on invasive species distributions. Journal of Applied Ecology. doi:10.1111/j.1365-2664.2009.01725.x

Dodds, W. K., & Gudder, D. A. (1992). The ecology of *Cladophora*. Journal of Phycology, 28(4), 415–427. doi:10.1111/j.0022-3646.1992.00415.x

Edwards, T. M, & Guillette, L. J. (2007). Reproductive characteristics of male mosquitofish (*Gambusia holbrooki*) from nitrate-contaminated springs in Florida. Aquatic Toxicology, 85, 40-47. doi: 10.1016/j.aquatox.2007.07.014

Edwards, T. M., Miller, H. D., & Guillette, L. J. (2006). Water Quality Influences Reproduction in Female Mosquitofish (*Gambusia holbrooki*) from Eight Florida Springs. Environmental Health Perspectives, 114(Suppl 1), 69–75. doi:10.1289/ehp.8056

Eilers, J. M., Loomis, D., St. Amand, A., Vogel, A., Jackson, L., Kann, J., … & Sweets, R. (2007). Biological effects of repeated fish introductions in a formerly fishless lake: Diamond Lake, Oregon, USA. Fundamental and Applied Limnology / Archiv Für Hydrobiologie, 169(4), 265–277. doi:10.1127/1863-9135/2007/0169-0265

Elosegui, A., & Sabater, S. (eds). (2009). Conceptos y técnicas en ecología fluvial. Fundación BBVA, Bilbao.

El-Sabaawi, R. W., Bassar, R. D., Rakowski, C., Marshall, M. C., Bryan, B. L., Thomas, S. N., … & Flecker, A. S. (2015). Intraspecific phenotypic differences in fish affect ecosystem processes as much as bottom-up factors. Oikos, 124(9), 1181–1191. doi:10.1111/oik.01769

European Commission. (2018). report from the Commission to the Council and the European Parilament on the implementation of Council Directive 91/676/EEC concerning the protection of waters against pollution caused by nitrates from agricultural sources based on Member State reports for the period 2012-2015.

Felip, M., & Catalan, J. (2000). The relationship between phytoplankton biovolume and chlorophyll in a deep oligotrophic lake: decoupling in their spatial and temporal maxima. Journal of Plankton Research, 22(1), 91–106. doi:10.1093/plankt/22.1.91

Fitzpatrick, S. W., Torres-Dowdall, J., Reznick, D. N., Ghalambor, C. K., & Chris Funk, W. (2014). Parallelism Isn't Perfect: Could Disease and Flooding Drive a Life-History Anomaly in Trinidadian Guppies? The American Naturalist, 183(2), 290–300. doi:10.1086/674611

Fox, J., & Weisberg, S. (2011). An R Companion to Applied Regression, 2nd ed. Sage, Thousand Oaks. Available at: http://socserv.socsci.mcmaster.ca/jfox/Books/Companion

Friberg, N., Bonada, N., Bradley, D. C., Dunbar, M. J., Edwards, F. K., Grey, J., … & Woodward, G. (2011). Biomonitoring of Human Impacts in Freshwater Ecosystems. Advances in Ecological Research, 1–68. doi:10.1016/b978-0-12-374794-5.00001-8

Gallardo, B., Clavero, M., Sánchez, M. I., & Vilà, M. (2015). Global ecological impacts of invasive species in aquatic ecosystems. Global Change Biology, 22(1), 151–163. doi:10.1111/gcb.13004

García-Berthou, E. (1999). Food of introduced mosquitofish: ontogenetic diet shift and prey selection. Journal of Fish Biology, 55(1), 135–147. doi:10.1006/jfbi.1999.0983

Garg, S. K., & Bhatnagar, A. (1996). Effect of varying closes of organic and inorganic fertilizers on plankton production and fish biomass in brackish water fish ponds. Aquaculture Research, 27(3), 157–166. doi:10.1111/j.1365-2109.1996.tb00980.x

Geyer, R. L., Smith, G. R., & Rettig, J. E. (2016). Effects of Roundup formulations, nutrient addition, and Western mosquitofish (*Gambusia affinis*) on aquatic communities. Environmental Science and Pollution Research, 23(12), 11729–11739. doi:10.1007/s11356-016-6381-2

Gobierno de España. (2011). Real Decreto 1628/2011, de 14 de noviembre, por el que se regula el listado y catálogo español de especies exóticas invasoras. BOE, 298.

Grup de Defensa del Ter. (2019). In: Nitrate concentration in Springs of Osona [Internet]. Available at: https://www.gdter.org/wp/wp-content/uploads/GDT-NITRATS-OSONA-01-2019.pdf

Hall, S. J., Huber, D. P., & Hughes, R. F. (2018). Invasion of Hawaiian rainforests by an introduced amphibian predator and N_2-fixing tree increases soil N_2O emissions. Ecosphere, 9(9), e02416. doi:10.1002/ecs2.2416

Hart, B. T., Bailey, P., Edwards, R., Hortle, K., James, K., McMahon, A., … & Swadling, K. (1991). A review of the salt sensitivity of the Australian freshwater biota. Hydrobiologia, 210(1-2), 105–144. doi:10.1007/bf00014327

Heino, J., & Soininen, J. (2007). Are higher taxa adequate surrogates for species-level assemblage patterns and species richness in stream organisms? Biological Conservation, 137(1), 78–89. doi:10.1016/j.biocon.2007.01.017

Hickey, C. W., & Martin, M. L. (2009). A review of nitrate toxicity to freshwater aquatic species. Environment Canterbury.

Hinchliffe, C., Atwood, T., Ollivier, Q., & Hammill, E. (2017). Presence of invasive *Gambusia* alters ecological communities and the functions they perform in lentic ecosystems. Marine and Freshwater Research, 68(10), 1867. doi:10.1071/mf16301

Jaeger, B. C., Edwards, L. J., Das, K., & Sen, P. K. (2016): An R^2 statistic for fixed effects in the generalized linear mixed model, Journal of Applied Statistics. doi: 10.1080/02664763.2016.1193725

Lancaster, H. F., & Drenner, R. W. (1990). Experimental Mesocosm Study of the Separate and Interaction Effects of Phosphorus and Mosquitofish (*Gambusia affinis*) on Plankton Community Structure. Canadian Journal of Fisheries and Aquatic Sciences, 47(3), 471–479. doi:10.1139/f90-051

Longhurst, A. R., & Glen Harrison, W. (1989). The biological pump: Profiles of plankton production and consumption in the upper ocean. Progress in Oceanography, 22(1), 47–123. doi:10.1016/0079-6611(89)90010-4

Maceda-Veiga, A., Webster, G., Canals, O., Salvadó, H., Weightman, A. J., & Cable, J. (2015). Chronic effects of temperature and nitrate pollution on *Daphnia magna*: Is this cladoceran suitable for widespread use as a tertiary treatment? Water Research, 83, 141–152. doi:10.1016/j.watres.2015.06.036

Martínez-Santos, P., de Stefano, L., Llamas, M. R., & Martínez-Alfaro, P. E. (2008). Wetland Restoration in the Mancha Occidental Aquifer, Spain: A Critical Perspective on Water, Agricultural, and Environmental Policies. Restoration Ecology, 16(3), 511–521. doi:10.1111/j.1526-100x.2008.00410.x

May, M. (2019). Odonata: Who They Are and What They Have Done for Us Lately: Classification and Ecosystem Services of Dragonflies. Insects, 10(3), 62. doi:10.3390/insects10030062

Naeem, S., Thompson, L. J., Lawler, S. P., Lawton, J. H., & Woodfin, R. M. (1994). Declining biodiversity can alter the performance of ecosystems. Nature, 368(6473), 734–737. doi:10.1038/368734a0

Nieman, C. L., Oppliger, A. L., McElwain, C. C., & Gray, S. M. (2018). Visual detection thresholds in two trophically distinct fishes are compromised in algal compared to sedimentary turbidity. Conservation Physiology, 6(1). doi:10.1093/conphys/coy044

Nürnberg, G. K. (1996). Trophic State of Clear and Colored, Soft- and Hardwater Lakes with Special Consideration of Nutrients, Anoxia, Phytoplankton and Fish. Lake and Reservoir Management, 12(4), 432–447. doi:10.1080/07438149609354283

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., … & Wagner, H. (2019). Vegan: community ecology package. Available at: https://CRAN.R-project.org/package=vegan

Pascoal, C., & Cassio, F. (2004). Contribution of Fungi and Bacteria to Leaf Litter Decomposition in a Polluted River. Applied and Environmental Microbiology, 70(9), 5266–5273. doi:10.1128/aem.70.9.5266-5273.2004

Peig, J., & Green, A. J. (2009). New perspectives for estimating body condition from mass/length data: the scaled mass index as an alternative method. Oikos, 118(12), 1883–1891. doi:10.1111/j.1600-0706.2009.17643.x

Preston, D. L., Hedman, H. D., & Johnson, P. T. J. (2018). Nutrient availability and invasive fish jointly drive community dynamics in an experimental aquatic system. Ecosphere, 9(3), e02153. doi:10.1002/ecs2.2153

Pyke, G. H. (2008). Plague Minnow or Mosquito Fish? A Review of the Biology and Impacts of Introduced *Gambusia* Species. Annual Review of Ecology, Evolution, and Systematics, 39(1), 171–191. doi:10.1146/annurev.ecolsys.39.110707.173451

R Core Team. (2016). In: R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria [software]. Available at: http://www.R-project.org/

Resh, V. H. (2008). Which group is best? Attributes of different biological assemblages used in freshwater biomonitoring programs. Environmental Monitoring and Assessment, 138(1-3), 131–138. doi:10.1007/s10661-007-9749-4

Romo, S., Miracle, M. R., Villena, M.-J., Rueda, J., Ferriol, C., & Vicente, E. (2004). Mesocosm experiments on nutrient and fish effects on shallow lake food webs in a Mediterranean climate. Freshwater Biology, 49(12), 1593–1607. doi:10.1111/j.1365-2427.2004.01305.x

Scheffer, M. (2004). Ecology of Shallow Lakes. doi:10.1007/978-1-4020-3154-0

Shulse, C. D., & Semlitsch, R. D. (2014). Western mosquitofish (*Gambusia affinis*) bolster the prevalence and severity of tadpole tail injuries in experimental wetlands. Hydrobiologia, 723(1), 131–144. doi:10.1007/s10750-013-1502-0

Sih, A., Bolnick, D. I., Luttbeg, B., Orrock, J. L., Peacor, S. D., Pintor, L. M., … & Vonesh, J. R. (2010). Predator-prey naïveté, antipredator behavior, and the ecology of predator invasions. Oikos, 119(4), 610–621. doi:10.1111/j.1600-0706.2009.18039.x

Simberloff, D., Martin, J. L., Genovesi, P., Maris, V., Wardle, D. A., Aronson, J., ... & Pyšek, P. (2013). Impacts of biological invasions: what's what and the way forward. Trends in ecology & evolution, 28(1), 58-66. doi:10.1016/j.tree.2012.07.013

Simon, K. S., & Townsend, C. R. (2003). Impacts of freshwater invaders at different levels of ecological organisation, with emphasis on salmonids and ecosystem consequences. Freshwater Biology, 48(6), 982–994. doi:10.1046/j.1365-2427.2003.01069.x

Smith, V. H., Tilman, G. D., & Nekola, J. C. (1999). Eutrophication: impacts of excess nutrient inputs on freshwater, marine, and terrestrial ecosystems. Environmental Pollution, 100(1-3), 179–196. doi:10.1016/s0269-7491(99)00091-3

Smith, C. C., & Sargent, R. C. (2006). Female fitness declines with increasing female density but not male harassment in the western mosquitofish, *Gambusia affinis*. Animal Behaviour, 71(2), 401–407. doi:10.1016/j.anbehav.2005.06.003

Tavera, R., Castillo, S. (2000). An eutrophication-induced shift in the composition, frequency and abundance of the phytoplankton in Lake Catemaco, Veracruz, Mexico. In: Munawar, M., Lawrence, S. G., Munawar, I. F., Malley, D. F. (eds.). Aquatic Ecosystems of Mexico: Status and Scope. Backhuys, Leiden, pp. 103-117.

Tesh, A. E. C., Wilby, O. K., & Tesh, J. M. (1990). Effects of inorganic nitrates on growth and development of *Hydra*. Toxicology in Vitro, 4(4-5), 614–615. doi:10.1016/0887-2333(90)90127-f

Vitousek, P. M. (1990). Biological Invasions and Ecosystem Processes: Towards an Integration of Population Biology and Ecosystem Studies. Ecosystem Management, 183–191. doi:10.1007/978-1-4612-4018-1_17

Vitousek, P. M., Aber, J. D., Howarth, R. W., Likens, G. E., Matson, P. A., Schindler, D. W., … & Tilman, D. G. (1997). Technical Report: Human Alteration of the Global Nitrogen Cycle: Sources and Consequences. Ecological Applications, 7(3), 737-750. doi:10.2307/2269431

Watt, P. J., & Ooldham, R. S. (1995). The effect of ammonium nitrate on the feeding and development of larvae of the smooth newt, *Triturus vulgaris* (L.), and on the behaviour of its food source, Daphnia. Freshwater Biology, 33(2), 319–324. doi:10.1111/j.1365-2427.1995.tb01170.x

Wilson, A. J., & Nussey, D. H. (2010). What is individual quality? An evolutionary perspective. Trends in Ecology & Evolution, 25(4), 207–214. doi:10.1016/j.tree.2009.10.002

Zulian, E., Bisazza, A., & Marin, G. (1995). Variations in male body size in natural populations of *Gambusia holbrooki*. Ethology Ecology & Evolution, 7(1), 1–10. doi:10.1080/08927014.1995.9522966

Zuur, A. F., Ieno, E. N., Walker, N., Saveliev, A. A., & Smith, G. M. (2009). Mixed effects models and extensions in ecology with R. Statistics for Biology and Health. doi:10.1007/978-0-387-87458-6

Supplementary materials

Table S1. References used to calculate the biomass of several taxa of phytoplankton, rotifers and ciliates recorded in mesocosms. We assigned the biovolume and dry weight of a phylogenetically closely related taxa (i.e. reference taxa) if the exact species was not found in the literature. For phytoplankton, we used the formula by Rocha and Duncan (1985, C *pg* = $0.1204 \cdot V^{1.051}$ μm^3) to calculate biomass from biovolume and to get dry mass assuming a dry weight – C content of 24.3% (Platt and Irwin, 1973).

Mesocosm taxa	Reference taxa	Author	$\log_{10}$ biovolume (μm^3)	Dry weight (mg)
Closterium moniliferum	*Closterium* spp.	Kremer et al. (2014)	2.665931	$4.11962 \cdot 10^{-7}$
Cosmarium formosulum	*Cosmarium* spp.	Kremer et al. (2014)	2.778151	$3.13988 \cdot 10^{-7}$
Cosmarium biretum				
Cosmarium bioculatum				
Chlorococcum spp.	*Chlorococcum* spp.	Kremer et al. (2014)	2.254282	$1.1595 \cdot 10^{-7}$
Oocystis elliptica	*Oocystis* spp.	Kremer et al. (2014)	1.974028	$5.88469 \cdot 10^{-8}$
Oocystis parva				
Tetraedron minimum	*Tetraedron* spp.	Kremer et al. (2014)	2.161368	$9.26015 \cdot 10^{-8}$
Scenedesmus abundans	*Scenedesmus* spp.	Kremer et al. (2014)	2.477121	$1.98827 \cdot 10^{-7}$
Pediastrum boryanum	*Pediastrum* spp.	Kremer et al. (2014)	3.293968	$1.43543 \cdot 10^{-6}$
Cladophora glomerata	*Cladophora* spp.	Kremer et al. (2014)	5.297749	$1.83212 \cdot 10^{-4}$
Keratella quadrata	*Keratella quadrata*	Dumont et al. (1975)	-	$3.2 \cdot 10^{-4}$
Keratella cochlearis				
Lecane luna	*Lecane* spp.	Bottrell et al. (1976)	-	$2.0 \cdot 10^{-4}$
Lecane corneta				
Lecane quadridentata				
Lecane lunaris				
Squatinella mutica	*Lepadella ovalis*	Dumont et al. (1975)	-	$3.1 \cdot 10^{-4}$
Euchlanis dilatata	*Euchlanis dilatata*	Bottrell et al. (1976)	-	$5.0 \cdot 10^{-4}$
Asplanchna spp.	*Asplanchna priodonta*	Malley et al. (1989)	-	$5.25 \cdot 10^{-4}$
Trichocerca spp.	*Trichocerca longiseta*	Dumont et al. (1975)	-	$3.6 \cdot 10^{-4}$
Lophocaris spp.	*Mytilina mucronata*	Dumont et al. (1975)	-	$5.0 \cdot 10^{-4}$
Ciliates	Ciliates	Bottrell et al. (1976)	-	$5 \cdot 10^{-5}$

Table S2. Literature from which we extract the equations to calculate the biomass of benthic invertebrates recorded in mesocosms. DW = dry weight, WW = wet weight, L = body length, W = body width (diameter maximum for nematodes, microturbellaria and *Hydra* spp., diameter at segment XI for Oligochaeta), SL = shell length. Wet weight was transformed to dry weight by applying a correction factor of 0.2 (Smit et al, 1993).

Mesocosm taxa	**Reference taxa**	**Author**	**Formula**
Hydra spp.	*Hydra* spp.	Feller and Warwick (1988)	$\mathrm{WW}\ \mu g = 1.13 \cdot \mathrm{L}\ mm \cdot \mathrm{W}^2 mm \cdot 385$
Microturbellaria	Turbellaria	Feller and Warwick (1988)	$\mathrm{WW}\ \mu g = 1.13 \cdot \mathrm{L}\ mm \cdot \mathrm{W}^2 mm \cdot 550$
Nematoda	Nematoda	Andrassy (1956)	$\mathrm{WW}\ \mu g = \frac{\mathrm{L}\ \mu m \cdot \mathrm{W}^2\ \mu m}{1 \cdot 10^6}$
Oligochaeta	Oligochaeta	Smit et al. (1993)	$\mathrm{WW}\ mg = 1.07 \cdot \mathrm{L}\ mm \cdot \pi \cdot (\mathrm{W}/2)^2\ mm$
Erpobdellidae	*Erpobdella octoculata*	Poepperl (1998)	$\mathrm{DW}\ mg = 0.0058 \cdot \mathrm{L}^{2.225}\ mm$
Helobdella spp.	*Helobdella stagnalis*	Poepperl (1998)	$\mathrm{DW}\ mg = 0.0294 \cdot \mathrm{L}^{1.752}\ mm$
Chironomini	Chironomini	Benke et al. (1999)	$\mathrm{DW}\ mg = 0.0007 \cdot \mathrm{L}^{2.952}\ mm$
Tanytarsini	Tanytarsini	Benke et al. (1999)	$\mathrm{DW}\ mg = 0.0008 \cdot \mathrm{L}^{2.728}\ mm$
Orthocladiinae	Orthocladiinae	Benke et al. (1999)	$\mathrm{DW}\ mg = 0.002 \cdot \mathrm{L}^{2.254}\ mm$
Tanypodinae	Tanypodinae	Benke et al. (1999)	$\mathrm{DW}\ mg = 0.0026 \cdot \mathrm{L}^{2.503}\ mm$
Chironomidae pupae	Chironominae	Benke et al. (1999)	$\mathrm{DW}\ mg = 0.0059 \cdot \mathrm{L}^{2.099}\ mm$
Caenidae	*Caenis* spp.	Benke et al. (1999)	$\mathrm{DW}\ mg = 0.0054 \cdot \mathrm{L}^{2.842}\ mm$
Ceratopogoninae	Ceratopogonidae	Benke et al. (1999)	$\mathrm{DW}\ mg = 0.00022 \cdot \mathrm{L}^{2.871}\ mm$
Anisoptera	*Celithemis fasciata*	Benke et al. (1999)	$\mathrm{DW}\ mg = 0.0058 \cdot \mathrm{L}^{2.877}\ mm$
Tipulidae	*Tipula abdominalis*	Benke et al. (1999)	$\mathrm{DW}\ mg = 0.0054 \cdot \mathrm{L}^{2.463}\ mm$
Ostracoda	Ostracoda	Stead et al. (2003)	$\mathrm{DW}\ \mu g = 16.45 \cdot \mathrm{L}^{1.943}\ mm$
Chydoridae	*Alona* spp.	Herzig (1984)	$\mathrm{DW}\ \mu g = 6 \cdot \mathrm{L}^{2.653}\ mm$
Daphniidae	*Alona* spp.	Herzig (1984)	$\mathrm{DW}\ \mu g = 6 \cdot \mathrm{L}^{2.653}\ mm$
Macrothricidae	*Alona* spp.	Herzig (1984)	$\mathrm{DW}\ \mu g = 6 \cdot \mathrm{L}^{2.653}\ mm$
Cyclopoida	Cyclopoida	Dumont et al. (1975)	$\mathrm{DW}\ \mu g = 1.1 \cdot 10^{-7} \cdot \mathrm{L}^{2.59}\ \mu m$
Harpacticoida	Cyclopoida	Dumont et al. (1975)	$\mathrm{DW}\ \mu g = 1.1 \cdot 10^{-7} \cdot \mathrm{L}^{2.59}\ \mu m$
Physa spp. < 3.5 mm	*Physa integra*	Eckblad (1971)	$\ln \mathrm{DW}\ mg = 0.867 \cdot \mathrm{SL}\ mm - 3.664$
Physa spp. > 3.5 mm	*Physa integra*	Eckblad (1971)	$\mathrm{DW}\ mg = 0.822 \cdot \mathrm{SL}\ mm - 2.734$
Planorbarius spp.	*Gyraulus parvus*	Eckblad (1971)	$\ln \mathrm{DW}\ mg = 0.962 \cdot \mathrm{SL}\ mm - 4.321$
Galba truncatula	*Lymnaea palustris*	Eckblad (1971)	$\mathrm{DW}\ mg = 0.257 \cdot \mathrm{SL}\ mm - 0.217$
Radix spp.	*Lymnaea palustris*	Eckblad (1971)	$\mathrm{DW}\ mg = 0.257 \cdot \mathrm{SL}\ mm - 0.217$

Table S3. Mean and minimum-maximum range of all community metrics and ecosystem properties recorded in mesocosms at four sampling times (t0, t1, t2 and t3) to explore the effects of fish and nitrate additions. Biomass data indicate dry weight in the whole mesocosm.

Variable	t_0	t_1	t_2	t_3
COMMUNITY METRICS				
Whole biomass	**11463** (5533 – 21205)	**9112** (5210 – 14216)	**7344** (3322 – 18678)	**5948** (1769 – 13916)
Pelagic biomass	**1410** (548 – 2501)	**1060** (229 – 2071)	**868** (125 – 1814)	**694** (81 – 3400)
Benthic biomass	**10053** (4755 – 18728)	**8052** (4496 – 13834)	**6476** (2258 – 17840)	**5320** (1040 – 13431)
Phytoplankton biomass	**0.5** (0.01 – 2.0)	**3.1** (0.2 – 7.6)	**346.0** (18.2 – 913.1)	**256.5** (51.2 – 771.2)
Zooplankton biomass	**1410** (548 – 2500)	**1057** (229 – 2070)	**522** (31 – 1753)	**438** (21 – 2628)
Whole richness	**16.1** (8 – 23)	**23.1** (19 – 29)	**19.0** (16 – 22)	**19.0** (13 – 25)
Pelagic richness	**6.4** (5 – 9)	**10.7** (7 – 14)	**7.9** (6 – 10)	**8.5** (6 – 12)
Benthic richness	**10.3** (4 – 16)	**12.6** (8 – 17)	**11.2** (9 – 14)	**10.6** (7 – 15)
Phytoplankton richness	**2.3** (1 – 4)	**5.6** (3 – 7)	**4.5** (4 – 6)	**5.1** (4 – 6)
Zooplankton richness	**4.1** (3 – 6)	**5.1** (3 – 7)	**3.4** (1 – 6)	**3.4** (2 – 6)
ECOSYSTEM PROPERTIES				
DOC (mg l^{-1})	**10.0** (8.3 – 13.7)	**11.5** (9.1 – 14.4)	**11.3** (8.5 – 14.2)	**11.4** (9.0 – 14.8)
pH	**8.7** (8.4 – 9.2)	**9.4** (8.7 – 10.2)	**9.6** (8.8 – 10.3)	**8.8** (8.1 – 9.6)
O_2 (mg l^{-1})	**8.6** (7.5 – 10.1)	**11.1** (8.0 – 14.9)	**6.3** (5.2 – 7.5)	**5.3** (4.0 – 6.3)
Turbidity (FTU)	**7.0** (5.2 – 9.0)	**7.0** (4.4 – 11.1)	**8.8** (3.8 – 20.0)	**12.0** (4.9 – 19.2)
Conductivity (μS cm^{-1})	**848.2** (797 – 892)	**1038.6** (806 – 1307)	**1368.0** (1023 – 1766)	**1860.7** (1443 – 2415)
NO_2^- (mg l^{-1})	**0.0** (0.0 – 0.0)	**0.9** (0.0 – 2.9)	**4.3** (0.0 – 11.5)	**4.8** (0.1 – 13.0)
NO_3^- (mg l^{-1})	**0.3** (0.1 – 3.2)	**162.9** (0.0 – 457.3)	**183.4** (0.0 – 444.6)	**190.2** (0.2 – 513.4)
Chl-a plankton (μg l^{-1})	**< d.l.**	**3.5** (1.2 – 5.8)	**3.9** (1.5 – 8.7)	**5.5** (2.3 – 11.4)
Chl-a benthos (mg m^{-2})	**0.086** (0.014 – 0.257)	**0.198** (0.023 – 0.724)	**0.095** (0.026 – 0.274)	**0.081** (0.032 – 0.338)
Cladophora (% cover)	**4.2** (1.6 – 7.7)	**18.8** (3.2 – 43.7)	**47.7** (21.4 – 72.2)	**55.4** (36.3 – 80.1)

Table S4. Statistics for the comparison of the effects of fish and nitrate additions on leaf litter decomposition over time. R^2_β indicates the coefficient of determination as established by Jaeger et al. (2016). Significance of factors was assessed by means of likelihood-ratio tests at $P \leq 0.05$, denoted by the asterisk (*). Remaining organic matter and C/N$_{molar}$ were log-transformed.

Function	Fixed structure	Effect size		χ^2	P	R^2_β
Remaining org. matter (% IOM)	Fish	0.016±0.054		0.09	0.76	0.86
	NO$_3^-$	-0.063±0.058		1.2	0.28	
	time	$-7.4\cdot10^{-3}\pm5.0\cdot10^{-4}$	*	225.4	<0.001	
	time:NO$_3^-$	$-3.8\cdot10^{-3}\pm7.4\cdot10^{-4}$	*	26.3	<0.001	
% N	Fish	-0.130±0.051	*	6.6	0.01	0.56
	NO$_3^-$	0.081±0.041	*	4.0	<0.05	
	time	$2.9\cdot10^{-3}\pm8.0\cdot10^{-4}$	*	13.2	<0.001	
	Fish:NO$_3^-$	0.126±0.058	*	4.8	0.03	
	Fish:time	$3.3\cdot10^{-3}\pm1.1\cdot10^{-3}$	*	8.7	<0.01	
% C	NO$_3^-$	-0.448±0.183	*	6.0	0.01	0.63
	time	-0.041±0.004	*	130.5	<0.001	
C/N$_{molar}$	Fish	0.091±0.035	*	6.9	<0.01	0.67
	NO$_3^-$	-0.071±0.028	*	6.5	0.01	
	Time	$-3.2\cdot10^{-3}\pm5.5\cdot10^{-4}$	*	33.7	<0.001	
	Fish:NO$_3^-$	-0.091±0.039	*	5.4	0.02	
	Fish:time	$-2.1\cdot10^{-3}\pm7.7\cdot10^{-4}$	*	7.2	<0.001	

Table S5. Statistics for the comparison of the effects of fish and nitrate additions on community metrics over time. R^2_β indicates the coefficient of determination as established by Jaeger et al. (2016). Significance of factors was assessed by means of likelihood-ratio tests at $P \leq 0.05$, denoted by the asterisk (*). ** Dependent variable was log-transformed

Metric	Factor	Effect size	χ^2	P-value		R^2_β
Total biomass	Fish	-1.34 ± 0.90	2.2	0.14		0.32
	Nitrate	-2.65 ± 0.92	8.3	<0.01	*	
	Time	-0.31 ± 0.10	9.5	<0.01	*	
Phytoplankton biomass **	Nitrate	1.36 ± 0.23	35.1	<0.001	*	0.91
	Time	1.73 ± 0.11	237.6	<0.001	*	
	Time^2	-0.11 ± 0.01	145.5	<0.001	*	
Zooplankton biomass	Fish	-0.21 ± 0.17	1.4	0.23		0.18
	Time	-0.06 ± 0.02	8.4	<0.01	*	
Total richness	Fish	0.32 ± 0.98	0.1	0.74		0.58
	Nitrate	-0.81 ± 1.20	0.5	0.50		
	Time	-1.50 ± 0.28	29.5	<0.001	*	
	Time^2	0.10 ± 0.02	22.8	<0.001	*	
	Time:Nitrate	-0.33 ± 0.12	7.5	<0.01	*	
Pelagic richness	Nitrate	-0.94 ± 0.54	3.0	0.08		0.43
	Time	-1.17 ± 0.22	27.7	<0.001	*	
	Time^2	0.08 ± 0.02	20.1	<0.001	*	
Zooplankton richness	Time	-0.66 ± 0.17	15.3	<0.001	*	0.39
	Time^2	0.04 ± 0.01	9.2	<0.01	*	

Table S6. Spearman rank correlation coefficients examining relationships among all community metrics. The lower panel shows the graphical values of bivariate correlations with a locally weighted scatterplot smoothing (*LOWESS*) curve. In the upper panel the size of the values are set according to the absolute value of Spearman's rho (ρ). P-values are indicated in red by: *** P<0.001, ** P<0.01; * P<0.05

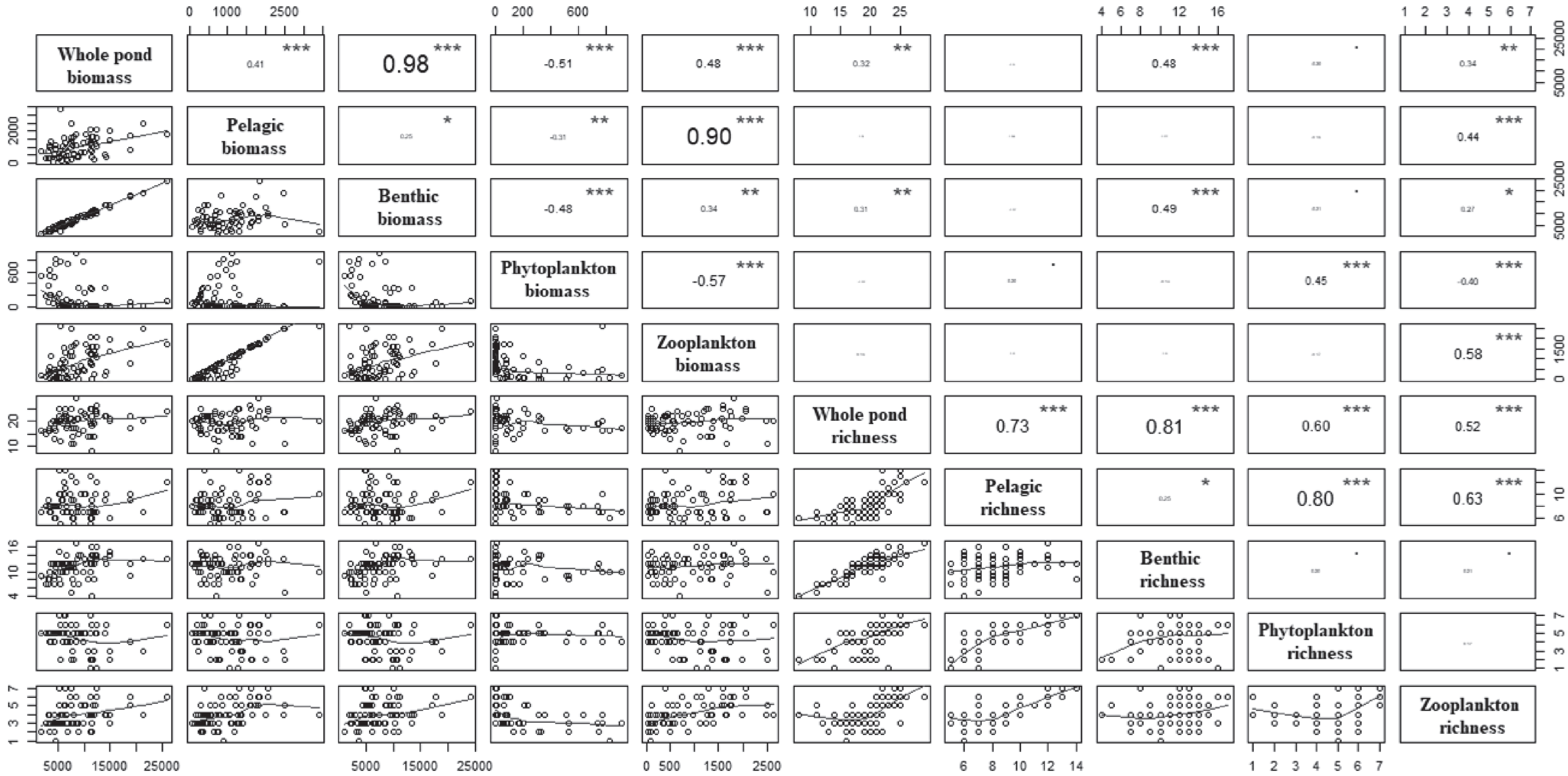
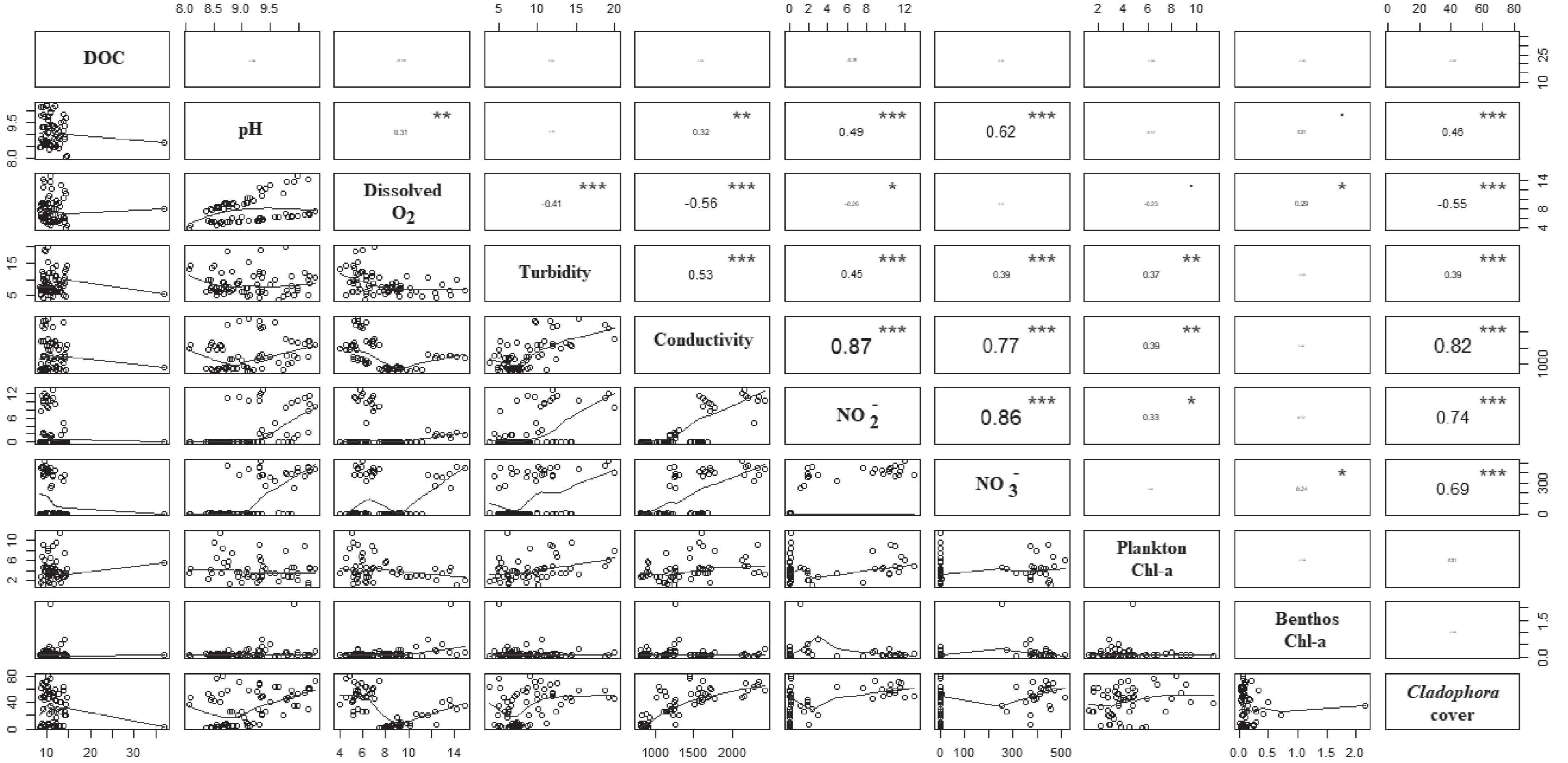


Table S7. Spearman rank correlation coefficients examining relationships among all ecosystem properties. The lower panel shows the graphical values of bivariate correlations with a locally weighted scatterplot smoothing (*LOWESS*) curve. In the upper panel the size of the values are set according to the absolute value of Spearman's rho (ρ). P-values are indicated in red by: *** P<0.001, ** P<0.01; * P<0.05



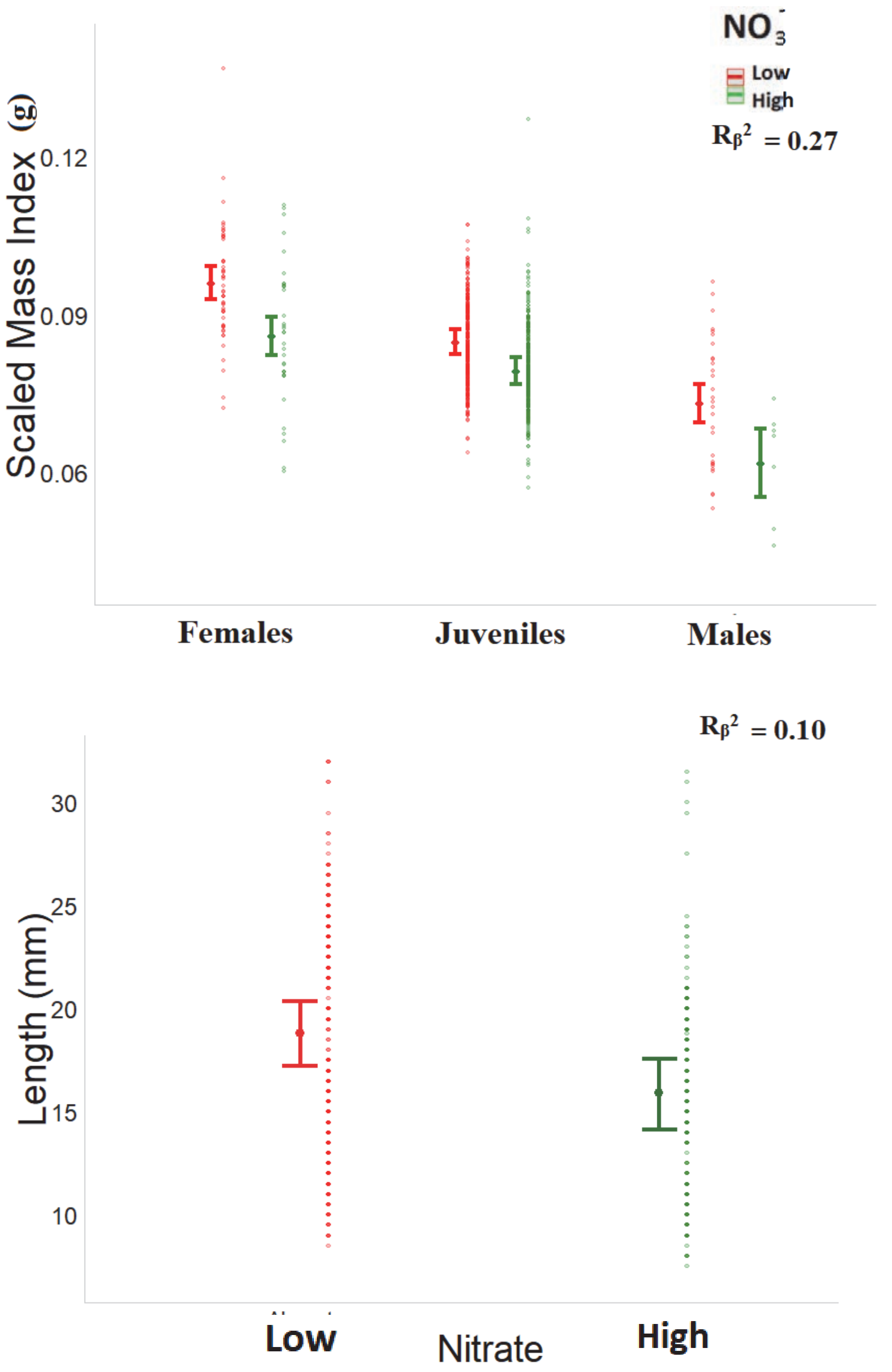
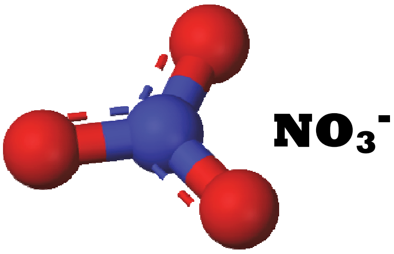
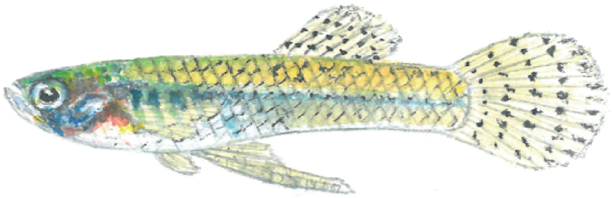


Figure S1. Predicted effects of nitrate addition on the body condition (g) of females, juveniles and males of eastern mosquitofish (*Gambusia holbrooki* at the end of the experiment (11 weeks). The effects of nitrate on the length of juveniles born during the experiment is also shown.

CAPÍTOL 2

Biomarcadors ecològicament rellevants revelen que els efectes crònics del nitrat depenen del sexe i l'estadi vital en el peix invasor *Gambusia holbrooki*





Oriol Cano-Rocabayera[a]; Adolfo de Sostoa[a]; Francesc Padrós[b]; Lorena Cárdenas[a]; Alberto Maceda-Veiga[a]

[a]Departament de Biologia Evolutiva, Ecologia i Ciències Ambientals – Institut de Recerca de la Biodiversitat (IRBio-UB), Universitat de Barcelona, Barcelona

[b]Servei de Diagnòstic Patològic en Peixos, Facultat de Veterinària, Universitat Autònoma de Barcelona, Bellaterra

PLOS | ONE

RESEARCH ARTICLE

Ecologically relevant biomarkers reveal that chronic effects of nitrate depend on sex and life stage in the invasive fish *Gambusia holbrooki*

Oriol Cano-Rocabayera[1]*, **Adolfo de Sostoa**[1], **Francesc Padrós**[2], **Lorena Cárdenas**[1], **Alberto Maceda-Veiga**[1]

1 Department of Evolutionary biology, Ecology and Environmental Sciences–Institute of Research in Biodiversity (IRBio-UB), Universitat de Barcelona, Spain, **2** Servei de Diagnòstic Patològic en Peixos, Facultat de Veterinària, Universitat Autònoma de Barcelona, Bellaterra, Spain

* canorocabayera@gmail.com

Abstract

Agricultural intensification and shifts in precipitation regimes due to global climate change are expected to increase nutrient concentrations in aquatic ecosystems. However, the direct effects of nutrients widely present in wastewaters, such as nitrate, are poorly studied. Here, we use multiple indicators of fish health to experimentally test the effects of three ecologically relevant nitrate concentrations (<10, 50 and 250 mg NO_3^-/l) on wild-collected mosquitofish (*Gambusia holbrooki*), a species widely introduced for mosquito biocontrol in often eutrophic waters. Overall, biomarkers (histopathology, feeding assays, growth and caloric content and stable isotopes as indicators of energy content) did not detect overt signs of serious disease in juveniles, males or females of mosquitofish. However, males reduced food intake at the highest nitrate concentration compared to the controls and females. Similarly, juveniles reduced energy reserves without significant changes in growth or food intake. Calorimetry was positively associated with the number of perivisceral fat cells in juveniles, and the growth rate of females was negatively associated with $\delta^{15}N$ signature in muscle. This study shows that females are more tolerant to nitrate than males and juveniles and illustrates the advantages of combing short- and long-term biomarkers in environmental risk assessment, including when testing for the adequacy of legal thresholds for pollutants.

OPEN ACCESS

Citation: Cano-Rocabayera O, de Sostoa A, Padrós F, Cárdenas L, Maceda-Veiga A (2019) Ecologically relevant biomarkers reveal that chronic effects of nitrate depend on sex and life stage in the invasive fish *Gambusia holbrooki*. PLoS ONE 14(1): e0211389. https://doi.org/10.1371/journal.pone.0211389

Editor: Amitava Mukherjee, VIT University, INDIA

Received: October 16, 2018

Accepted: January 11, 2019

Published: January 28, 2019

Copyright: © 2019 Cano-Rocabayera et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All excel data files are available in the supporting file "S1 File."

Funding: This work was finacially supported by Fundació Barcelona Zoo (PRIC grant 3RB 057 for labware and analysis costs). OCR received a grant by the University of Barcelona (APIF) during the period 2015-2017. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Introduction

Nutrient pollution results in man-made eutrophication, which is amongst the most pernicious forms of global change affecting aquatic ecosystems around the world [1,2]. Human causes of eutrophication are the inefficient use of fertilizers, aquaculture and urban outflows and atmospheric nitrogen deposition from combustion [3–5]. The ecological effects of eutrophication are well-known, including toxic algal blooms and high mortality of animals due to dissolved oxygen depletion at night [6–8]. Water authorities attempt to mitigate eutrophication by establishing safe nutrient concentrations (e.g. OECD, 1982; Directive 91/676/ECC [9,10]).

However, the direct toxicity of nutrients to wildlife under chronic exposure is still poorly studied [11,12]. Considering agricultural intensification continues unabated and water purification is costly [13], there is the pressing need to get better insight into the health effects that environmentally relevant nutrient concentrations have on wildlife.

Nitrate (NO_3^-) is a widely distributed nutrient that naturally occurs at a low environmental concentration [3]. However, it can reach up to 2000 mg NO_3^-/l in aquaculture tanks and 345 mg NO_3^-/l in surface waters in nitrate vulnerable zones [14,15]. From 2012 to 2015 the surface area vulnerable to nitrate pollution increased from 1951898 km^2 to 2175861 km^2 just in Europe, representing 61% of the total agricultural area [16]. Alongside surface waters, nitrate pollution degrades groundwater, with reported concentrations of more than 395 mg NO_3^-/l [17], which exceeds the legal thresholds for Europe (50 mg NO_3^-/l; Directive 91/676/ECC [10]) and U.S. (44 mg NO_3^-/l; USEPA SWDA [18]). Ground and surface waters are linked, buffering groundwater against shortages of surface water during drought [7]. Moreover, climate change may intensify the effects of nitrate pollution on temperate rivers if shifts in precipitation regimes increase agricultural run-off [2].

Nitrate toxicity has long attracted the attention of public health agencies after nitrate-induced oxidation of respiratory pigment (methemoglobinemia) was recorded in U.S. babies [19]. Studies have since reported diseases other than respiratory issues in humans and in laboratory and domestic animals after drinking nitrate-polluted water, including mortality, oxidative stress, hypertension, birth defects, diabetes, impaired thyroid function, spontaneous abortions or cancer [11,20,21]. For water-breathing animals, nitrate was generally considered of little concern, possibly because nitrate has low branchial permeability compared to the highly toxic ammonia and nitrite [22,23]. This view changed after experimental evidence showed methemoglobinemia and alterations in hormone levels, behaviour, growth or in vulnerability to diseases in aquatic taxa under chronic nitrate exposure [11,24,25]. However, these studies used eggs, juveniles or adults of one sex of different species, all of which are factors that may affect the toxic response [26]. Moreover, toxic responses can be delayed, so that the combined use of short- (e.g. feeding assays) and long-term biomarkers (e.g. growth) will provide a more holistic view of nitrate toxicity to wildlife than the often used single-type biomarker approach [27].

The eastern mosquitofish (*Gambusia holbrooki*) is one of the world's worst piscine invaders, which has been introduced in many temperate regions due to a misguided strategy for mosquito control [28]. Although extensively used in ecotoxicology, nitrate toxicity to mosquitofish has not been examined in detail. Reduced sperm counts and increased testicular weight in male mosquitofish were associated with concentrations of up to 22 mg NO_3^-/l in U.S. streams [29]. However, there is no experimental evidence for other nitrate-induced alterations in mosquitofish. The effects of nitrate on other fish species are negative [11,24,30], almost neutral [31] and even protective against a disease [32]. Nevertheless, these studies were mostly conducted on captive-reared species, which may have a higher tolerance to nitrate than wild fish because nitrate accumulates in aquaculture tanks and nitrate pre-exposure increases tolerance [33]. This rationale may apply to wild taxa if pre-exposure to the many pollutants occurring in natural waters induces co-tolerance [34].

The present experimental study monitored over 8 weeks the effects of three ecologically relevant nitrate concentrations on wild males, females and juveniles of mosquitofish using endpoints associated with their ecological impact. If males are the sicker sex [35,36,37] and juveniles are more vulnerable than adults to pollution [26,38], then we expected female mosquitofish to be the most tolerant to nitrate pollution. If the effects of nitrate pollution are subtle, then we expected nitrate effects to be more apparent in short- than in long-term biomarkers. Finally, responses in nitrate treatments should be comparable to those in controls if mosquitofish can cope with nitrate toxicity. Given the ecological relevance of the biomarkers used, our work will explore whether the ecological impact of the mosquitofish can be modulated by changing water-nutrient concentrations.

Materials and methods

Fish origin and general fish maintenance

The male and female mosquitofish used in this study were captured with dip nets in November 2012 in channels draining an agricultural area in the Llobregat river, Barcelona, Spain (41˚ 16'52"N, 2˚02'04"E). Fish were brought to the University of Barcelona in opaque plastic tanks provided with air-pumps and were acclimatised for one week to the laboratory conditions in two mixed sex stock 500 L tanks provided with an external filter, artificial plants and flowerpots for refugee. Fish were maintained in acclimation and experimental conditions as follows. A malaquite green/formaline bath was applied at a prophylactic dose upon arrival (see [39]). Water was then fully renewed by using dechlorinated tap water as we did to maintain the experimental environmental conditions (see section 2.3). Water properties in the laboratory tap were: pH = 7.7, mg/l, ammonia <0.5 mg/l, nitrite <0.03 mg/l, nitrate = 7.4 mg/l, sulphates = 81.2 mg/l, chloride = 130 mg/l, bicarbonate = 221 mg/l and conductivity = 784 μS/cm. Pregnant females (*N* = 15) with overt signs of giving birth soon were introduced in batches of three in 100 L tanks provided with nets to collect recently newborn juveniles (N = 165). Both adults and newborn were kept under 22±1˚C and 12 h light:12 h dark cycle and fed daily with crushed commercial Sera Vipan flakes and weekly with frozen bloodworms for adults and live *Artemia* nauplii for newborns. Fish were fed once daily until satiety and uneaten food and faeces were removed daily with a dipnet. Each tank had a biological filter to prevent metabolic waste built-up (NH_4^+ and NO_2^-) and ensure water oxygenation.

Ethics statement

The experimental procedure was authorised by the Natural Environment and Biodiversity Division at the Catalan Department of Agriculture and Fisheries (Num. DAAM 8290). Fish capture and maintenance were approved by the Committee for an Ethical use of Experimental Animals at the University of Barcelona (Num. 87/15). All fish were humanely euthanized on the termination of the experiment in compliance with Spanish legislation for the management of invasive species (Real Decreto 1628/2011 [40]).

Experimental nitrate concentrations and exposure conditions

Sodium nitrate ($NaNO_3$, CAS Number: 7631-99-4) was used to make two nitrate solutions (50 and 250 mg NO_3^-/l, equivalent to 11.5 and 57 mg NO_3^-N/l, respectively) using dechlorinated tap water, which was also used in the control treatment (<10 mg NO_3^-/l). The lowest nitrate concentration is the safety nitrate threshold for European waters (Directive 91/676/ECC [10]) and the highest level is within the range reported in aquaculture [24] and in rivers draining nitrate vulnerable zones in Europe [41] and tropical countries [42]. Experimental nitrate concentrations represented a 0, 5- and 25-fold increase, respectively, for mosquitofish in relation to the nitrate concentration at the collection site (9.9 ± 3.0 mg NO_3^-/l, based on quarterly water analyses over one year).

Male and female mosquitofish were visually size-matched per sex (total length, female: TL = 37.6 ± 0.4 and male: 25.9 ± 0.2 mm) and exposed for 8 weeks to the experimental nitrate solutions in 20 L aquaria (N = 5 tanks per treatment and sex) with six males or females in each replicate. For juveniles, the same experimental setting was used but these were exposed in batches of 11 siblings per tank. The exposure started by increasing nitrate concentrations in

Table 1. Water quality properties measured in the experimental tanks.

	Control	50 mg NO_3^-/l	250 mg NO_3^-/l
Temperature (°C)	22.5 ± 0.7	22.4 ± 0.7	22.5 ± 0.8
pH	7.8 ± 0.2	7.8 ± 0.2	7.8 ± 0.2
Carbonate hardness (KH)	9.2 ± 0.9	8.9 ± 1.0	9.3 ± 0.9
Total hardness (GH)	12.3 ± 0.8	11.9 ± 0.6	11.8 ± 1.0
Dissolved O_2 (mg/l)	8.73 ± 0.17	8.78 ± 0.16	8.71 ± 0.16
Dissolved O_2 (%)	98.3 ± 1.5	98.8 ± 1.1	98.1 ± 0.7
Ammonium (mg NH_4^+ /l)	0.08 ± 0.03	0.03 ± 0.02	0.08 ± 0.03
Nitrite (mg NO_2^-/l)	0.23 ± 0.07	0.26 ± 0.06	0.33 ± 0.08
Nitrate (mg NO_3^-/l)	14 ± 2	47 ± 3	216 ± 11
Phosphate (mg P-PO_4/l)	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.2

Values represent mean (±Standard Error). Data from aquaria with males, females and juveniles of mosquitofish (*Gambusia holbrooki*) combined.

https://doi.org/10.1371/journal.pone.0211389.t001

each aquarium drop by drop (~3 h) via a 5 mm ø tube connected to a tank with clean water or one of the two nitrate solutions. This drop-by-drop system was used to refill each tank with fresh water from each experimental condition after 50% of water was changed every three days. Water samples randomly analysed from the different treatments using the colorimetric kit VISOCOLOR indicated that the water quality conditions remained constant through the experiment at 24 h of the next water change (Table 1).

Overview of the biomarkers measured to appraise nitrate toxicity

The effects of chronic nitrate exposure on males, females and juveniles of mosquitofish were examined using 13 variables (Table 2). All of them are indicators of fish health, but calorimetry and stable isotope signatures inform the quality of fish for piscivores (see section 2.4.2).

Table 2. List of the biomarkers used to assess the effects of nitrate on mosquitofish (*Gambusia holbrooki*).

Biomarker	Rationale	Sample size
Histopathology Liver, gill	Liver is involved in the detoxification of many toxicants and these mostly entry through the gills, including nitrate. Medium to long-term response.	15M / 15F / 15J per treatment
Calorimetry [*] Energy density (J/g)	An overall measure of energy content based on the amount of heat released from the combustion of mostly lipids and glycogen. Medium to long-term response.	15M / 15F / 40J per treatment
Stable isotopes $\delta^{13}C$, $\delta^{15}N$, C/N	A measure of tissue composition mostly based on the effect that a change in lipid content has on the C/N ratio. Medium to long-term response.	15M / 15F per treatment
Mass–length measures Scaled Mass Index (SMI) Specific growth rate	Mass-length relationships, such as the SMI, indicate changes in the weight of an individual in relation to another of the same length. Specific growth rate informs the success of an individual in energy allocation to cope with pollution. Long-term response biomarkers.	30M / 30F / 55J per treatment
Feeding behaviour Feeding latency Voracity, Satiety	Alterations in food intake often occur in fish under stress, such as pollution exposure. Short-term response; over the long term it can translate to effects on fish survival and ecological networks.	30M / 30F / 55J per treatment

Values indicate the sample size (*N*) of male (M), female (F) and juveniles (J). Since histopatology, stable isotopes and calorimetry biomarkers implied destructive sampling; their sample size was half of the non destructive analyses.

[*] Males and juveniles were pooled within aquarium to meet the equipment minimum mass requirements. For juveniles, this did not allow us to use them for stable isotope analysis.

https://doi.org/10.1371/journal.pone.0211389.t002

Alterations in the quantity of food eaten or in the reaction time to a stimulus show how nitrate may alter mosquitofish performance in ecosystems. Indicators related to fish growth, body condition based on mass-length relationships, energetic content and histopathology were recorded on the termination of the experiment (8 weeks). However, the feeding behaviour of mosquitofish was monitored at 0, 4, 6 and 8 weeks. All fish were euthanized at 8 weeks using an overdose of the anaesthetic MS-222.

Growth and body condition based on mass-length relationships. Male and female mosquitofish were anesthetised with MS-222 (0.02%), measured (TL, mm) and weighted (0.001g) at Time 0 and at 8 weeks. Newborns other than those used in the experiment were used to estimate the size of experiment juveniles at Time 0 (TL = 8.9 ± 1.2 mm) to avoid compromising the health of the tiny tested individuals due to handling.

Fish size measures were used to calculate the specific fish growth rate (G) using the equation $G = (\ln L_t - \ln L_0) / t_n$ [43], where $\ln L_t$ is the natural logarithm of fish length at 8 weeks, $\ln L_0$ is that of fish length at Time 0 and t_n is the duration of the experiment (8 weeks). We ranked fish in each tank by body length at Time 0 and t_n to identify fish individuals and be able to calculate G because our tagging equipment (e.g. elastomer) is not suitable for such small fish. Moreover, we calculated the Scaled Mass Index (SMI) as an index of body condition: $SMI = W_i (L_o/L_i)^{bSMA}$ [44], where W_i and L_i are the weight and length of each fish individual, respectively, L_0 is the arithmetic mean length of all the tested mosquitofish and b_{SMA} is the slope of a standardised major axis regression of the mass-length relationship. The SMI is regarded as a correlate of energy and fitness measures [44].

Energetic reserves. Changes in fish $\delta^{13}C$ and $\delta^{15}N$ stable isotope signatures and calorimetry were used as two complementary measures of energetic reserves. Stable isotopes are widely used in studies of trophic ecology because the isotopic composition of predator tissue is naturally altered by the type and amount of food assimilated [45]. Moreover, tissue isotopic differences are due to changes in metabolism, including increased lipid storage [46,47], because lipids are about 6–7% depleted in ^{13}C relative to protein [48]. We used the $\delta^{13}C$ and $\delta^{15}N$ ratio as proxy for lipid content in white muscle because this is the tissue widely used in fisheries (e.g. [47,49]). However, lipid content varies amongst fish tissues [50], so that we used the caloric content of the whole fish as an additional measure of energy content.

For stable isotope analyses, we freeze-dried fish muscle samples from below the dorsal fin of 3 adult fish from each tank and we ground them to fine power. Two sub-samples of 0.30 mg each were placed into tin buckets and crimped for combustion to determine $\delta^{13}C$ and $\delta^{15}N$ using a Flash EA1112 and TC/EA coupled to a stable isotope mass spectrometer Delta C through a Conflo III interface (ThermoFinnigan). Analytical accuracy was controlled using replicate assays of certified standards indicating an analytical error of ±0.1‰ and ±0.3‰ for $\delta^{13}C$ and $\delta^{15}N$, respectively. Isotope ratios are expressed conventionally as δ values in ppt (‰) according to the following equation: $\delta X = ((R_{sample}/R_{standard}) - 1) \cdot 1000$, where X (‰) is ^{13}C, ^{15}N, and R are the corresponding ratios $^{13}C/^{12}C$ $^{15}N/^{14}N$, related to the standard values: R standard for ^{13}C is Pee Dee Belemnite, for ^{15}N is atmospheric nitrogen.

For calorimetry, we used three adults per tank and we pooled all juveniles from each tank to reach the detection limits of the IKA Calorimeter c7000 (Germany). Samples were oven-dried at 60°C for 48 h, weighted and the caloric content was expressed as joules per gram (J/g).

Histopathology. A random sample of three fish from each tank was processed for histology. The head and viscera of adult fish were fixed individually in 10% buffered formalin, dehydrated in ethanol, cleared in xylene and embedded in paraffin wax [51]. Juveniles were processed as a whole due to small size.

Sagittal sections of 5 μm thick were cut in all fish at the same position and stained with conventional haematoxylin-eosin [51]. Observations were made under an Olympus BH light microscope at 400x magnification. We focused on liver and gills because nitrate uptake is through gills [52] and liver is the target of many toxicants [27]. Gill alterations and the number of mucous cells in the gills were recorded and expressed as a ratio out of the 50 secondary lamellae and spaces examined. Liver alterations and the number of melanomacrophague centres were expressed as a ratio out of the number of microscope fields examined. We took photographs with a Jenoptik ProgRes C3 camera and used the ImageJ software to quantify the area (μm^2) of perivisceral fat in 3 sections of each juvenile fish as an additional measure of energy storage. Outcomes were expressed as average per juvenile.

Feeding behaviour. Temporal changes in feeding behaviour were quantified in all experimental fish, which were not fed 24 h before the assay to ensure all fish were hungry. The assay was conducted in an aquarium with a plastic sheet in one side, where the tested fish were left for 3 minutes to acclimatise before the trial (Fig 1). The trial began when the sheet was gently removed and ten size-matched unfrozen *Artemia* adults in 200 ml of water were gently released into the experimental area on the opposed side of the tested fish. Live *Artemia* nauplii were used for juveniles. The assay was repeated individually for all fish and the same observer, sat in front of the aquarium, recorded: i) latency defined as the time spent to capture the first item, ii) voracity defined as the time needed to capture the first items (four in adults and ten in juveniles), and iii) satiety defined as the total number of items eaten without stopping > 2 minutes. If a fish ate all 10 prey items, then we added *Artemia* individuals on the water surface until satiety.

Statistical analyses

All analyses were conducted using the R software [53] and the functions outlined below. Spearman rank correlation coefficients were used to examine associations amongst all biomarker responses. The effects of chronic nitrate exposure on mortality, fish growth, SMI, caloric content, the C/N ratio, histopathological and behavioural measures were assessed using generalized linear mixed models (GLMMs). Data on males, females and juveniles were analysed separately because there were significant differences in all response variables (S5 Table). To avoid pseudoreplication, aquarium ID was included as random intercept in all models to account for the fact that fish were exposed to nitrate in batches (6 adults or 11 juveniles). For juveniles, aquarium ID was nested within mother ID as random intercept to account for systematic differences amongst clutches. Nitrate was included as fixed effect in all models. The interaction between nitrate and time was included in the behaviour model to test whether the effects of nitrate on fish varied with exposure time. The distribution of all response variables was visually inspected and the error distribution in GLMMs was chosen accordingly (e.g. Gaussian for fish growth, Poisson for the number of prey eaten). Model assumptions were checked by inspecting diagnostic plots of residuals [54]. The function *Anova* within the package *car* [55] was used to assess significance at $P \leq 0.05$.

Results

Mosquitofish were evenly distributed by size and sex amongst treatments and fish did not differ in size amongst aquaria at Time 0, either for males ($F = 0.54$; $P = 0.90$) or females ($F = 1.22$; $P = 0.28$). However, females (Mean ± S.E. = 37.6 ± 0.42 mm) were significantly bigger than males (25.9 ± 0.18 mm; $t = 25.8$; $P < 0.001$). There was no mortality in males due to nitrate and only minor mortality was recorded for juveniles (3.6%) and females (3.3%). Nonetheless, mortality did not differ significantly between treatments (Nitrate: $\chi^2 = 1.59$, $P = 0.66$) or sexes (Sex: $\chi^2 = 0.43$, $P = 0.93$).

Growth, body condition and energetic reserves

An eight-week nitrate exposure did not alter significantly the growth rate or body condition, as defined by the SMI, of males, females and juveniles (Table 3). The caloric content of juveniles at 50 mg NO_3^-/l and in the controls at <10 mg NO_3^-/l was markedly higher than at 250 mg NO_3^-/l (Table 3). However, males significantly increased in caloric content at 50 mg NO_3^-/l compared to the other two concentrations. Females showed no significant differences amongst treatments (Table 3). Nitrate also did not affect the $\delta^{13}C$ and $\delta^{15}N$ measures in the white muscle of all tested fish (Table 3).

Histopathology

A detailed examination of all slides did not reveal overt clinical signs of disease, but some tissular changes were observed in liver and gills (Table 3, S2 and S3 Figs). Occasional telangiectasia and

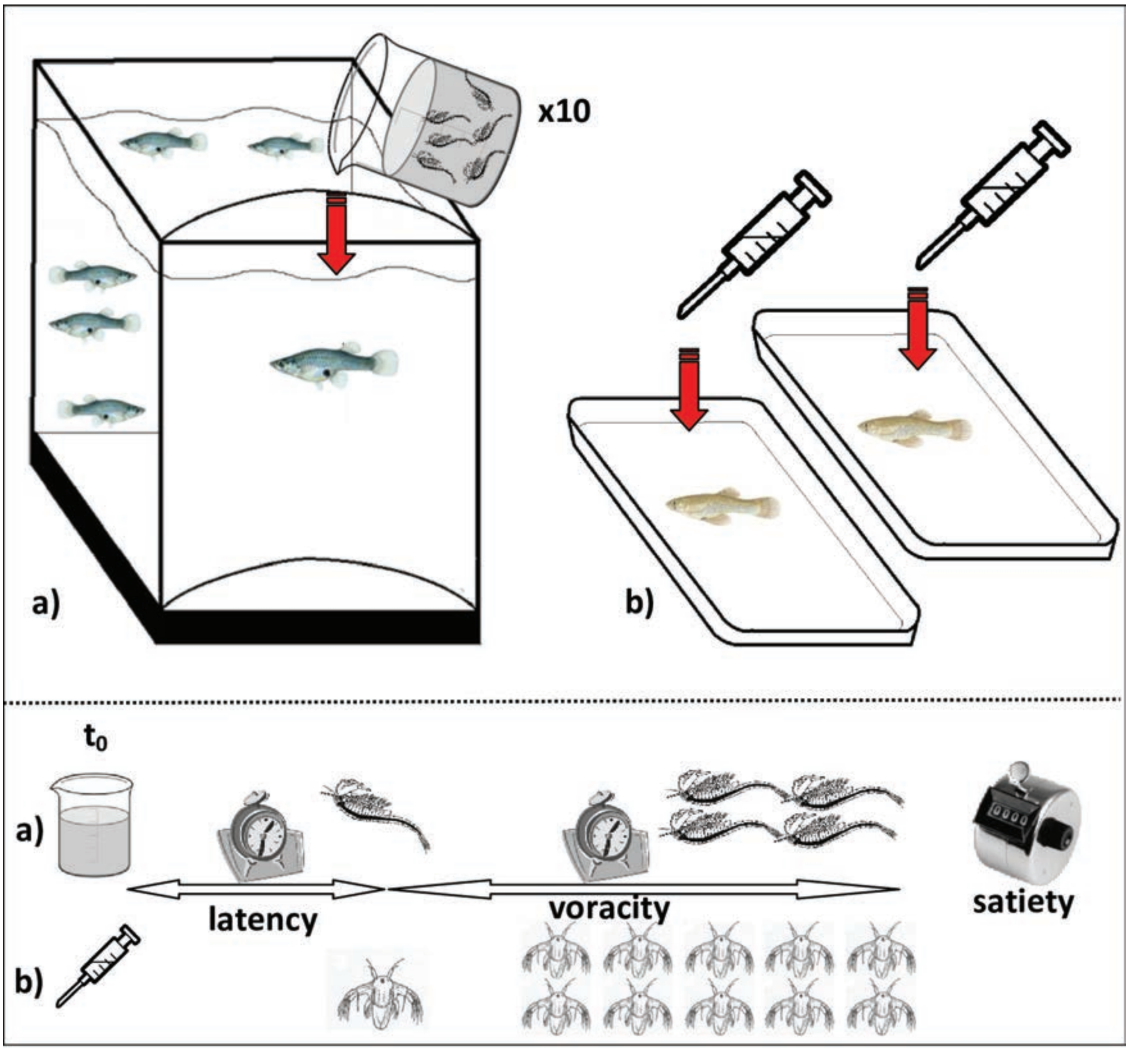


Fig 1. Experimental setting for the feeding behaviour assay of adults and juveniles of mosquitofish. a) 10 prey items are offered to an isolated adult mosquitofish. b) Live brine shrimp nauplii are offered with a syringe to an isolated juvenile mosquitofish in a tray. After adding food (t_0) we quantified: feeding latency time (time to capture the first prey, t_{LAT}), voracity time (time to capture 4 preys in adults and 10 in juveniles, t_{VOR}) and satiety.

https://doi.org/10.1371/journal.pone.0211389.g001

Table 3. Biomarkers used to appraise the health status (energy content, mass–length measures, histopathology) of males, females and juveniles of mosquitofish (*Gambusia holbrooki*) exposed to three experimental nitrate concentrations (<10 mg/l, 50 mg/l, 250 mg/l).

			Control	50 mg NO_3^-/l	250 mg NO_3^-/l
Histopathology	Juveniles *	MMC	0	0	0
		Lam%	6.8±1.2	5.7±1.7	5.8±1.2
		GMC	0.3±0.2	0.7±0.3	0.9±0.3
		PVFC	$3.51E^{5}\pm5.47E^{4}$	$2.84E^{5}\pm4.50E^{4}$	**$2.18E^{5}\pm1.62E^{4}$**
	Males	MMC	1.1±0.5	0.9±0.2	1.2±0.2
		Lam%	1.6±0.6	1.6±0.6	0.7±0.3
		GMC	0.3±0.2	0.5±0.2	0.7±0.3
	Females	MMC	15.5±7.3	8.0±1.8	23.7±12.3
		Lam%	7.5±2.2	8.8±3.3	9.7 ±2.1
		GMC	2.1±0.6	2.3±0.8	2.2±0.6
Calorimetry	Juveniles	J/g	23008.5±279.8	22540.2±547.0	**21895.1±483.1**
	Males	J/g	15820.8±832.3	**17920.3±403.3**	16165.0±625.9
	Females	J/g	18471.4±409.2	18770.9±218.5	18696.0±171.4
Stable isotope analysis **	Males	$\delta^{13}C$	-24.1±0.2	-24.5±0.2	-24.5±0.1
		$\delta^{15}N$	13.3±0.4	13.4±0.4	13.0±0.2
		C/N_m	4.29±0.06	4.32±0.08	4.27±0.05
	Females	$\delta^{13}C$	-21.3±0.1	-21.2±0.1	-21.3±0.1
		$\delta^{15}N$	15.8±0.3	15.5±0.2	15.6±0.2
		C/N_m	3.97±0.03	3.96±0.02	4.00±0.03
Mass–length measures	Juveniles	SMI	0.192±0.002	0.189±0.002	0.189±0.002
		G	$1.11E^{-2}\pm3.17E^{-4}$	$1.16E^{-2}\pm2.71E^{-4}$	$1.06E^{-2}\pm2.40E^{-4}$
	Males	SMI	0.188±0.006	0.193±0.004	0.217±0.012
		G	$6.21E^{-4}\pm1.23E^{-4}$	$6.90E^{-4}\pm1.16E^{-4}$	$6.39E^{-4}\pm1.40E^{-4}$
	Females	SMI	0.193±0.002	0.192±0.003	0.193±0.002
		G	$6.48E^{-4}\pm9.32E^{-5}$	$7.15E^{-4}\pm6.79E^{-5}$	$6.86E^{-4}\pm7.47E^{-5}$

Values represent mean ± standard Error. MMC: melanomacrophague centers per microscope field; Lam%: % of gill secondary lamellae with alterations; GMC: number of gill mucous cells per section. PVFC: perivisceral fat content as μm^2 per section; J/g: energy density; $\delta^{13}C/\delta^{15}N$: carbon or nitrogen fractionation; C/N_m: molar carbon to nitrogen ratio; SMI: scaled mass index, computed using fresh weight including viscera; G: specific growth rate. Bold values indicate a significant effect compared to control values at $\alpha = 0.05$.

* Perivisceral fat content was analysed only in juveniles because the quantification method we used was only reliable in sagittal sections of the whole body.

** Stable isotopes in juveniles were not analysed.

https://doi.org/10.1371/journal.pone.0211389.t003

slight epithelial lifting were observed in secondary lamellae. However, we did not observe other tissue alterations such as hyperplasia, hypertrophy or increased number of mucous cells. All liver samples had regularly aligned cords of hepatocytes (S3 Fig). However, slight changes in the staining intensity of the cytoplasm were observed in liver tissue, probably related to glycogen deposits and occasional lipid droplets. The presence of macrophage aggregates was restricted to adult fish, with females having a larger number than males, but without significant changes due to nitrate (Table 3). The amount of perivisceral adipose tissue in juveniles at the highest nitrate concentration was lower than in juveniles in the other treatments (Table 3, S4 Table).

Feeding behaviour

Males, females and juveniles showed differences in latency, voracity and satiety, but without significant changes due to nitrate apart from males from the sixth week onwards (Fig 2, S1

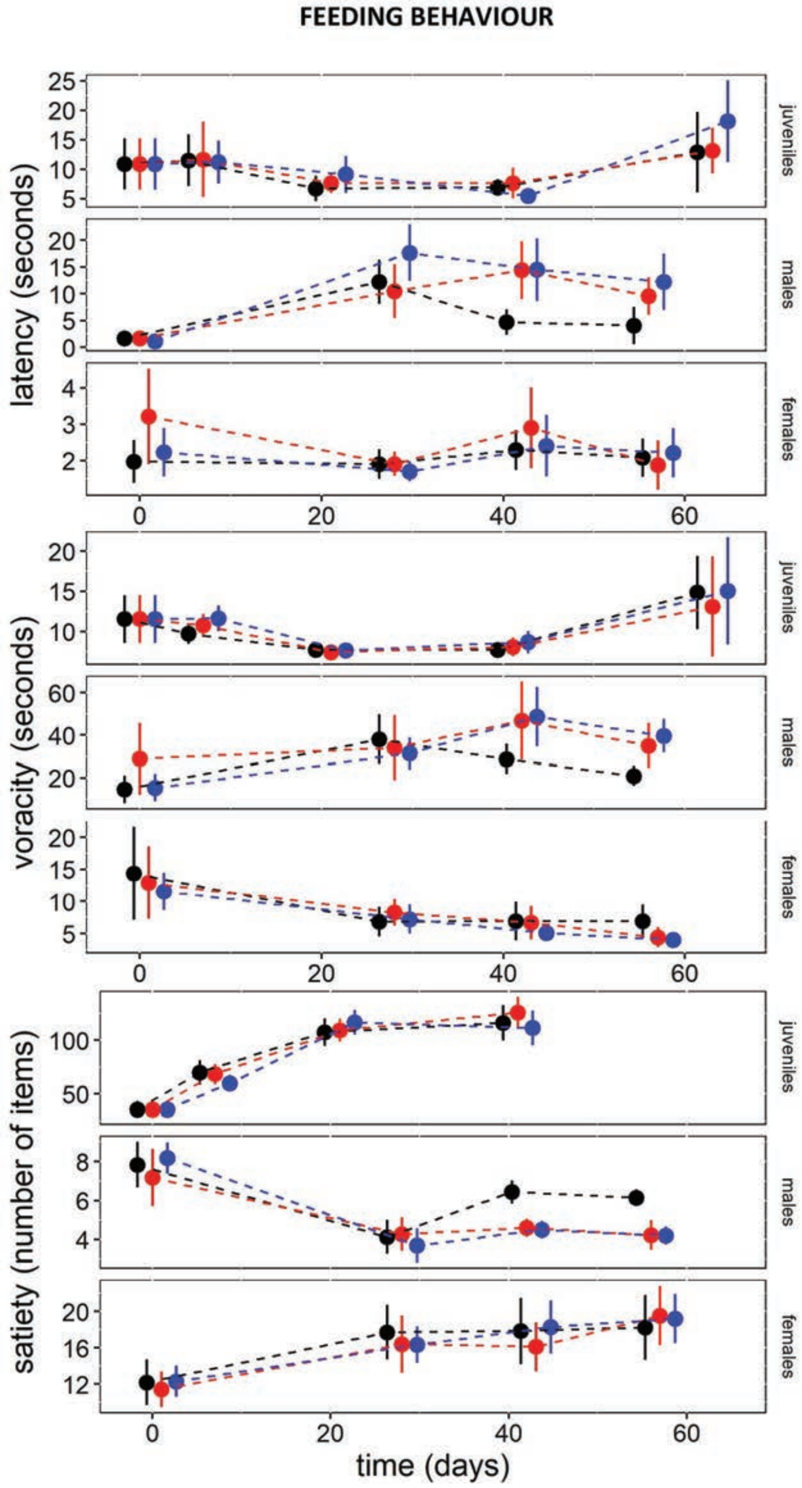


Fig 2. Feeding behaviour variables along the experiment. Symbols and bars represent means ± 95% confidence intervals for each variable and assigned for each treatment (black: control, red: 50 mg NO_3^-/l, blue: 250 mg NO_3^-/l). a) Latency is the time spent to capture the first food item. b) Voracity is the time to capture a given number of food items. c) Satiety is the total number of eaten food items.

https://doi.org/10.1371/journal.pone.0211389.g002

Table). Males at 50 and 250 mg NO_3^-/l exhibited lower satiety values and lower voracity (i.e. > time to capture prey) than those in the controls at <10 mg NO_3^-/l (Fig 2). Overall, females and juveniles had a higher voracity and satiety than males, and juveniles had greater latency times than adults (Fig 2). Females and juveniles also tended to increase voracity and satiety and to reduce latency times throughout the experiment compared to males (Fig 2).

Pair-wise correlations amongst biomarkers

Spearman rank correlation coefficients were generally low amongst all biomarkers measured in juveniles, males and females of mosquitofish. There was a strong positive correlation between calorimetry and growth ($\rho = 0.73$, $P = 0.002$) and between calorimetry and the amount of perivisceral fat cells in juveniles ($\rho = 0.72$, $P = 0.003$, S6 Table). However, a marked negative correlation was found between satiety and latency time of males ($\rho = -0.78$, $P < 0.001$, S7 Table). A strong negative association was observed in females for $d^{15}N$ and growth ($\rho = -0.74$, $P = 0.002$), even though the relationship was positive between C/N and growth ($\rho = 0.77$, $P < 0.001$, S8 Table).

Discussion

This is the first comprehensive study examining the chronic effects of nitrate on a widely introduced fish species, as exemplified by the eastern mosquitofish (*Gambusia holbrooki*) [28]. Moreover, this is one of the few ecotoxicological studies using short- and long-term biomarkers (e.g. growth, histopathology, feeding assays) in females, males and juveniles of the same species. Overall, we did not find overt clinical signs of disease, which supports the prevailing idea that many invasive species, including mosquitofish, have wide tolerance to changes in water quality [28,56]. However, the fact that nitrate altered food intake or energetic reserves in males and juveniles suggests that concentrations >50 mg NO_3^-/l cannot be considered completely safe [11,21].

Many studies have shown that males are more likely to acquire diseases than females, including fish [37,57–59]. We did not find gross pathological alterations in any fish, but the more marked effects of nitrate on males provide some support for males being the sicker sex [35,36]. The weaker response of the tested fish to nitrate is unlikely to be much attributed to pre-acclimation to nitrate at the collection site (9.9 ± 3.0 mg NO_3^-/l), as reported for amphibians [33]. However, this outcome does not exclude the possibility of nitrate tolerance being increased due to pre-exposure to other ions, including those of water hardness (see [60]), which is high at the collection site and in the laboratory dechlorinated tap water due to a calcareous geology. The effects of metals and chlorine compounds on fish in the animal facility probably were negligible because tap water is filtered through active charcoal and the aquarium product Sera Aquatan is used to further guarantee water is free of metals and chlorine (see [39]). The fact that juveniles had a greater tolerance to nitrate than males was unexpected because young fish are generally more sensitive to chronic pollution than adults [26,38]. However, 96-h LC50 tests revealed that susceptibility to nitrate increases with body size in the Siberian sturgeon *Acipenser baeri* [61]. Although we cannot reveal the mechanisms for the mild effects of nitrate on juveniles because nitrate metabolites in tissues were not measured (e.g. nitric oxide [62]), differences in space and behaviour between adults and juveniles might partially explain outcomes. Adults were kept at lower densities (6 fish per tank) than juveniles (11 fish per tank) and not surprisingly, we observed more agonistic interactions among adults due to confinement.

Mosquitofish populations are often confined in small water bodies and female-biased [63,64], including in the collection site of the studied fish (authors *pers. observ.*). The biased sex-ratio has been attributed to the high life-span of females compared to males [65]. We built on this knowledge by showing that females may dominate in number because they are more tolerant than males to polluted waters, where mosquitofish often occur (e.g. [66]). Female mosquitofish had higher feeding rates than males regardless of the nitrate treatment, which is consistent with previous data in clean water [64]. Given that higher nitrogen excretion rates have been reported in females [64], it is possible that tolerance to environmental nitrate can be predicted from nitrogen excretion rates in fish. Nonetheless, sensitivity to nitrate probably depends on many factors in wild fish, including temperature, predation, and the fact that a parasite with more severe effects on males than females [37] is more sensitive to nitrate than the fish host [32]. In contrast to mainstream literature in which external factors other than pollutants are often not included in toxicological assays [27], our study accounted for intraspecific interactions; that is, several individuals were exposed to nitrate in the same tank instead of fish being exposed individually. Agonistic interactions probably are amongst the most important factors to explain *G. holbrooki* performance alongside sex because females are more aggressive towards conspecifics than males [67].This might explain why more females died during the experiment than males, although mortality did not differ significantly amongst treatments.

Feeding traits were more affected by nitrate than other biomarkers measured in male mosquitofish, which supports that food intake is amongst the most sensitive biomarkers in ecotoxicology [68,69]. Although we cannot identify the mechanisms, it might be a response-mediated by stress hormones (e.g. cortisol) because high levels of these hormones often reduce appetite in fish and other animals [70]. However, cortisol levels remained stable in females of Siberian sturgeon (*Acipenser baeri*) after 30-day exposure to 250 mg NO_3^-/l, as opposed to the reproductive hormones testosterone and estradiol [24]. Moreover, there is correlative evidence for reduced sperm count in male mosquitofish at < 22 mg NO_3^-/l [29]. These studies illustrate that nitrate is an endocrine disruptor through *in-vivo* conversion to nitric oxide, which is involved in many metabolic pathways [71], suggesting that it is possible that the effects of nitrate on fish probably would have been stronger than observed if we had used biochemical biomarkers. Nevertheless, many biochemical alterations often do not have far reaching impacts on individuals, reason for which they are considered of less ecological relevance than behavioural assays, including the feeding traits we measured [27].

Even though fish differed in food intake amongst nitrate treatments, we did not observe overt signs of disease, including reduced fish growth. Reduced food ingestion in males may be attributed to fatigue because nitrate forms methaemoglobin, which transports oxygen worse than haemoglobin [19]. However, fish can cope with moderate methaemoglobinemia [72], especially in hard water, such as ours in the laboratory, which may have mitigated nitrate adverse effects [60]. Growth was expected to decrease in mosquitofish because iodine uptake, which is needed for thyroid functions and animal development, is altered by nitrate, but concentrations up to 11 mg NO_3^-/l did not impair the thyroid function in perch (*Perca fluviatilis*) and Crucian carp (*Carassius carassius*) [73]. The neutral effect of nitrate we saw on mosquitofish growth agrees with Freitag et al. [74], who found that concentrations up to 450 mg NO_3^-/l had no effect on the thyroid hormone levels in Atlantic salmon (*Salmo salar*). Our outcome is also consistent with studies in other freshwater taxa showing that nitrate effects on growth and survival occur at > 500 mg NO_3^-/l (e.g. [31,75–77]). However, the neutral effect of nitrate on mosquitofish does not exclude the possibility that fish exposed to nitrate may reduce their ability to cope with other pollutants if nitrate alters the internal ionic composition of fish at an osmoregulation cost and probably impairs important enzymatic complexes, such as those involved in detoxification [62].

Histopathological analyses revealed no relevant tissue alterations because slight epithelial lifting and other alterations we saw are not pathological but tissue processing artifacts (see [78]). Changes in caloric content only matched with histological data for juveniles at 250 mg

NO_3^-/l, which reduced energy reserves as also exemplified by peripheral fat content, but no major changes in $\delta^{13}C$ and $\delta^{15}N$ measures of muscle occurred in fish from any treatment combination. These findings suggest that no single tissue can be a good proxy of overall fish energy reserves because they vary greatly amongst tissues [50]. Moreover, our findings confirmed that no biomarker, including mass-length relationship indices such as the SMI, can be assumed to accurately reflect 'true condition' without analysing body composition [27,44]. The lack of response of the SMI may be attributed to the fact that nitrate did not markedly change fish weight, possibly because although fish reduced food intake, fish were fed daily until satiety. However, the biochemical composition of fish tissues might have changed due to nitrate because pollutants often alter tissue stoichiometry [52] with potential far reaching impacts for fish predators. In this regard, juvenile mosquitofish altered energy content in tissues, but food intake or growth were not affected, which suggests that growth is prioritised over lipid storage, probably to reduce size-dependent predation mortality [79].

In our study, energy costs are likely to be mostly attributed to intraspecific interactions and osmoregulation due to nitrate. Osmoregulation cost probably was caused mainly by the anion nitrate (NO_3^-, see [80]) and, to a minor degree, by the cation sodium (Na^+) of the salt ($NaNO_3$). The sodium concentration in the highest nitrate concentration was below 1 parts per thousand (ppt) and there is no experimental evidence for major changes in mosquitofish metabolism at 20 ppt [81] or in mosquitofish plasma osmotic concentration at 10 ppt [82]. Surprisingly, we found that the caloric content of males was higher at 50 mg NO_3^-/l than at 250 mg NO_3^-/l and in the controls at <10 mg NO_3^-/l. Reduced caloric content in males at 250 mg NO_3^-/l compared to 50 mg NO_3^-/l can be due to osmoregulation cost increasing nitrate concentration. However, this rationale does not explain why males at 250 mg NO_3^-/l had a similar caloric content to those in the controls at <10 mg NO_3^-/l, although the latter had the highest feeding rates in the study. Less energy stored implies that males at <10 mg NO_3^-/l had an additional cost than those at 50 mg NO_3^-/l, which may be the courtship display. Control males were reproductive active and we observed copulation attempts with other males, a behaviour that often occurs in male poeciliids in the absence of females [83]. Courtship display has an energetic cost [84,85], which probably was reduced at 50 mg NO_3^-/l because nitrate, even at lower concentrations, reduces testosterone [62,86] and this hormone promotes the sexual characteristics of males.

Conclusions

Our study shows that females of the invasive fish *G. holbrooki* are more tolerant to nitrate pollution than males and juveniles, but that there are all weak effects combining short- and long-term biomarkers. Therefore, the ecological impact of this invasive fish seems not to be much affected by nitrate pollution, especially if populations are female-biased. However, our study cannot inform the indirect effects that nitrate may have on *G. holbrooki* through the alteration of aquatic food-webs, including a possible reduction in prey numbers accompanied by impaired food intake in males. There is the pressing need for an–omics screening (e.g. transcriptomics) to identify simultaneously all the metabolic pathways that are altered in fish exposed to nitrate in order to improve the mechanistic understanding of the effects of this widely distributed subsidy and pollutant in aquatic ecosystems.

Supporting information

S1 Table. Mixed models analysis of variance of satiety, latency to eat and voracity of juveniles, males and females along the experiment.
(PDF)

S2 Table. Mixed models analysis of variance of calorimetry and stable isotopes of juveniles, males and females at the end of the experiment.
(PDF)

S3 Table. Mixed models analysis of variance of growth and body condition of juveniles, males and females at the end of the experiment.
(PDF)

S4 Table. Mixed models analysis of variance of histopathology variables of juveniles, males and females at the end of the experiment.
(PDF)

S5 Table. Mixed models analysis of variance of all biomarkers with sex and length as principal explicative variables.
(PDF)

S6 Table. Spearman rank correlation coefficients examining the associations amongst all biomarkers in juveniles.
(PDF)

S7 Table. Spearman rank correlation coefficients examining the associations amongst all biomarkers in males.
(PDF)

S8 Table. Spearman rank correlation coefficients examining the associations amongst all biomarkers in females.
(PDF)

S9 Table. Mean (±Standard Error) of the variables used to appraise the feeding rates of males, females and juveniles of mosquitofish.
(PDF)

S1 Fig. Histological samples of the abdominal cavity.
(PDF)

S2 Fig. Histological samples of the gill tissue examined.
(PDF)

S3 Fig. Histological samples of the hepatic tissue.
(PDF)

S1 File. Compressed file including separate datasets for juveniles, females and males data.
(7Z)

Acknowledgments

We thank the staff at the animal house facilities, especially Jordi Guinea. We also thank lab technician Cèlia Arcarons for histology sample processing, as well as Mercè Durfort and Jordi Correas for histology methods guidance. Bomb calorimetry analysis was done with the guidance of Xavier Remesar and Rosa M. Marimon. We also thank Pili Rubio for stable isotopes processing, Helena Satorras and Mònica Utjés for image editing and Mario Monroy for support in fish collection and lab procedures. We would also like to thank the two anonymous reviewers for their suggestions on this text.

Author Contributions

Conceptualization: Oriol Cano-Rocabayera, Adolfo de Sostoa, Alberto Maceda-Veiga.

Formal analysis: Oriol Cano-Rocabayera, Lorena Cárdenas.

Funding acquisition: Oriol Cano-Rocabayera, Adolfo de Sostoa, Alberto Maceda-Veiga.

Investigation: Oriol Cano-Rocabayera, Lorena Cárdenas, Alberto Maceda-Veiga.

Methodology: Oriol Cano-Rocabayera, Francesc Padrós, Lorena Cárdenas.

Project administration: Adolfo de Sostoa.

Supervision: Adolfo de Sostoa, Francesc Padrós, Alberto Maceda-Veiga.

Validation: Francesc Padrós, Alberto Maceda-Veiga.

Writing – original draft: Oriol Cano-Rocabayera.

Writing – review & editing: Adolfo de Sostoa, Francesc Padrós, Alberto Maceda-Veiga.

References

1. Smith VH, Tilman GD, Nekola JC. Eutrophication: impacts of excess nutrient inputs on freshwater, marine, and terrestrial ecosystems. Environmental Pollution 1999; 100(1–3): 179–196. https://doi.org/10.1016/s0269-7491(99)00091-3 PMID: 15093117
2. Sinha E, Michalak AM, Balaji V. Eutrophication will increase during the 21st century as a result of precipitation changes. Science 2017; 357(6349): 405–408. https://doi.org/10.1126/science.aan2409 PMID: 28751610
3. Galloway JN, Schlesinger WH, Levy H, Michaels A, Schnoor JL. Nitrogen fixation: Anthropogenic enhancement-environmental response. Global Biogeochemical Cycles 1995; 9(2): 235–252. https://doi.org/10.1029/95gb00158
4. Puckett LJ. Identifying the major sources of nutrient water pollution. Environmental Science & Technology 1995; 29(9): 408A–414A. https://doi.org/10.1021/es00009a743
5. Fields S. Global nitrogen: cycling out of control. Environmental Health Perspectives 2004; 112(10): A556–A563. https://doi.org/10.1289/ehp.112-a556 PMID: 15238298
6. Kolkwitz R, Marsson M. Ökologie der tierischen Saprobien. Beiträge zur Lehre von der biologischen Gewässerbeurteilung. Internationale Revue der Gesamten Hydrobiologie und Hydrographie 1909; 2 (1–2): 126–152. https://doi.org/10.1002/iroh.19090020108
7. Margalef R. Limnología. Barcelona: Ediciones Omega; 1983.
8. Smith VH, Schindler DW. Eutrophication science: where do we go from here? Trends in ecology & evolution 2009; 24(4): 201–207. https://doi.org/10.1016/j.tree.2008.11.009 PMID: 19246117
9. OECD. Eutrophication of waters: monitoring, assessment and control. Final report. Paris, France: Organisation for Economic Cooperation and Development; 1982.
10. European Commission. Council Directive 91/676/EEC of 12 December 1991 concerning the protection of waters against pollution caused by nitrates from agricultural sources. Official Journal of the European Communities 1991; 375, 1–8.
11. Camargo JA, Alonso Á. Ecological and toxicological effects of inorganic nitrogen pollution in aquatic ecosystems: a global assessment. Environment international 2006; 32(6): 831–849. https://doi.org/10.1016/j.envint.2006.05.002 PMID: 16781774
12. Moore AP, Bringolf RB. Effects of nitrate on freshwater mussel glochidia attachment and metamorphosis success to the juvenile stage. Environmental Pollution 2018; 242: 807–813. https://doi.org/10.1016/j.envpol.2018.07.047 PMID: 30032077
13. Lee DR, Barrett CB. Tradeoffs or synergies? Agricultural intensification, economic development and the environment. CABI; 2001. https://doi.org/10.1079/9780851994352.0000
14. Honda H, Watanabe Y, Kikuchi K, Iwata N, Takeda S, Uemoto H, et al. High density rearing of Japanese flounder, *Paralichthys Olivaceus* with a closed seawater recirculation system equipped with a denitrification unit. Aquaculture Science 1993; 41(1): 19–26. https://doi.org/10.11233/aquaculturesci1953.41.19

15. Agència Catalana de l'Aigua. 2014 Sep 20. In: Catalan Water Agency. Interactive applications [Internet]. Available from: http://aca-web.gencat.cat/sdim21/seleccioXarxes.do.

16. European Commission. 2018 Sep 20. In: Report from the commission to the council and the European Parlament on the implementation of Council Directive 91/676/EEC concerning the protection of waters against pollution caused by nitrates from agricultural sources based on member states reports from the period 2012–2015 [Internet]. Available from: https://eur-lex.europa.eu/legal-content/en/TXT/?uri=CELEX:52018DC0257.

17. Grup de Defensa del Ter. 2018 Sep 20. In: Nitrate concentration in springs of Osona [Internet]. Available from: https://www.gdter.org/wp/wp-content/uploads/GDT-NITRATS-OSONA-01-2018.pdf.

18. EPA US. The Safe Drinking Water Act. Public law 1974; 93–523.

19. Craun GF, Greathouse DG, Gunderson DH. Methaemoglobin levels in young children consuming high nitrate well water in the United States. International journal of epidemiology 1981; 10(4): 309–317. https://doi.org/10.1093/ije/10.4.309 PMID: 7327829

20. Rodríguez-Estival J, Martínez-Haro M, Martín-Hernando MP, Mateo R. Sub-chronic effects of nitrate in drinking water on red-legged partridge (*Alectoris rufa*): Oxidative stress and T-cell mediated immune function. Environmental Research 2010; 110(5): 469–475. https://doi.org/10.1016/j.envres.2010.03.008 PMID: 20382380

21. Poulsen R, Cedergreen N, Hayes T, Hansen M. Nitrate: An Environmental Endocrine Disruptor? A Review of Evidence and Research Needs. Environmental science & technology 2018; 52(7): 3869–3887. https://doi.org/10.1021/acs.est.7b06419 PMID: 29494771

22. Thurston RV, Russo RC, Vinogradov GA. Ammonia toxicity to fishes. Effect of pH on the toxicity of the unionized ammonia species. Environmental science & technology 1981; 15(7): 837–840. https://doi.org/10.1021/es00089a012

23. Jensen FB. Uptake and effects of nitrite and nitrate in animals. Nitrogen metabolism and excretion. In: Walsh PJ, Wright P, editors. Nitrogen metabolism and excretion. Boca Raton: CRC Press; 1995. pp 289–303.

24. Hamlin HJ, Moore BC, Edwards TM, Larkin IL, Boggs A, High WJ, et al. Nitrate-induced elevations in circulating sex steroid concentrations in female Siberian sturgeon (*Acipenser baeri*) in commercial aquaculture. Aquaculture 2008; 281(1–4): 118–125. https://doi.org/10.1016/j.aquaculture.2008.05.030

25. Secondi J, Lepetz V, Cossard G, Sourice S. Nitrate affects courting and breathing but not escape performance in adult newts. Behavioral ecology and socio-biology 2013; 67(11): 1757–1765. https://doi.org/10.1007/s00265-013-1583-9

26. Férard JF, Blaise C. Encyclopedia of Aquatic Ecotoxicology. Dordrecht, Netherlands: Springer; 2013.

27. Colin N, Porte C, Fernandes D, Barata C, Padrós F, Carrassón M, et al. Ecological relevance of biomarkers in monitoring studies of macro-invertebrates and fish in Mediterranean rivers. Science of the Total Environment 2016; 540: 307–323. https://doi.org/10.1016/j.scitotenv.2015.06.099 PMID: 26148426

28. Pyke GH. Plague minnow or mosquito fish? A review of the biology and impacts of introduced Gambusia species. Annual review of ecology, evolution, and systematic 2008: 39(1): 171–191. https://doi.org/10.1146/annurev.ecolsys.39.110707.173451

29. Edwards TM, Guillette LJ Jr. Reproductive characteristics of male mosquitofish (*Gambusia holbrooki*) from nitrate-contaminated springs in Florida. Aquatic Toxicology 2007; 85(1): 40–47. https://doi.org/10.1016/j.aquatox.2007.07.014 PMID: 17767965

30. Hord NG, Beaver L, Axton E, Truong L, St. Marie L, Tanguay R, et al. Nitrate and nitrite exposure affect cognitive behavior and oxygen consumption during exercise in zebrafish. The FASEB Journal 2017; 31 (1_supplement): lb277.

31. Pereira A, Carvalho AP, Cruz C, Saraiva A. Histopathological changes and zootechnical performance in juvenile zebrafish (*Danio rerio*) under chronic exposure to nitrate. Aquaculture 2017; 473: 197–205. https://doi.org/10.1016/j.aquaculture.2017.02.018

32. Smallbone W, Cable J, Maceda-Veiga A. Chronic nitrate enrichment decreases severity and induces protection against an infectious disease. Environment international 2016; 91: 265–270. https://doi.org/10.1016/j.envint.2016.03.008 PMID: 26995268

33. Johansson M, Räsänen K, Merilä J. Comparison of nitrate tolerance between different populations of the common frog, *Rana temporaria*. Aquatic Toxicology 2001; 54(1–2): 1–14. https://doi.org/10.1016/s0166-445x(00)00182-x PMID: 11451421

34. Coutellec MA, Barata C. An introduction to evolutionary processes in ecotoxicology. Ecotoxicology 2011; 20(3): 493–496. https://doi.org/10.1007/s10646-011-0637-x PMID: 21416110

35. Bateman AJ. Intra-sexual selection in Drosophila. Heredity 1948; 2: 349–368. https://doi.org/10.1038/hdy.1948.21 PMID: 18103134

36. Rolff J. Bateman's principle and immunity. Proceedings of the Royal Society B: Biological Sciences 2002; 269(1493): 867–872. https://doi.org/10.1098/rspb.2002.1959 PMID: 11958720

37. Stephenson JF, Kinsella C, Cable J, Van Oosterhout C. A further cost for the sicker sex? Evidence for male-biased parasite-induced vulnerability to predation. Ecology and Evolution 2016; 6(8): 2506–2515. https://doi.org/10.1002/ece3.2049 PMID: 27066240

38. McKim JM. Evaluation of tests with early life stages of fish for predicting long-term toxicity. Journal of the Fisheries Research Board of Canada 1977; 34(8): 1148–1154. https://doi.org/10.1139/f77-172

39. Noga EJ. Fish disease: diagnosis and treatment. John Wiley & Sons; 2011.

40. Gobierno de España. Real Decreto 1628/2011, de 14 de noviembre, por el que se regula el listado y catálogo español de especies exóticas invasoras. BOE 2011; 298.

41. Bouraoui F, Grizzetti B, Aloe A. Nutrient discharge from rivers to seas for year 2000. JRC Scientific and Technical Reports, European Commission; 2009. https://doi.org/10.2788/38971

42. WHO. Guidelines for drinking-water quality: first addendum to third edition. Vol. 1, Recommendations. World Health Organization; 2006.

43. Hopkins KD. Reporting fish growth: A review of the Basics. Journal of the world aquaculture society 1992; 23(3): 173–179. https://doi.org/10.1111/j.1749-7345.1992.tb00766.x

44. Peig J, Green AJ. New perspectives for estimating body condition from mass/length data: the scaled mass index as an alternative method. Oikos 2009; 118(12): 1883–1891. https://doi.org/10.1111/j.1600-0706.2009.17643.x

45. Post DM. Using stable isotopes to estimate trophic position: models, methods, and assumptions. Ecology 2002; 83(3): 703–718. https://doi.org/10.2307/3071875

46. Gannes LZ, O'Brien DM, Del Rio CM. Stable isotopes in animal ecology: assumptions, caveats, and a call for more laboratory experiments. Ecology 1997; 78(4): 1271–1276. https://doi.org/10.2307/2265878

47. Gaye-Siessegger J, Focken U, Muetzel S, Abel H, Becker K. Feeding level and individual metabolic rate affect $\delta^{13}C$ and $\delta^{15}N$ values in carp: implications for food web studies. Oecologia 2004; 138(2): 175–183. https://doi.org/10.1007/s00442-003-1429-7 PMID: 14608500

48. Focken U, Becker K. Metabolic fractionation of stable carbon isotopes: implications of different proximate compositions for studies of the aquatic food webs using $\delta^{13}C$ data. Oecologia 1998; 115(3): 337–343. https://doi.org/10.1007/s004420050525 PMID: 28308424

49. Cano-Rocabayera O, Maceda-Veiga A, de Sostoa A. Fish fins and scales as non-lethally sampled tissues for stable isotope analysis in five fish species of north–eastern Spain. Environmental biology of fishes 2015; 98(3): 925–932. https://doi.org/10.1007/s10641-014-0328-6

50. Wallace PD, Hulme TJ. The fat/water Relationship in the Mackerel, *Scomber scombrus* (L.), pilchard, *Sardina pilchardus* (Walbaum) and sprat, *Sprattus sprattus* (L.) and the seasonal variations in fat content by size and maturity. Directorate of Fisheries Research, Lowestoft 1977; 35: 1–10

51. Durfort M. Iniciació a les tècniques histològiques vegetals i animals. Barcelona: Institut d'Estudis Catalans; 2006.

52. Stormer J, Jensen FB, Rankin JC. Uptake of nitrite, nitrate, and bromide in rainbow trout,(Oncorhynchus mykiss): effects on ionic balance. Canadian Journal of Fisheries and Aquatic Sciences 1996; 53(9): 1943–1950. https://doi.org/10.1139/cjfas-53-9-1943

53. R Core Team. 2018 Sep 20. In: R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria [software]. Available from: http://www.R-project.org/.

54. Zuur AF, Ieno EN, Walker N, Saveliev AA, Smith GM. Mixed effects models and extensions in ecology with R. New York: Springer; 2009. https://doi.org/10.1007/978-0-387-87458-6

55. Fox J, Weisberg S. In: An R companion to applied regression, 2nd edition [software]. Thousand Oaks CA: Sage; 2011. Available from: http://socserv.socsci.mcmaster.ca/jfox/Books/Companion

56. Marchetti MP, Moyle PB, Levine R. Alien fishes in California watersheds: characteristics of successful and failed invaders. Ecological Applications 2004; 14(2): 587–596. https://doi.org/10.1890/02-5301

57. Nakazawa T, Hamaguchi S, Kyono-Hamaguchi Y. Histochemistry of liver tumors induced by diethylnitrosamine and differential sex susceptibility to carcinogenesis in *Oryzias latipes*. Journal of the National Cancer Institute 1985; 75(3): 567–573. https://doi.org/10.1093/jnci/75.3.567 PMID: 2993731

58. Perkins EJ, Griffin B, Hobbs M, Gollon J, Wolford L, Schlenk D. Sexual differences in mortality and sublethal stress in channel catfish following a 10 week exposure to copper sulfate. Aquatic toxicology 1997; 37(4): 327–339. https://doi.org/10.1016/s0166-445x(96)00825-9

59. Klein SL, Flanagan KL. Sex differences in immune responses. Nature Reviews Immunology 2016; 16 (10): 626–638. https://doi.org/10.1038/nri.2016.90 PMID: 27546235

60. Baker JA, Gilron G, Chalmers BA, Elphick JR. Evaluation of the effect of water type on the toxicity of nitrate to aquatic organisms. Chemosphere 2017; 168: 435–440. https://doi.org/10.1016/j.chemosphere.2016.10.059 PMID: 27810544

61. Hamlin HJ. Nitrate toxicity in Siberian sturgeon (*Acipenser baeri).* Aquaculture 2006; 253: 688–693. https://doi.org/10.1016/j.aquaculture.2005.08.025

62. Edwards TM, Hamlin HJ. Reproductive endocrinology of environmental nitrate. General and Comparative Endocrinology 2018; 265: 31–40. https://doi.org/10.1016/j.ygcen.2018.03.021 PMID: 29577898

63. Vargas MJ, de Sostoa A. Life history of *Gambusia holbrooki* (Pisces, Poeciliidae) in the Ebro delta (NE Iberian peninsula). Hydrobiologia 1996; 341(3): 215–224. https://doi.org/10.1007/bf00014686

64. Fryxell DC, Arnett HA, Apgar TM, Kinnison MT, Palkovacs EP. Sex ratio variation shapes the ecological effects of a globally introduced freshwater fish. Proceedings of the Royal Society B: Biological Sciences 2015; 282(1817): 20151970. https://doi.org/10.1098/rspb.2015.1970 PMID: 26490793

65. Haynes JL, Cashner RC. Life history and population dynamics of the western mosquitofish: a comparison of natural and introduced populations. Journal of Fish Biology 1995; 46(6): 1026–1041. https://doi.org/10.1006/jfbi.1995.0098

66. Maceda-Veiga A, Baselga A, Sousa R, Vilà M, Doadrio I, de Sostoa A. Fine-scale determinants of conservation value of river reaches in a hotspot of native and non-native species diversity. Science of the Total Environment 2017; 574: 455–466. https://doi.org/10.1016/j.scitotenv.2016.09.097 PMID: 27644023

67. Smith CC, Sargent RC. Female fitness declines with increasing female density but not male harassment in the western mosquitofish, *Gambusia affinis*. Animal Behaviour 2006; 71: 401–407. https://doi.org/10.1016/j.anbehav.2005.06.003

68. Kestemont P, Baras E. Environmental factors and feed intake: mechanisms and interactions. In: Houlihan D, Boujard T, Jobling M, editors. Food intake in fish. Blackwell Science Ltd; 2001. pp. 131–156. https://doi.org/10.1002/9780470999516.ch6

69. Lall SP. Disorders of nutrition and metabolism. In: Leatherland JF, Woo TF, editors. Fish diseases and disorders, Volume 2. University of Guelph, Canada: CABI; 2010. pp. 202–237. https://doi.org/10.1079/9781845935535.0202

70. Bernier NJ. Food intake regulation and disorders. In: Leatherland JF, Woo TF, editors. Fish diseases and disorders, Volume 2. University of Guelph, Canada: CABI; 2010. pp. 238–266. https://doi.org/10.1079/9781845935535.0238

71. Guillette LJ Jr, Edwards TM. Is Nitrate an Ecologically Relevant Endocrine Disruptor in Vertebrates? Integrative and Comparative Biology 2005; 45(1): 19–27. https://doi.org/10.1093/icb/45.1.19 PMID: 21676740

72. Eddy FB, Williams EM. Nitrite and freshwater fish. Chemistry and Ecology 1987; 3(1): 1–38. https://doi.org/10.1080/02757548708070832

73. Lahti E, Harri M, Lindqvist OV. Uptake and distribution of radioiodine, and the effect of ambient nitrate, in some fish species. Comparative Biochemistry and Physiology Part A: Physiology 1985; 80(3): 337–342. https://doi.org/10.1016/0300-9629(85)90047-7

74. Freitag AR, Thayer LR, Leonetti C, Stapleton HM, Hamlin HJ. Effects of elevated nitrate on endocrine function in Atlantic salmon, *Salmo salar*. Aquaculture 2015; 436: 8–12. https://doi.org/10.1016/j.aquaculture.2014.10.041

75. Hrubec TC, Smith SA, Robertson JL. Nitrate toxicity: a potential problem of recirculating systems. Northeast regional agricultural engineering service Ithaca—Publications–NRAES 1996; 1(98): 41–48.

76. Scott G, Crunkilton RL. Acute and chronic toxicity of nitrate to fathead minnows (*Pimephales promelas*), *Ceriodaphnia dubia*, and *Daphnia magna*. Environmental Toxicology and Chemistry 2000; 19(12): 2918–2922. https://doi.org/10.1002/etc.5620191211

77. Shimura R, Ijiri K, Mizuno R, Nagaoka S. Aquatic animal research in space station and its issues—focus on support technology on nitrate toxicity-. Advances in space research 2002; 30(4): 803–808. https://doi.org/10.1016/s0273-1177(02)00399-x PMID: 12530377

78. Wolf JC, Baumgartner WA, Blazer VS, Camus AC, Engelhardt JA, Fournie JW, et al. Nonlesions, Misdiagnoses, Missed Diagnoses, and Other Interpretive Challenges in Fish Histopathology Studies: A Guide for Investigators, Authors, Reviewers, and Readers. Toxicologic Pathology 2015; 43: 297–325. https://doi.org/10.1177/0192623314540229 PMID: 25112278

79. Martin BT, Heintz R, Danner EM, Nisbet RM. Integrating lipid storage into general representations of fish energetic. Journal of Animal Ecology 2017; 86: 812–825. https://doi.org/10.1111/1365-2656.12667 PMID: 28326538

80. Jensen FB. Nitrite and red cell function in carp: control factors for nitrite entry, membrane potassium ion permeation, oxygen affinity and methaemoglobin formation. Journal of Experimental Biology 1990; 152 (1): 149–166.

81. Akin S, Neill WH. Routine metabolism of mosquitofish (*Gambusia affinis*) at three different salinities. Texas Journal of Science 2003; 55: 255–226.

82. Nordlie FG, Mirandi A. Salinity relationships in a freshwater population of eastern mosquitofish. Journal of Fish Biology 1996; 49: 1226–1232. https://doi.org/10.1111/j.1095-8649.1996.tb01791.x

83. Field KL, Waite TA. Absence of female conspecifics induces homosexual behaviour in male guppies. Animal Behaviour 2004; 68: 1381–1389. https://doi.org/10.1016/j.anbehav.2003.12.022

84. Christiansen JS, Jorgensen EH, Jobling M. Oxygen consumption in relation to sustained exercise and social stress in Arctic Charr (*Salvelinus alpinus* L.). Journal of Experimental Zoology 1991; 260(2): 149–156. https://doi.org/10.1002/jez.1402600203

85. Haller J. Biochemical background for an analysis of cost-benefit interrelations in aggression. Neuroseience and Biobehavioral Reviews 1995; 19(4): 599–604. https://doi.org/10.1016/0149-7634(95)00053-4

86. Panesar NS, Chan KW. Decreased steroid hormone synthesis from inorganic nitrite and nitrate: studies *in vitro* and *in vivo*. Toxicology and Applied Pharmacology 2000; 169: 222–230. https://doi.org/10.1006/taap.2000.9079 PMID: 11133344

Supplementary materials

S1 Table. Mixed models analysis of variance of satiety, latency to eat and voracity of juveniles, males and females along the experiment. Asterisk (*) denotes significant factors and interactions at $\alpha = 0.05$. Satiety was square-root transformed except for males, while latency and voracity was $\log_e$ transformed in all fish.

Function	Sex	Effect size			t-value	P
Satiety	J	Intercept	11.1±0.40	*	27.7	<0.001
		time	-0.012±0.019		-0.651	0.515
		$50NO_3^-$	-0.089±0.328		-0.271	0.793
		$250NO_3^-$	-0.524±0.332		-1.577	0.149
		$time^2$	$-4.8E^{-3}\pm8.2E^{-4}$	*	-5.888	<0.001
		$time{:}50NO_3^-$	0.012±0.018		0.678	0.498
		$time{:}250NO_3^-$	$7.1E^{-3}\pm0.018$		0.386	0.700
	M	Intercept	5.21±0.32	*	16.1	<0.001
		time	0.05±0.01	*	3.42	<0.001
		$50NO_3^-$	-1.12±0.44	*	-2.52	0.027
		$250NO_3^-$	-1.31±0.44	*	-2.94	0.012
		$time^2$	$2.2E^{-3}\pm3.8E^{-4}$	*	5.81	<0.001
		$time{:}50NO_3^-$	-0.059±0.019	*	-3.19	0.002
		$time{:}250NO_3^-$	-0.057±0.019	*	-3.08	0.002
	F	Intercept	3.38±0.30	*	11.1	<0.001
		time	0.015±0.004	*	3.90	<0.001
		$50NO_3^-$	-0.063±0.432		-0.146	0.886
		$250NO_3^-$	0.088±0.431		0.205	0.841
		$time{:}50NO_3^-$	$1.2E^{-3}\pm5.6E^{-3}$		0.211	0.833
		$time{:}250NO_3$	$9.5E^{-4}\pm5.5E^{-3}$		0.172	0.864
Feeding latency	J	Intercept	1.62±0.12	*	13.4	<0.001
		time	$-5.5E^{-3}\pm2.9E^{-3}$		-1.906	0.057
		$50NO_3^-$	0.053±0.116		0.463	0.643
		$250NO_3^-$	0.112±0.120		0.934	0.351
		$time^2$	$5.1E^{-4}\pm9.7E^{-5}$	*	5.18	<0.001
		$time{:}50NO_3^-$	$6.6E^{-3}\pm3.8E^{-3}$		1.75	0.080
		$time{:}250NO_3$	$4.1E^{-3}\pm3.7E^{-3}$		1.097	0.273
	M	Intercept	1.72±0.13	*	13.04	<0.001
		time	-0.026±0.007	*	-3.68	<0.001
		$50NO_3^-$	0.52±0.18	*	2.92	0.013
		$250NO_3^-$	0.65±0.18	*	3.68	0.003
		$time^2$	$-1.1E^{-3}\pm1.9E^{-4}$	*	-5.54	<0.001
		$time{:}50NO_3^-$	0.027±0.010	*	2.86	0.005
		$time{:}250NO_3$	0.020±0.010	*	2.09	0.038
	F	Intercept	0.47±0.12	*	3.91	<0.001
		time	$2.1E^{-3}\pm2.7E^{-3}$		0.764	0.446
		$50NO_3^-$	0.32±0.17		1.90	0.082
		$250NO_3^-$	0.10±0.17		0.591	0.565
		$time{:}50NO_3^-$	$-7.3E^{-3}\pm3.8E^{-3}$		-1.91	0.058
		$time{:}250NO_3$	$-2.8E^{-3}\pm3.8E^{-3}$		-0.74	0.458

Voracity	J**	Intercept	1.990 ± 0.042	*	46.9	<0.001
		time	$-1.8E^{-3}\pm1.4E^{-3}$		-1.30	0.193
		$50NO_3^-$	-0.032 ± 0.047		-0.676	0.500
		$250NO_3^-$	0.047 ± 0.048		0.986	0.325
		$time^2$	$3.4E^{-4}\pm4.6E^{-5}$	*	7.38	<0.001
		$time:50NO_3^-$	$-3.8E^{-3}\pm1.9E^{-3}$		-2.02	0.044
		$time:250NO_3$	$-2.1E^{-3}\pm1.9E^{-3}$		-1.12	0.264
	M	Intercept	3.17 ± 0.13	*	24.13	<0.001
		time	$-9.4E^{-3}\pm5.6E^{-3}$		-1.66	0.098
		$50NO_3^-$	0.206 ± 0.180		1.14	0.276
		$250NO_3^-$	0.339 ± 0.179		1.89	0.083
		$time^2$	$-3.1E^{-4}\pm1.6E^{-4}$	*	-1.97	<0.050
		$time:50NO_3^-$	$9.5E^{-3}\pm7.6E^{-3}$		1.25	0.214
		$time:250NO_3$	0.021 ± 0.008	*	2.83	0.005
	F	Intercept	2.17 ± 0.16	*	13.7	<0.001
		time	-0.011 ± 0.003	*	-3.65	<0.001
		$50NO_3^-$	0.14 ± 0.22		0.640	0.534
		$250NO_3^-$	0.048 ± 0.219		0.221	0.829
		$time:50NO_3^-$	$-6.3E^{-3}\pm4.4E^{-3}$		-1.44	0.152
		$time:250NO_3$	$-5.5E^{-3}\pm4.3E^{-3}$		-1.28	0.201

** Although the t-test gives significant results for the interaction $time:50NO_3^-$ in juveniles, the overall F-test gave P=0.129.

S2 Table. Mixed models analysis of variance of calorimetry and stable isotopes of juveniles, males and females at the end of the experiment. The following abbreviatures are used: J/g, energy density; $\delta^{13}C/\delta^{15}N$, carbon or nitrogen fractionation; C/N_m, molar carbon to nitrogen ratio. Asterisk (*) denotes significant factors and interactions at $\alpha = 0.05$.

Function	Sex	Effect size			t-value	P
J/g	J	Intercept	23132±463	*	49.95	<0.001
		$50NO_3^-$	-706±381		-1.85	0.101
		$250NO_3^-$	-1366±391	*	-3.50	0.008
	M	Intercept	15084±838	*	18.0	<0.001
		$50NO_3^-$	2114±937	*	2.26	0.044
		$250NO_3^-$	335±1054		0.32	0.756
	F	Intercept	18471±409	*	45.14	<0.001
		$50NO_3^-$	299±464		0.645	0.531
		$250NO_3^-$	225±444		0.506	0.622
$\delta^{13}C$	M	Intercept	-24.11±0.23	*	-106.4	<0.001
		$50NO_3^-$	-0.43±0.32		-1.35	0.201
		$250NO_3^-$	-0.37±0.32		1.17	0.267
	F	Intercept	-21.27±0.06	*	-371.3	<0.001
		$50NO_3^-$	0.08±0.10		0.79	0.447
		$250NO_3^-$	-0.07±0.11		-0.66	0.524
$\delta^{15}N$	M	Intercept	13.33±0.39	*	34.0	<0.001
		$50NO_3^-$	0.09±0.55		0.16	0.878
		$250NO_3^-$	-0.37±0.55		-0.66	0.521
	F	Intercept	15.78±0.28	*	55.9	<0.001
		$50NO_3^-$	-0.29±0.40		-0.74	0.476
		$250NO_3^-$	-0.20±0.40		-0.50	0.625
C/N_m	M	Intercept	4.29±0.07	*	65.6	<0.001
		$50NO_3^-$	0.03±0.09		0.34	0.740
		$250NO_3^-$	-0.01±0.09		-0.13	0.901
	F	Intercept	3.97±0.03	*	115.4	<0.001
		$50NO_3^-$	-0.02±0.05		-0.35	0.731
		$250NO_3^-$	0.02±0.05		0.49	0.630

S3 Table. Mixed models analysis of variance of growth and body condition of juveniles, males and females at the end of the experiment. The following abbreviatures are used: SMI_t: scaled mass index, computed using fresh weight including viscera; G: specific growth rate. Asterisk (*) denotes significant factors and interactions at $\alpha = 0.05$.

Function	**Sex**	**Effect size**			**t-value**	**P**
G	J	Intercept	$1.1E^{-2}\pm3.8E^{-4}$	*	29.61	<0.001
		$50NO_3^-$	$2.0E^{-4}\pm4.1E^{-4}$		0.49	0.640
		$250NO_3^-$	$-6.6E^{-4}\pm4.1E^{-4}$		-1.61	0.145
	M	Intercept	$6.2E^{-4}\pm1.5E^{-4}$	*	4.19	<0.001
		$50NO_3^-$	$6.9E^{-5}\pm2.1E^{-4}$		0.33	0.748
		$250NO_3^-$	$1.9E^{-5}\pm2.1E^{-4}$		0.09	0.931
	F	Intercept	$6.5E^{-4}\pm1.2E^{-4}$	*	5.19	<0.001
		$50NO_3^-$	$6.1E^{-5}\pm1.8E^{-4}$		0.34	0.736
		$250NO_3^-$	$3.2E^{-5}\pm1.8E^{-4}$		0.18	0.862
SMI_t	J	Intercept	0.193±0.004	*	49.60	<0.001
		$50NO_3^-$	-0.004±0.003		-1.25	0.248
		$250NO_3^-$	-0.006±0.003		-2.05	0.074
	M	Intercept	0.188±0.006	*	31.6	<0.001
		$50NO_3^-$	0.004±0.007		0.62	0.548
		$250NO_3^-$	0.028±0.013	*	2.18	<0.050
	F	Intercept	0.193±0.003	*	74.57	<0.001
		$50NO_3^-$	-0.001±0.004		-0.159	0.876
		$250NO_3^-$	0.000±0.004		0.068	0.947

S4 Table. Mixed models analysis of variance of histopathology variables of juveniles, males and females at the end of the experiment. The following abbreviatures are used: MMC, melanomacrophagues centers; MF, microscope fields. Asterisk (*) denotes significant factors and interactions at α = 0.05.

Function	Sex	Effect size			t-value	P
MMC **	M	Intercept	0.809±0.509		1.59	0.126
		$50NO_3^-$	0.045±0.400		0.11	0.913
		$250NO_3^-$	0.334±0.360		0.93	0.372
		MF	0.114±0.083		1.37	0.185
	F	Intercept	3.835±0.500	*	7.68	<0.001
		$50NO_3^-$	-0.202±0.495		-0.41	0.691
		$250NO_3^-$	0.401±0.429		0.93	0.368
		MF	-0.022±0.073		-0.30	0.765
Gill secondary lamellae Alterations ***	J	Intercept	-0.328±0.503		-0.65	0.520
		$50NO_3^-$	-0.162±0.300		-0.54	0.603
		$250NO_3^-$	-0.042±0.297		-0.14	0.890
	M	Intercept	-1.258±1.584		-0.79	0.434
		$50NO_3^-$	-0.056±0.663		-0.08	0.934
		$250NO_3^-$	-0.703±0.713		-0.99	0.344
	F	Intercept	1.317±0.325	*	4.06	<0.001
		$50NO_3^-$	0.164±0.442		0.37	0.716
		$250NO_3^-$	0.265±0.432		0.61	0.551
Gill mucous cells	J	Intercept	-1.386±0.646	*	-2.15	0.032
		$50NO_3^-$	0.693±0.847		0.82	0.413
		$250NO_3^-$	1.520±0.827		1.84	0.066
	M	Intercept	-1.386±0.646	*	-2.15	0.032
		$50NO_3^-$	1.099±0.842		1.31	0.192
		$250NO_3^-$	0.693±0.847		0.82	0.413
	F	Intercept	1.012±0.584		1.73	0.083
		$50NO_3^-$	0.288±0.875		0.33	0.742
		$250NO_3^-$	-0.319±0.801		-0.40	0.691
Perivisceral fat content	J	Intercept	12.68±0.15	*	83.65	<0.001
		$50NO_3^-$	-0.216±0.143		-1.518	0.168
		$250NO_3^-$	-0.386±0.146	*	-2.643	0.030

** Melanomacrophagues centers were not present in juveniles and no statistical model was computed.

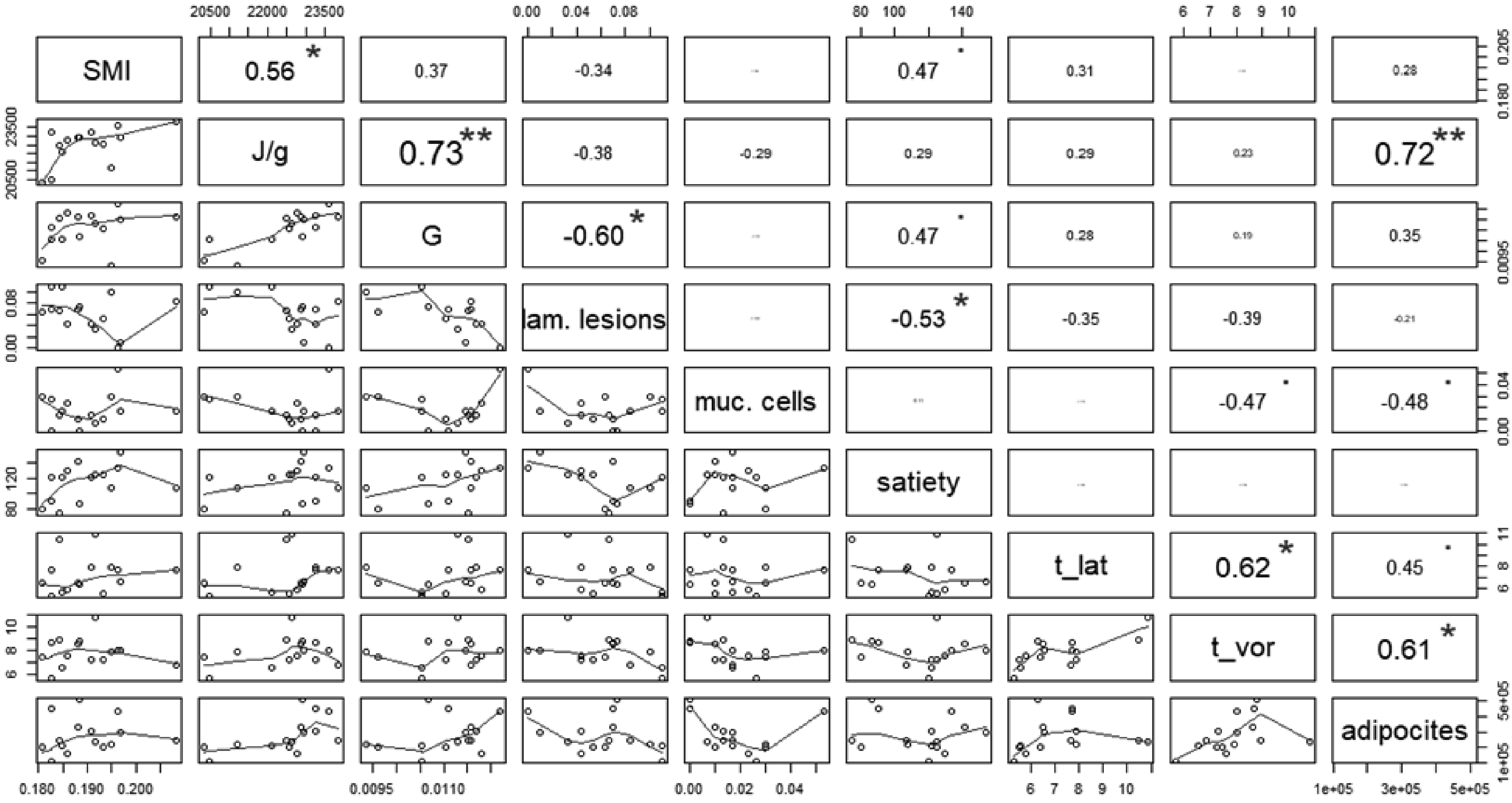
*** In gill mucous cells analysis it was necessary to do a zero-inflated model due to excess of zeros. The values shown are the statistics for the binomial part (models the absence and presence of 0’s). The counts are not shown for easiness of the reader; no statistical differences occurred between treatments.

S5 Table. Mixed models analysis of variance of all biomarkers with sex and length as principal explicative variables. The following abbreviatures are used: MMC, melanomacrophagues centers; MF, microscope fields; 2Lam%: % of gill secondary lamellae with alterations; GMC: number of gill mucous cells; J/g, energy density; δ^{13}C/δ^{15}N, carbon or nitrogen fractionation; C/N$_m$, molar carbon to nitrogen ratio; SMI$_t$: scaled mass index, computed using fresh weight including viscera; G: specific growth rate.

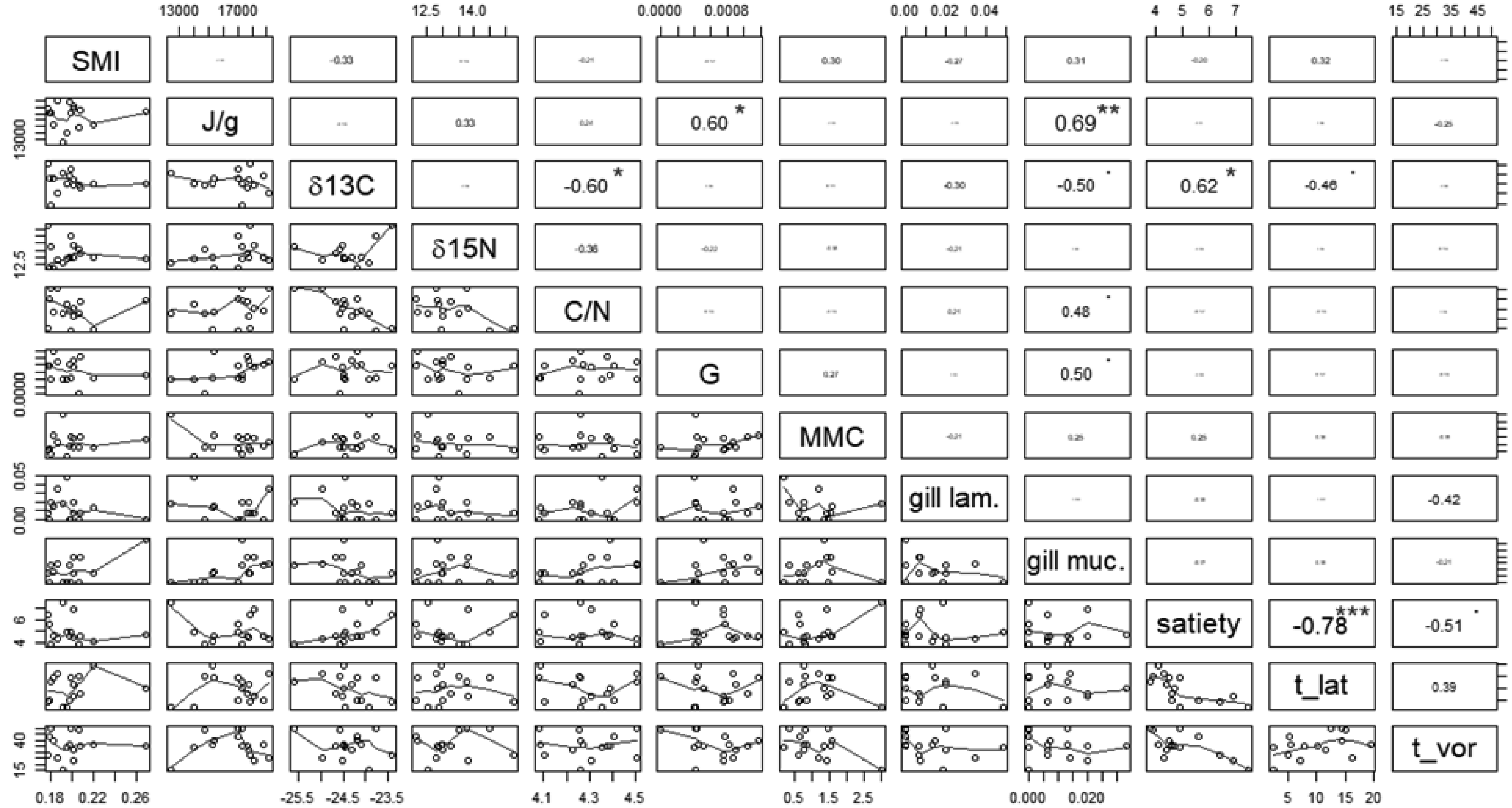
Function	Param.	F-value			P
Histopathology	MMC	Sex	40.28	*	<0.001
		length	192.16	*	<0.001
		MF	53.17	*	<0.001
	2Lam%	Sex	101.01	*	<0.001
		length	1.96		0.161
		lamellae	15.03	*	<0.001
	GMC	Sex	11.39	*	0.003
		length	9.62	*	0.002
Calorimetry	J/g	Sex	59.85	*	<0.001
		length	0.00		0.965
Stable isotopes	δ^{13}C	Sex	120.75	*	<0.001
		length	1.32		0.253
	δ^{15}N	Sex	4.640	*	0.034
		length	3.483		0.065
	C/N$_m$	Sex	3.88		0.052
		length	1.06		0.306
Mass – length variables	SMI	Sex	40.18	*	<0.001
		length	84.53	*	<0.001
	G	Sex	309.35	*	<0.001
		length	5.30	*	0.022
Feeding Behaviour **	Satiety	Sex	1617.47	*	<0.001
		length	59.25	*	0.011
		time	87.67.3	*	<0.001
		time:Sex	54.84	*	<0.001
	Latency	Sex	3.69	*	0.025
		length	0.57		0.450
		time	1.85		0.174
		time:Sex	1.32		0.267
	Voracity	Sex	443.91	*	<0.001
		length	9.38	*	0.002
		time	8.36	*	0.004
		time:Sex	38.17	*	<0.001

** Satiety was square-root transformed; latency and voracity was log-transformed.

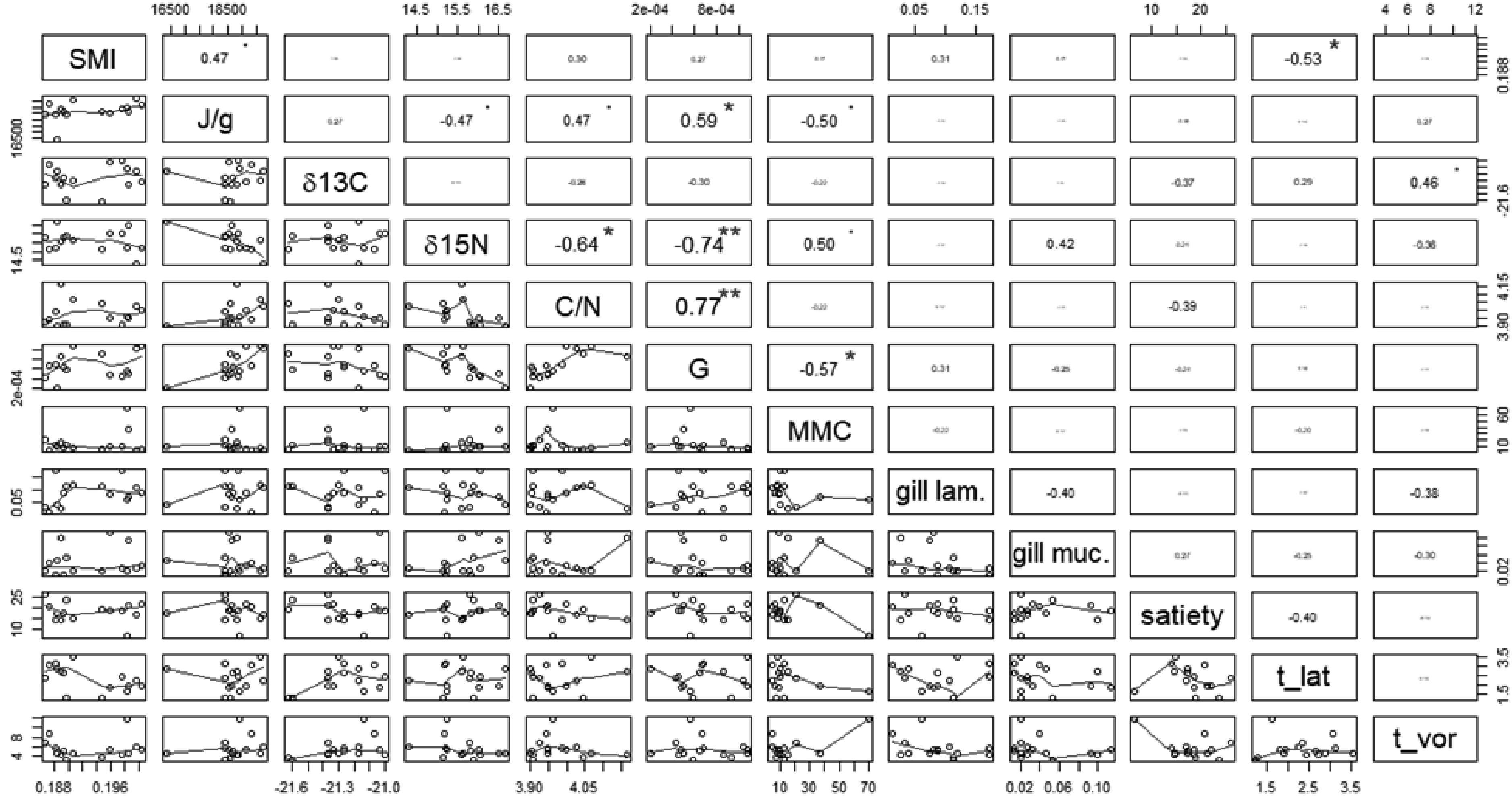
S6 Table. Spearman rank correlation coefficients examining the associations amongst all biomarkers in juveniles. The lower panel shows the graphical values of bivariate correlations with a locally weighted scatterplot smoothing (*LOWESS*) curve. In the upper panel the size of the values are set according to the absolute value of Spearman's rho (ρ). P-values are indicated in red by: ** P<0.001, * P<0.01; ● P<0.05



S7 Table. Spearman rank correlation coefficients examining the associations amongst all biomarkers in males. The lower panel shows the graphical values of bivariate correlations with a locally weighted scatterplot smoothing (*LOWESS*) curve. In the upper panel the size of the values are set according to the absolute value of Spearman's rho (ρ). P-values are indicated in red by: ** P<0.001, * P<0.01; ● P<0.05

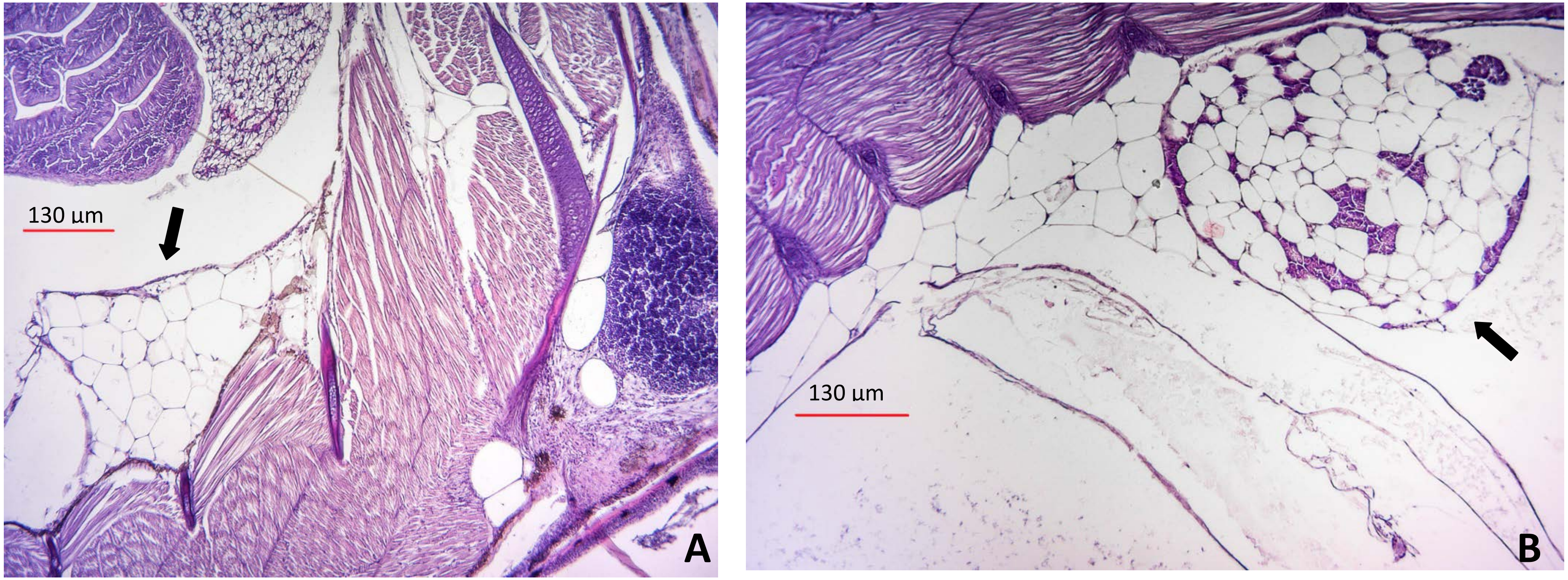


S8 Table. Spearman rank correlation coefficients examining the associations amongst all biomarkers in females. The lower panel shows the graphical values of bivariate correlations with a locally weighted scatterplot smoothing (*LOWESS*) curve. In the upper panel the size of the values are set according to the absolute value of Spearman's rho (ρ). P-values are indicated in red by: ** P<0.001, * P<0.01; ● P<0.05

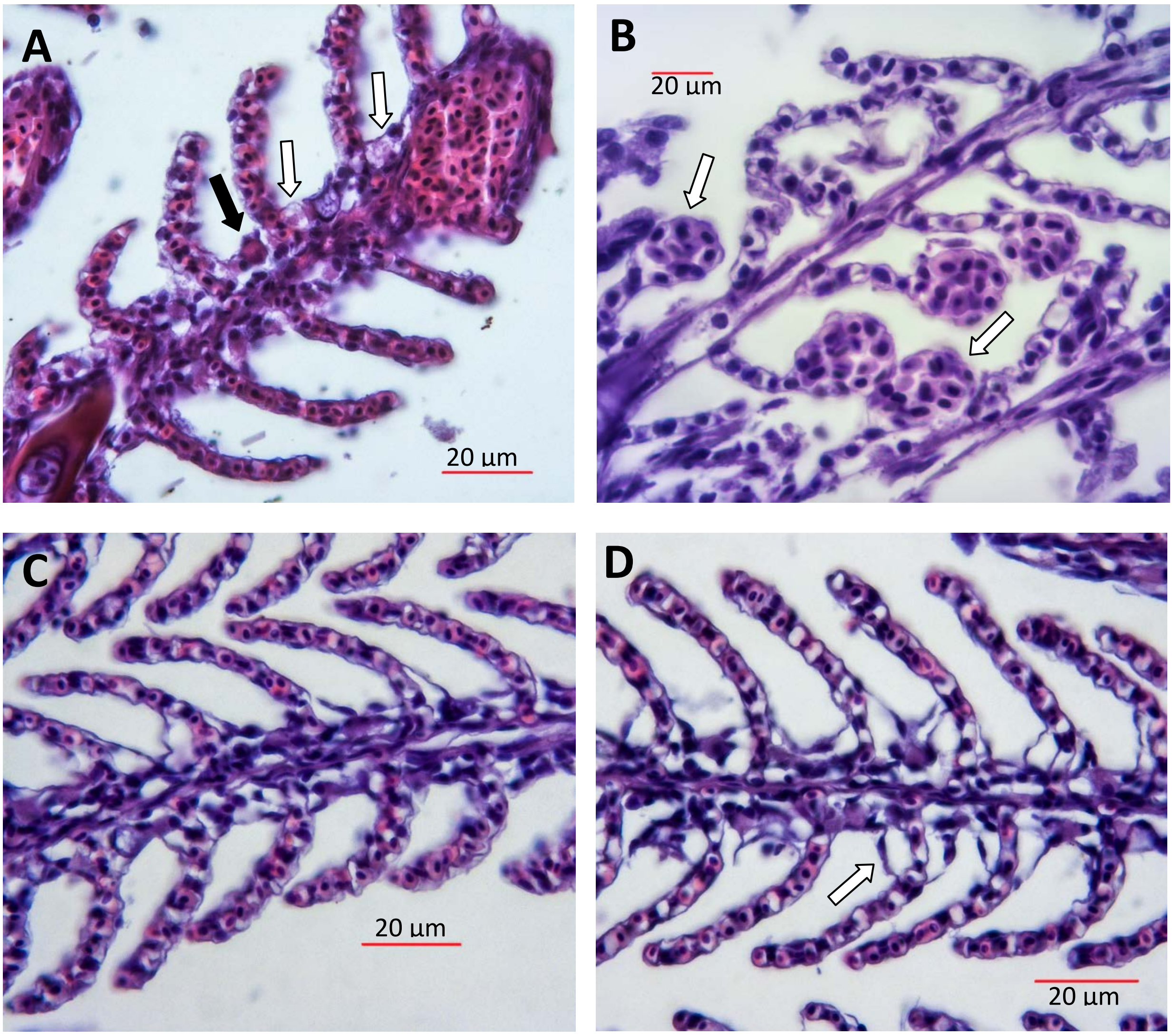


S9 Table. Mean (±Standard Error) of the variables used to appraise the feeding rates of males, females and juveniles of mosquitofish. Juveniles had an extra measurement (T1) to account for feeding changes in rapidly growing fish. T0 in juveniles was measured with a different non-experimental set of fish to avoid extra manipulation.

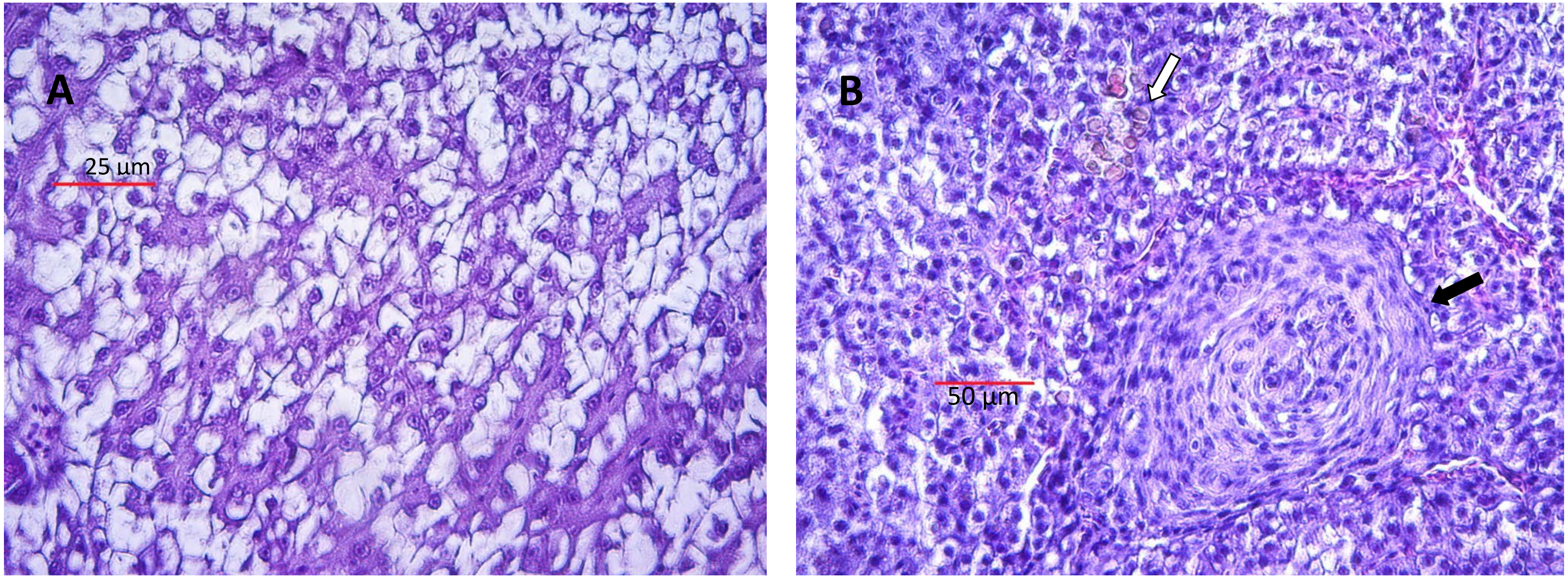
Function	Sex	NO_3^-	T0	T1	T2	T3	T4
Latency time	**J**	Control	10.9±2.2	11.5±2.2	6.7±1.1	6.9±0.7	12.9±3.5
		50		11.7±3.3	7.6±0.8	7.7±1.3	13.2±2.0
		250		11.2±1.9	9.1±1.6	5.4±0.5	18.2±3.5
	M	Control	1.7±0.3	No data	12.2±2.1	4.7±1.2	4.1±1.8
		50	1.7±0.6		10.5±2.6	14.4±2.7	9.6±1.8
		250	1.2±0.4		17.7±2.7	14.5±3.0	12.2±2.7
	F	Control	2.0±0.3	No data	1.9±0.2	2.3±0.3	2.1±0.3
		50	3.2±0.7		1.9±0.2	2.9±0.6	1.9±0.3
		250	2.2±0.3		1.7±0.1	2.4±0.4	2.2±0.3
Voracity time	**J**	Control	11.5±1.5	9.8±0.7	7.7±0.5	7.7±0.3	14.9±2.3
		50		10.8±0.7	7.4±0.4	8.1±0.6	13.1±3.2
		250		11.6±0.8	7.6±0.3	8.7±0.7	15.1±3.4
	M	Control	14.5±3.3	No data	38.1±6.0	28.8±3.6	20.9±2.4
		50	28.9±8.6		34.0±7.8	46.9±9.3	35.1±5.4
		250	15.3±3.3		31.5±3.8	48.7±7.1	39.7±3.9
	F	Control	14.4±3.7	No data	6.8±1.2	6.9±1.5	6.9±1.3
		50	12.9±2.9		8.3±1.0	6.7±1.3	4.4±0.8
		250	11.6±1.5		7.2±1.1	5.1±0.6	4.0±0.4
Satiety	**J**	Control	35.4±4.0	69.7±5.8	107.5±6.6	116.3±8.4	No data
		50		68.3±4.8	109.4±5.4	125.8±7.5	
		250		60.0±4.0	116.5±5.9	111.5±8.3	
	M	Control	7.8±0.6	No data	4.1±0.4	6.4±0.3	6.1±0.2
		50	7.2±0.7		4.3±0.4	4.6±0.2	4.2±0.4
		250	8.2±0.4		3.7±0.5	4.5±0.2	4.2±0.2
	F	Control	12.2±1.3	No data	17.7±1.5	17.8±1.9	18.2±1.8
		50	11.4±1.0		16.4±1.6	16.1±1.4	19.5±1.7
		250	12.3±0.9		16.3±1.0	18.3±1.5	19.2±1.4



S1 Figure. Histological samples of the abdominal cavity. Adipocytes (black arrows), sometimes with pancreatic tissue associated, conform visceral mass surrounding intestine, liver (A) and swim bladder (B) in mosquitofish juveniles. Magnification x100.



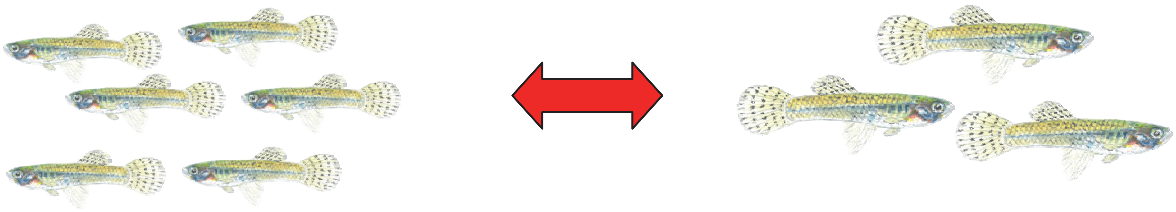
S2 Figure. Histological samples of the gill tissue examined. No differences were found between control and nitrate exposed mosquitofish individuals at 50 and 250 mg/l. A) Normal gill structure, with slight congestion in the blood vessels. Black arrow: mucous cell in the gill epithelium. White arrow: chloride cells at the base of the secondary lamella. No hyperplasia or changes of both cell types were found in nitrate treatments compared to control. B) Small telangiectasias (white arrows) occasionally appeared in few individuals. These small dilations of blood vessels are likely attributable to the euthanasia methods. C and D) Normal aspect of gill filament with secondary lamellae in females. Slight epithelial lifting at the base (white arrow) can be occasionally found in fish at different exposure levels. These changes are considered fixation artefacts. Magnification x400.



S3 Figure. Histological samples of the hepatic tissue. A) Normal layout of hepatocytes with white areas within the cytoplasm. B) Occasional presence of granulomas (black arrow) in few individuals of adult mosquitofish. We could not ascertain their origin, possibly after Microsporidia or parasite infection. Macrophage aggregates (white arrow) in the vicinity of the granuloma. These aggregates, which tend to increase in number and size in different organs as fish grows, were also present in males and especially females. Magnification x400, x200.

CAPÍTOL 3

La gestió d'espècies invasores aquàtiques de mida petita i altament prolífiques: explorant una aproximació ecosistèmica per a la gambúsia (*Gambusia holbrooki*)



Oriol Cano-Rocabayera[a]; Adolfo de Sostoa[a]; Lluís Coll[a]; Alberto Maceda-Veiga[a,b]

[a] *Departament de Biologia Evolutiva, Ecologia i Ciències Ambientals – Institut de Recerca de la Biodiversitat (IRBio-UB), Universitat de Barcelona, Barcelona*

[b] *Departamento de Ecología Integrativa, Estación Biológica de Doñana (EBD-CSIC), Sevilla*

Science of the Total Environment 673 (2019) 594–604

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Managing small, highly prolific invasive aquatic species: Exploring an ecosystem approach for the eastern mosquitofish (*Gambusia holbrooki*)

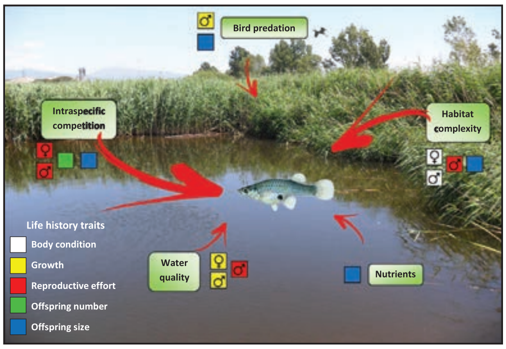
Oriol Cano-Rocabayera [a,*], Adolfo de Sostoa [a], Lluís Coll [a], Alberto Maceda-Veiga [a,b]

[a] *Department of Evolutionary Biology, Ecology and Environmental Sciences, Institute of Research in Biodiversity (IRBio-UB), Universitat de Barcelona, Spain*
[b] *Department of Integrative Ecology, Estación Biológica de Doñana (EBD-CSIC), Sevilla, Spain*

HIGHLIGHTS

- Invasive species threaten native diversity and their spread must be controlled.
- Life-history variation in *Gambusia holbrooki* was mostly explained by its density.
- Weaker associations were observed for bird abundance and water-quality conditions.
- The best-preserved sampling sites had the fish with the best body condition.
- Direct removal seems to be the best action to control *G. holbrooki* invasion.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 17 January 2019
Received in revised form 14 February 2019
Accepted 15 February 2019
Available online 06 April 2019

Editor: Damia Barcelo

Keywords:
Invasion success
Life-history traits
Mosquitofish
Ecosystem approach
Density-dependent limiting factors
Ecological management

ABSTRACT

Invasive exotic species threaten native biodiversity worldwide and their management is on the agenda of an increasing number of countries. We explored the potential of an ecosystem approach for the natural control of *Gambusia holbrooki*, which is among the most pernicious and widely distributed fish invaders. Individual-based linear mixed models were used to identify the ecosystem factors (conspecific density, environment and piscivorous birds) that most influenced life-history variation in male and female *G. holbrooki* ($N = 654$). All traits (body condition, growth, length, gonad weight, offspring size and number, real and potential fertility) were associated with at least one ecosystem factor from the 18 water bodies surveyed in north-eastern Spain. Models for female reproductive traits had the highest fit ($R^2 = 0.89$) and those for body condition in both sexes the lowest (0.12). The life history of *G. holbrooki* was mostly affected by its density; increasing offspring number at the expense of offspring size at the sites with the highest fish density. Weaker effects on *G. holbrooki* life history were observed for the abundance of piscivorous birds and water-quality conditions, including turbidity and nutrient concentrations. Although effects were not consistent between traits, outputs supported that *G. holbrooki* has a wide tolerance to changes in water quality. Therefore, actions based solely on environmental changes within the range tested probably will fail in reducing the proliferation of *G. holbrooki*, especially if its body condition improved at the most naturalised sites. Overall, this study suggests that the management of *G. holbrooki* using ecologically sound treatments is likely to be very difficult in stagnant waters. Preventing new introductions and direct removal once established are the most appropriate actions for the management of this small, highly prolific fish invader.

© 2019 Elsevier B.V. All rights reserved.

---

[*] Corresponding author at: Departament de Biologia Evolutiva, Ecologia i Ciències Ambientals, Facultat de Biologia, Universitat de Barcelona, Av. Diagonal 643, 08028 Barcelona, Spain.
E-mail address: canorocabayera@gmail.com (O. Cano-Rocabayera).

https://doi.org/10.1016/j.scitotenv.2019.02.460
0048-9697/© 2019 Elsevier B.V. All rights reserved.

1. Introduction

Invasive exotic species threaten native biodiversity worldwide and their management is on the agenda of an increasing number of countries (Perrings et al., 2010; Essl et al., 2018; Roy et al., 2018). However, the management of invasive species is complex and expensive, with annual costs of $120 billion in the USA and €20 billion in Europe alone (Pimentel et al., 2005). These figures are likely to be underestimated because the monetary cost of many invasions is unknown and the number of new introductions continues unabated (Pimentel et al., 2005; Seebens et al., 2017). Once an exotic species invades a region, its eradication is often extremely difficult (Genovesi, 2011). This particularly applies to invasions by small animals, which are difficult to detect due to size and to control due to high fecundity (Prior et al., 2018). It is therefore not surprising that large mammals account for the highest number of successful eradications, especially in spatially restricted ecosystems such as islands (Genovesi, 2011; DIISE, 2018). However, many invasions are caused by small body-size animals, including in aquatic ecosystems, where pernicious invaders, such as zebra mussel *Dreissena polymorpha* and mosquitofish *Gambusia* spp., are proliferating (Lowe et al., 2000). Moreover, up to 50% of eradication schemes do not restore altered habitats simultaneously and, consequently, native communities do not recover (Prior et al., 2018). Therefore, research into more ecologically sound control methods for invasive species is needed, especially given the new animal welfare standards (Cowan and Warburton, 2011).

An ecosystem approach can be defined as a way to regulate invasive populations by altering the natural mechanisms governing the structure of communities (Zavaleta et al., 2001; Hulme, 2006). The disproportional abundance of an invasive species is due to altered top-down (predator mediated) or bottom-up (resource mediated) controls (Hulme, 2006). Invasive species often occur in human-altered habitats, in part due to the lack of functionally equivalent native species (i.e. empty niches, Fridley and Sax, 2014). Predation may offset this bottom-up effect, but invasive species are often regarded as free of natural enemies (e.g. Torchin et al., 2003). However, structurally simplified ecosystems may facilitate predation because prey are more difficult to locate and to catch in complex habitats (Hartel et al., 2007). Nonetheless, poor environmental conditions, which are typically associated with the success of invasive species, often reduce the abundance of aquatic predators (e.g. Maceda-Veiga et al., 2018). Therefore, understanding how changes in predation and abiotic conditions affect the life history strategy of a species is the first-step towards developing an ecosystem approach for its management.

The widely distributed eastern mosquitofish *Gambusia holbrooki* exemplifies the difficulties in the management of a small (total length = 1–5 cm), prolific invasive species (Pyke, 2005). Originating from the U.S.A. and Mexico, this thermophilous poeciliid has been introduced in 34 countries for mosquito biocontrol (Pyke, 2005; Froese and Pauly, 2018), including Spain in 1921 (Doadrio, 2002). However, declines in the abundance of native invertebrates, fish and amphibians followed *G. holbrooki* introductions (e.g. Pyke, 2005; Vannini et al., 2018), including globally threatened taxa such as the endemic cyprinodontid fish *Aphanius iberus* and *Valencia hispanica* (Doadrio, 2002). Populations of *G. holbrooki*, like those of other fast-living species (Ricklefs and Wikelski, 2002, formerly r-strategist *sensu* Pianka, 1970; Fig. 1), increase in size fast because juveniles grow quickly and females give birth every 4 weeks (Vargas, 1993; Pyke, 2005). Even though the environmental determinants of *G. holbrooki* life history are relatively well studied (Meffe, 1992; Fernández-Delgado and Rossomanno, 1997; Pyke, 2005; Murphy et al., 2015), most research has focused on just one or two factors (e.g. salinity, temperature) (e.g. Meffe, 1992; Martin et al., 2009; Alcaraz and García-Berthou, 2007). Piscivores are also known to feed upon *G. holbrooki* in invaded areas (e.g. Bredin, 1984; Ashoori et al., 2017). However, no study has explored whether the life-history strategy of *G. holbrooki* changes as function of the environmental conditions and predation, as occurs with other highly prolific species (e.g. Servanty et al., 2011). Density-dependent limiting factors, including intraspecific competition, may also influence the life-history strategy of *G. holbrooki* because of its high fecundity (Bassar et al., 2013). Nonetheless, this question has not yet been investigated.

Our study aims to identify the ecosystem factors at which management actions can act to reduce the invasion success of *G. holbrooki*. Murphy et al. (2015) reported that the occurrence of *G. holbrooki* was more determined by geography-related variables than by environmental factors associated with human impacts. However, geographical differences often obscure associations with local conditions and better inferences are expected in studies at finer scales, especially if life-history traits are examined (e.g. Alcaraz and García-Berthou, 2007; Lee

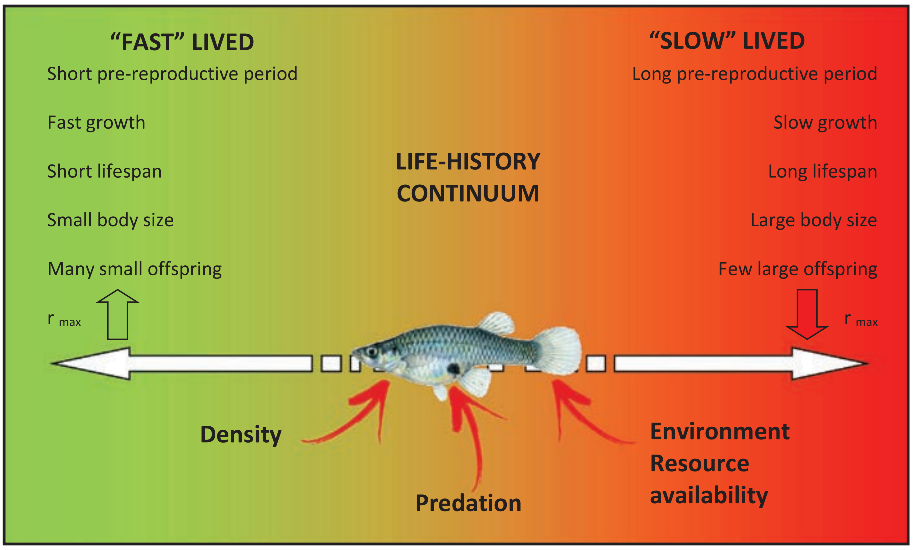


Fig. 1. Hypothetised fast-slow continuum gradient in the life-history strategy of the eastern mosquitofish (*Gambusia holbrooki*) as function of ecosystem factors, including intrinsic constraints, such as conspecific density. Inspired from the classical r- and K living strategies by Pianka (1970), it was later expanded in the form of pace-of-life hypothesis Ricklefs and Wikelski (2002).

et al., 2017). We used the latter approach to study life-history variation in *G. holbrooki* across 18 waterbodies mostly surrounded by agroecosystems. These are important habitats for many native species (Davies et al., 2016), and are among the most at risk due to human actions, including species invasions (Paini et al., 2016). If an ecosystem approach has potential to regulate *G. holbrooki* invasion, then predation pressure and habitat quality should be associated with life history traits modulating invasion success, such as fertility, growth rate and body condition. However, we expected these associations to be weak if *G. holbrooki* has a wide tolerance to changes in water and physical habitat conditions (Pyke, 2005). If so, we should find that *G. holbrooki* life-history is primarily modulated by density-dependent limiting factors, including intra-specific competition and cannibalism.

2. Materials and methods

2.1. Study system

We studied changes in the growth rate, body condition and fertility of *G. holbrooki* as function of predator abundance, environmental conditions and *G. holbrooki* density in 18 water bodies from five relatively closed regions in north-eastern Spain (Fig. 2). Most sampling sites were ponds, but there were also six irrigation channels and 1 stream; all low flow conditions. All sampling sites were confined-like experimental settings, ranging from simple man-made to complex natural habitats and represented relatively wide ranges of water quality (Table 1). To facilitate the establishment of associations between life-

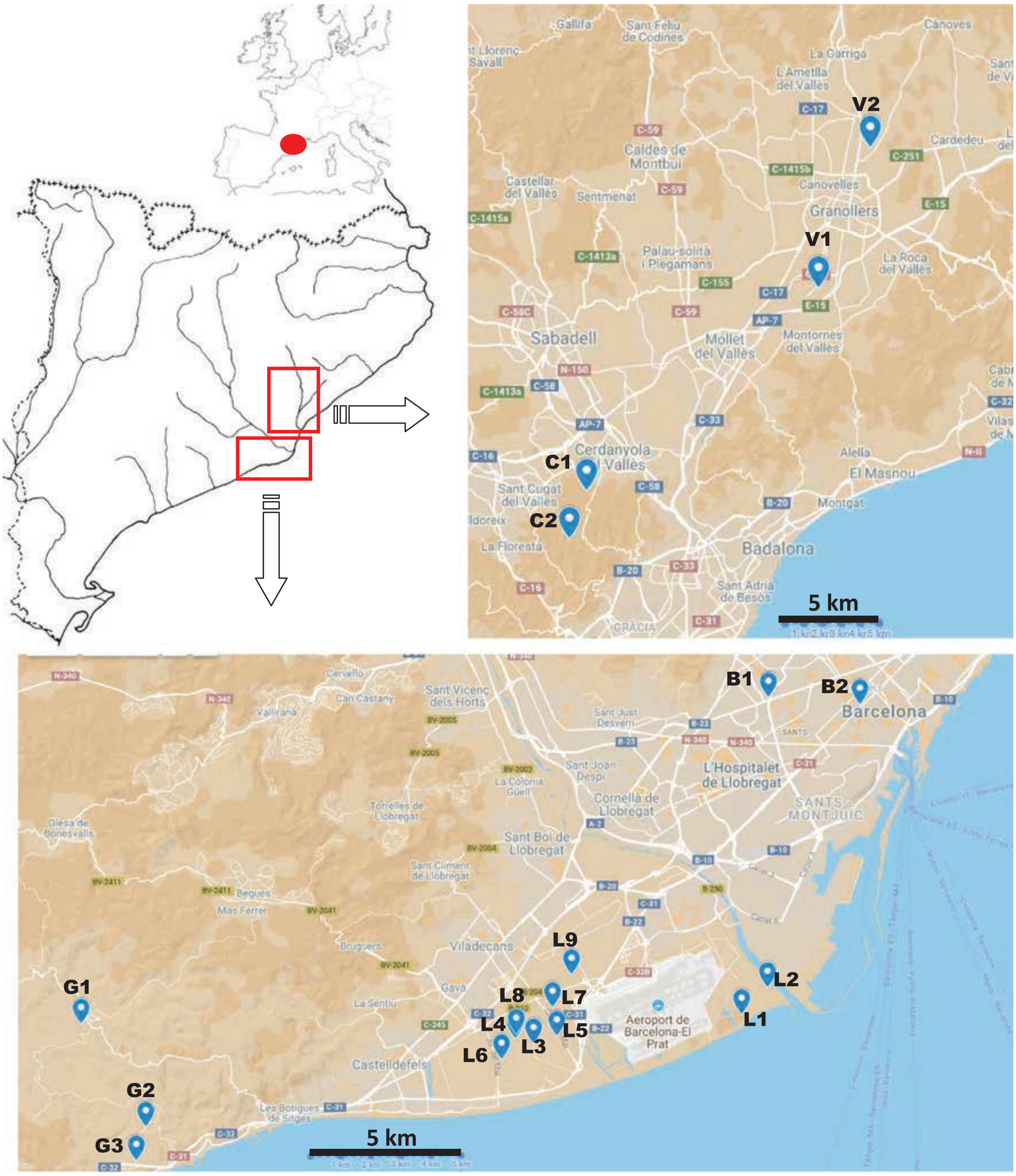


Fig. 2. Distribution of the 18 water bodies surveyed in north-eastern Spain to explore life-history variation in *Gambusia holbrooki*. Letters grouped sampling sites according to five main clutches (G, Garraf; L, Llobregat delta; B, Barcelona city; C, Collserola Natural Park and V, Vallès Oriental). 'Naturalised' complex localities: L1, L5, L6, L7, C1, C2, V1; 'man-made' simple localities: G1, G2, G3, L2, L3, L4, L8, L9, B1, B2, V2.

Table 1
List of water quality and environmental sampled parameters of the sampling locations. The values represent the median (min–max) of the 18 locations. Each location had 4 sampling dates, and their median was used as the representative value for each location. KH and GH stand for carbonate and general hardness, respectively. Parameters used in the models are highlighted in bold.

Parameter	Median (range)	Parameter	Median (range)
Surface area (m^2)	151.7 (7.4–1339.2)	**NH_4^+ (mg/l)**	0.2 (0.0–3.0)
Temperature (°C)	19.92 (16.36–23.54)	**NO_2^- (mg/l)**	0.08 (0.05–1.00)
Conductivity (μS/cm)	1508 (203–25,355)	**NO_3^- (mg/l)**	0.7 (0.0–48.0)
Salinity (‰)	2 (0–18)	**PO_4-P (mg/l)**	0.4 (0.0–1.8)
pH	8.0 (7.5–9.0)	KH (mg $CaCO_3$/l)	232.2 (71.4–723.3)
Dissolved O2 (%)	78.28 (23.65–135.85)	GH (mg $CaCO_3$/l)	401.9 (98.2–857.3)
Turbidity (FTU)	48.25 (12.8–155.6)	**Fish density**	3 (1–4)
Chlorophyll-a (μg/l)	0.02 (0.00–0.22)	**Estimated predation**	0.054 (0–0.990)

history traits and local conditions, we surveyed from 7 to 20 of May 2015, when most females of *G. holbrooki* had not yet given birth to the first new-borns of the year (e.g. Alcaraz and García-Berthou, 2007). Other fish species than *G. holbrooki* did not occur in the study sites apart from *Cyprinus carpio* and *Carassius* spp. in the localities G1, C1 and C2 (*authors pers. observ.*). The selected sites were placed in regions frequently visited by users of the citizen-science project Ornithocat (http://www.ornitho.cat) in order to use its records to infer bird predation on fish. We focused on birds because they are the most important fish predators in biomass in the study area.

2.2. Environmental conditions

Standard water properties known to affect fish health were measured in all water bodies prior to fish surveys and water analyses were repeated in October 2015, February 2016 and July 2016 to account for seasonal variation. The colorimetric test kit VISOCOLOR® was used to quantify carbonate and total water hardness (° dK and °dGH, respectively) and the concentrations of ammonium (mg L^{-1} NH_4^+; detection limit, d.l. < 0.2 mg L^{-1}), nitrite (mg L^{-1} NO_2^-, d.l. < 0.02 mg L^{-1}), nitrate (mg L^{-1} NO_3^-, d.l. < 1 mg L^{-1}) and phosphate (mg L^{-1} PO_4^{3}-P, d.l. < 0.2 mg L^{-1}). The digital probe YSI MPS 556 was used to measure water conductivity (μS/cm) and the probe CRISON OXI 45P determined water temperature (°C) and dissolved oxygen concentration (mg L^{-1} O_2 and % saturation).

Ammonium, nitrite, nitrate and phosphate are subsidies (i.e. nutrients for primary producers) but at high concentrations, they are toxic to fish and all four can profoundly alter the structure of aquatic ecosystems because of eutrophication (Wetzel, 2001). The concentration of chlorophyll-a was used as a complementary indicator of eutrophication, coupled to pH and the concentration of dissolved O_2. In February 2016 (winter) and July 2016 (summer), a water sample from each pond was filtered through a glass fibre Whatman GF/F filter in situ and was processed following standard procedures (Parsons and Strickland, 1963). Sampling sites were selected to minimise the influence of seawater because salinity is already known to reduce invasion success in *G. holbrooki* (Alcaraz and García-Berthou, 2007). Therefore, our conductivity measures are mostly due to pollution and natural mineralization and we accounted for the latter through water hardness. Water turbidity (FTU) was quantified using the turbidimeter Orbeco-Hellige MP-975 as another factor that may affect *G. holbrooki*, e.g. foraging efficiency (Horppila et al., 2004).

Water bodies were grouped into ‘man-made simple habitats’ and ‘natural complex habitats’ because these were the two categories that best represented the variation in habitat complexity in the study area. ‘Man-made simple habitats’ ($N = 11$) were ponds made of concrete used for irrigation or ornament, which offer limited refugee to fish and predators. ‘Natural complex habitats’ ($N = 7$) were natural or naturalised water bodies with relatively well-developed riparian vegetation and often dead wood at the bottom, rock cavities and submerged aquatic plants. We used Google Earth® to calculate the surface area (m^2) of all sampling sites because prey and predators are more diverse and abundant in larger basins (Blakely and Didham, 2010).

2.3. Piscivorous birds

Citizen science projects are a new avenue to obtain biological data with documented benefits on conservation (e.g. Cornell Lab of Ornithology's eBird), especially if a scientific advisory board validates the records as occurs with Ornithocat from the Catalan Ornithological Institute. We downloaded all bird observations in the studied sites from 21/05/2013 to 20/05/2016. Species of Ardeidae, Podicipedidae, Alcedinidae and Rallidae were considered potential fish predators (see details in Table S1) apart from coots (*Fulica atra*), which are mostly herbivorous (Ashoori et al., 2017). Predation pressure was expressed as a ratio between the total number of observations of piscivorous birds and the total number of bird observations at each site to account for differences in sampling effort across the study area. In Ornithocat, users report the scientific name and the number of individuals of each species in 1 × 1 UTM.

2.4. Fish sampling and life-history traits

We captured *G. holbrooki* with dip nets using a variable sampling effort. Deep ponds or large wetlands required using waders and the sampling time increased with habitat complexity. However, shallow ponds with high or low habitat complexity were surveyed relatively quickly from the shore. This situation makes difficult to calculate captures per unit of effort and we instead used a visual semi-quantitative estimation of fish density as did Pilcher and Copp (1997). *Gambusia holbrooki* mostly swims close to the surface (Pyke, 2005), so that abundance can be assumed to be proportional to the surface area occupied by the fish prior to capture the fish. We ranked abundance on a 1–4 scale such as 1, 10, 100 or >100 individuals/m^2. Minnow traps were not used because very few fish were captured in a 48 h pilot survey. We did capture 20 males and 20 females in each site relatively quick to fit all surveys early in the breeding season (see above). Fish were euthanized with MS-222® in situ, fixed in 10% formalin and transported to the University of Barcelona.

2.4.1. Fish body mass, length and reproductive traits

Euthanized fish were measured (total length, mm) and their total and eviscerated weight were recorded (±0.1 mg) in the laboratory. The number of oocytes in each development stage was counted under an Olympus VMT dissecting microscope. Oocyte classification followed the six categories of Reznick (1981): *I*, yolked non-fertilized eggs (no discernible development, pre-vitellogenic ones are not counted); *II*, uneyed (primitive streak embryos); *III*, early-eyed (some retinal pigment appears); *IV*, middle-eyed (melanophores appear on the dorsum and fin rudiments present); *V*, late-eyed (fin rays present, tail overlaps head); *VI*, mature embryos (yolk is virtually absent). Stages *II*–*VI* were considered as fertilized eggs. The total weight of gonads (intra-ovarian tissue was discarded) and that of oocytes in each development stage was recorded. All weight measures were recorded after 48 h drying at 60 °C. We calculated a ratio between gonad and total fish eviscerated weight (i.e. reproductive allotment, RA) as an integrated measure of total gonad development in males and females of *G. holbrooki*.

The oocyte classification was used to calculate potential fertility (stage I) and real fertility (from stages *II* to *VI*), both of which are complementary indicators of the breeding strategy of *G. holbrooki*. High real but low potential fertility suggests that the experimental factors might promote early reproduction, which is typical of a fast-living organism (Fig. 1). However, real fertility is expected to decline at the expense of embryo size if bigger new-borns have better competitive abilities, which mirrors a slow-living strategy. Nature is placed in a continuum and *G. holbrooki* is unlikely to be an exception, so that its living strategy might vary depending on the local conditions in order to improve invasion success.

2.4.2. Fish body condition and growth rate

Fish body mass and length were used to calculate the Scaled Mass body condition Index, such that: $SMI = W_i\,[L_o/L_i]^{bSMA}$ (Peig and Green, 2009; Maceda-Veiga et al., 2014), where W_i and L_i were the weight and length of each fish individual, L_o was the arithmetic mean length of all individuals and *bSMA* was the slope of a standardized major axis regression between the body mass and length measures of all fish.

Fish growth rate was estimated using scale rings (annuli), which are formed one per year in fish from temperate latitudes (Lea, 1910; Vargas, 1993). We collected 5–8 scales from the same dorsal skin area and side of each fish. Scales were mounted on slides and rings were examined under a dissecting microscope. The first annulum close to the centre of the scale is the "date of birth" (focus) and the number of annuli is fish age. We measured the distance between the focus and the anterior margin of the scale (S_c), and between the focus and each annulus (S_i). These radii alongside fish length at the time of sampling (L_c) were used to back-calculate fish size at each age using the equation of Whitney and Carlander (1956):

$$L_i = \frac{c + d\,S_i}{c + d\,S_c}\,\mathrm{L_c}$$

where, *c* and *d* are the intercept (*c*) and slope (*d*) of L_c regressed on S_c and L_i is the estimated fish size at the time of formation of the *i*th scale annulus. The fish length at the sampling date and at the last formed annulus were used to calculate specific growth rate (SGR) during the sampling season such that: $SGR\,(\%\cdot \mathrm{day}^{-1}) = 100\cdot(\ln L_c - \ln L_i)\,/\,\Delta t$. $\mathrm{Ln}L_c$ and $\ln L_i$ is the natural logarithm of L_c and L_i, and Δt is the number of days between the sampling date and the last 'birthday' of each fish, that is when fish began to grow after winter.

2.5. Statistical analyses

The distribution of all variables was visually inspected and series of generalized linear mixed models (GLMMs) were built to explore the associations between fish life-history traits and explanatory factors using the most appropriate error distributions and link functions in R (see below). Gradients of variation in local conditions were used in models instead of grouping water bodies according to a binary condition (e.g. oligotrophic vs eutrophic waters) because gradients are more realistic and objective representations of ecosystems due to natural covariation among explanatory variables. The life-history traits (dependent variables) were the Scaled Mass Index (SMI), specific growth rate, back-calculated length and reproductive effort, as defined by the reproductive allotment, potential and real fertility, total number of oocytes (offspring number) and mean oocyte size (offspring size). Models were built separately for each sex because females are much bigger than males in *G. holbrooki*.

2.5.1. Pair-wise correlations and multicollinearity among predictors

Spearman rank correlation coefficients were used to examine redundancy among the 16 ecosystem factors (predictors) prior to modelling. The least correlated predictors ($|\rho| < 0.60$) were inspected for multicollinearity (i.e. shared variance among predictors, see Zuur et al., 2009) using variation inflation factors (VIF, Table S2). Variables with the highest VIF were sequentially removed until VIF was <3, which is the 'safe' threshold to accurately interpret regression estimates in models (Zuur et al., 2009). Ecosystem factors in the final set were grouped as nutrient concentrations, other water quality variables, bird predation, physical habitat features and fish density. These five groupings tend to be managed simultaneously by resource managers and were associated with our a priori hypotheses to explain invasion success in *G. holbrooki* (Table 2). All predictors were scaled (z-scores).

2.5.2. Associations between life-history traits and ecosystem factors

The relative importance of the 5 sets of predictors to explain variation in the 8 life-history traits of *G. holbrooki* was examined using the function *glmer* in R. Gaussian errors with identity link were used for all life-history traits apart from fertility data and offspring number. The latter was modelled using the Poisson error distribution with log-link. Models for fertility were built in two steps because of the many 0 s. The presence (1) and absence (0) of oocytes in an early or late development stage was modelled assuming the binomial error distribution. Then, negative binomial models were used to explore variation in each of the two development stages across the study area.

All predictors were included individually in GLMMs (e.g. SMI ~ pH + turbidity + temperature) but combined in sets (e.g. water quality). There were 32 nested models, one for each combination of sets, including the null model (just with random effects and intercept) and the full model including all predictors. Site ID was included as random effect in all models to account for the fact that multiple fish were surveyed in each sampling site. For oocyte data, female ID was nested to site ID as random effect to account for the mother effect. The random factor region was excluded from final models because its standard deviation was very low (e.g. 0.007 for reproductive allotment) and did not have a marked effect on the estimates for ecosystem factors. All were random intercept models.

Table 2
Rationale for using five sets of predictors (general water quality, nutrient concentrations, predation, density-dependent limiting factors and physical habitat) to explore their associations with life-history variation in *Gambusia holbrooki*. Predictors other than those listed were excluded to avoid excessive collinearity (see Materials and methods). All predictors were included in models as individual variables.

Sets	Ecosystem factor	Rationale
General water quality variables	Temperature	Both fish and ecosystem metabolism depend on water temperature.
	pH	pH increases with the photosynthetic activity and reduces with the accumulation of dead organic matter. Eutrophication increases pH in daylight.
	Turbidity	Indicator of eutrophication, siltation and agricultural run-off.
Nutrients	Chlorophyll-a	Indicator of eutrophication (green water originating from algae blooms).
	Ammonium, nitrite, nitrate and phosphate	Subsidies causing eutrophication at high concentrations. Ammonium and nitrite are highly toxic to aquatic fauna at low levels.
Predation pressure	Piscivorous bird abundance	Piscivorous birds were often seen in the study area feeding upon fish, so that the life-history of *G. holbrooki* might be affected by their abundance.
Density-dependent factors	Conspecific density	*G. holbrooki* populations rapidly increase in size, so that density-dependent limiting factors (e.g. intraspecific competition, cannibalism) are likely to affect the life-history of this fish species.
Physical habitat	Habitat size	The physical space determines the abundance and diversity of species in an ecosystem. Larger surface water bodies correspond to more resources available.
	Habitat complexity	Man-made ecosystems are subject to high disturbance, and so, the structure of their biological assemblages is often kept at early successional stages. This contrasts with natural water bodies, which harbour more diverse habitat structures and biological assemblages.

We used three criteria to assess the adequacy of models for response variables: (i) the 32 models for each life-history trait were ranked using the Akaike Information Criterion corrected for small sample size (AICc) and those with the lowest AICc were identified as the best models; (ii) these best models, including combinations of the five sets of explanatory variables, were assessed for model fit (AUC for binomial; R^2_β by Jaeger et al., 2016 for others). The R^2_β compares a model with fixed and random effects to a null model with only the random effects and an intercept. Fit was the criterion used to discern between two models considered equally plausible (deviation of <4 units of AICc); and (iii) the function *dredge* within the MuMin package (Bartoń, 2018) was then applied to the models to delete irrelevant individual predictors. We therefore obtained the simplest (the lowest AICc) and most informative (the highest fit) final model for each life-history trait. Diagnostic plots of model residuals were used to check for statistical assumptions (e.g. normality, homogeneity of variances). Significance of individual explanatory variables in the final models was assessed using likelihood-ratio χ^2 tests at $P \leq 0.05$ (function *Anova* in R, Fox and Weisberg, 2011).

3. Results

A total of 654 *G. holbrooki* were captured in the selected 18 water bodies with variable habitat complexity that spanned the intended broad gradients of eutrophication, piscivorous bird abundance and *G. holbrooki* density to address our hypotheses (Table 1). However, there was unavoidable variation in covariates, such as temperature (Table 1). The citizen project recorded 15 potentially piscivorous birds from four families, being the cattle egret *Bulbucus ibis* the species most frequently seen (Table S1). We only studied main effects of ecosystem factors on *G. holbrooki* life-history traits because we did not find 'true' replicates for testing interactive effects.

3.1. Description of G. holbrooki life-history traits

All 307 males (mean ± standard error = 28.7 ± 0.1 mm) and 347 females (39.7 ± 0.3 mm) were sexually mature and both sexes were captured in enough numbers in all 18 water bodies. Both females and males had a maximum age ≤ 3 years. Females had better body condition than males, as defined by the Scaled Mass Index (SMI, Table 3). The growth rate, calculated from scale rings, was slower in females than in males (Table 3). The estimated size of fish at the age +1 (back-calculated size) was 25.4 ± 0.1 mm for males and 28 ± 0.2 mm for females. Female had embryos in all six development stages, showing a hump-back relationship (Fig. 3). The offspring number and size greatly varied among sites (Table 3), which provided a pertinent case study to identify the ecosystem factors that were responsible for such variation. There were no strong correlations (|ρ| < 0.60) among *G. holbrooki* life-history traits except for offspring size and potential fertility (Tables S3 and S4).

3.2. Had ecosystem factors discernible effects on life-history variation in G. holbrooki?

Life history variation in both sexes was explained by at least one ecosystem factor and model fits ranged from 0.12 to 0.89. The highest model fits were for female reproductive traits and the lowest were for body-condition models in both sexes. Of the 14 final best models, seven models (50%) identified *G. holbrooki* density as significant predictor to explain variation in any of the eight life-history traits, both sexes combined (Table 4). Habitat complexity was highlighted in five models (36%), piscivorous bird abundance and nutrient concentrations did so in two models (14%), and other water variables, including temperature, were highlighted in five models (36%, Table 4). Effect sizes (i.e. standardized regression coefficients in GLMMs) showed the direction and strength of the associations between ecosystem factors and life history traits (Figs. 4, 5 and Tables 5, 6). When multiple ecosystem factors were highlighted as important predictors to explain life-history variation in *G. holbrooki*, conspecific density and physical habitat complexity had the largest effect sizes after accounting for the effects of covariates (e.g. fish size, the surface area of water bodies).

The SMI in males and females was positively associated with habitat complexity, but this was not significantly correlated with other ecosystem factors (Fig. 4). Fish growth also had one significant association, a negative relationship with the covariate water temperature just for females (Tables 4 and 5). Other ecosystem factors than temperature neither significantly influenced the estimated back-calculated size of females, but the association with water temperature was positive. A positive relationship with bird abundance was the only significant association for the back-calculated size of males. The back-calculated size of both sexes was positively associated with pH (Table 6).

Most reproductive traits had negative associations with conspecific density, which had greater effect size than other ecosystem factors in both sexes (Table 4). The exception was offspring size for which the relationship with conspecific density was positive following a quadratic relationship with embryo development (Fig. 3). There was a strong positive association between habitat complexity and offspring size, which had a weaker inverse association with bird abundance (Fig. 5). Other significant associations between female reproductive traits and ecosystem factors were positive, including indicators of eutrophication (turbidity, nutrient concentrations or chlorophyll-a, Tables 4, 6 and Fig. 5). For males, the only discernible ecosystem effect, together with conspecific density, was a positive association between gonad size and turbidity (Fig. 5). The associations between habitat complexity and

Table 3
List of measured life history traits of the sampling locations. The values represent the median (min–max) of the 18 locations. Life history traits analysed are highlighted in bold.

Trait	Median (range)	Trait	Median (range)
Length (mm)		**RA**	
♂♂	29 (22–38)	♂♂	3.79 (0.35–9.14)
♀♀	39 (25–57)	♀♀	43.6 (0.16–175.39)
SMI		**PF (n° oocyte)**	4 (0–124)
♂♂	0.270 (0.148–0.473)	**RF (n°embryo)**	13 (0–121)
♀♀	0.276 (0.169–0.508)		
SGR (%·day $^{-1}$)		**Off. number**	26 (0–163)
♂♂	0.273 (0.064–0.475)	**Off. size (g)**	
♀♀	0.229 (0.057–0.672)	Stage I	0.0014 (0.0001–0.0036)
B–C lengths (mm)		Stage II	0.0017 (0.0011–0.0027)
♂♂ +1	25.5 (20.7–31.7)	Stage III	0.0015 (0.0008–0.0029)
+2	29.9 (27.3–35.6)	Stage IV	0.0016 (0.0006–0.0023)
♀♀ +1	27.5 (20.1–42.9)	Stage V	0.0014 (0.0008–0.0022)
+2	33.9 (25.6–45.8)	Stage VI	0.0012 (0.0008–0.0013)
+3	41.6 (35.3–53.1)		

Scaled mass index, SMI; Specific growth rate, SGR; Back-calculated fish length, B–C lengths; Reproductive allotment, RA; Potential fertility, PF; Real fertility, RF.

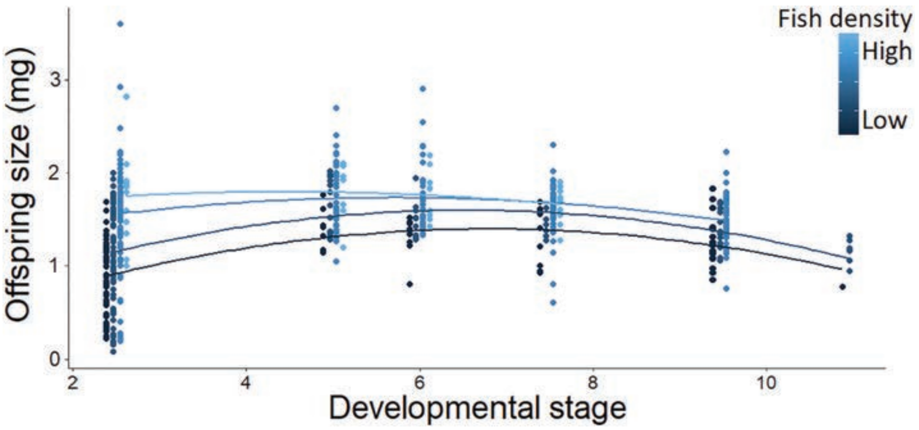


Fig. 3. Plot of offspring size (mg) on developmental stage. Different lines are fitted for each fish density in the pond. Although we used Reznick (1981) classification of developmental stages (*I*–*VI*), they were aligned to Haynes (1995) stages for a better developmental time matching and a better goodness of fit.

reproductive traits, if significant, were always positive for males and females (Table 4).

4. Discussion

This study is among the few comprehensive works (e.g. Lee et al., 2017) exploring life-history variation in an invasive species as function of multiple biotic and abiotic factors, even though studies of life-history have long attracted the attention of ecologists (MacArthur and Wilson, 1967; Stearns, 1992; Ricklefs and Wikelski, 2002). Short-lived invasive species, such as the eastern mosquitofish (*Gambusia holbrooki*), usually have rapid population dynamics, including fast-growth rates and great reproductive effort, making them difficult to control (Winemiller and Rose, 1992). We built on this issue by showing that *G. holbrooki* may adjust the living strategy as function of its density, bird abundance, eutrophication or habitat complexity. We used such life-history variation to suggest factors that may reduce invasion success in *G. holbrooki* and to discuss the overall potential of an ecosystem approach for the management of this globally invasive fish (see Froese and Pauly, 2018).

A detailed understanding of how a species responds to environmental changes can aid in management, yet these associations are often difficult to establish in highly mobile taxa, such fish (Runge et al., 2014). Even small-sized species, such as *G. holbrooki*, can disperse relatively fast within rivers (e.g. 800 m/day, Alemadi and Jenkins, 2007). However, we studied confined populations, such as those in ponds, which effectively acted as experimental units (see Hartel et al., 2007; Bassar et al., 2013). Nonetheless, our study did not account for the potential confounding effect of human-assisted translocations. The introduction of *G. holbrooki* is now forbidden in Spain (Real Decreto 1628/, 2011), but illegal introductions may have occurred after decades of using this species in mosquito control (Nico and Fuller, 2019). Outputs go beyond the studied ponds because *G. holbrooki* is widely distributed, including rivers (Courtenay Jr and Meffe, 1989). Moreover, the water and physical habitat conditions in our study (Table 1) are within the range reported in other freshwater ecosystems, including elsewhere in Spain (e.g. Maceda-Veiga et al., 2017).

Table 4
Sets of ecosystem factors identified in the final best models as important to explain life-history variation in *Gambusia holbrooki* using likelihood-ratio χ^2 tests at $P \leq 0.05$. Standardized regression coefficients determined the direction of their effects (+, positive; −, negative; and 0, non-significant effect). The individual predictors within each set are shown in Table 1 and +/− indicate that the direction of effects was not consistent among them. Potential and real fertility were modelled using presence/absence data and variation in oocyte numbers when present because of the highly skewed distribution (see Materials and methods).

Life-history traits		General WQ	Nutrients	Predation	Conspecific density	Physical habitat
SMI	♂♂	0	0	0	0	+
	♀♀	0	0	0	0	+
SGR	♂♂	0	0	0	0	0
	♀♀	−	0	0	0	0
BCL	♂♂	−	0	−	0	0
	♀♀	+/−	0	0	0	0
RA	♂♂	+	0	0	−	+
	♀♀	0	0	0	−	0
PF	0/1	0	0	0	−	0
	>0	0	+	0	−	+
RF	0/1	+	0	0	0	0
	>0	0	0	0	−	0
ON	♀♀	0	0	0	−	0
OS	♀♀	0	+	−	+	+/−

Scaled mass index, SMI; Specific growth rate, SGR; Back-calculated fish length, BCL; Reproductive allotment, RA; Potential fertility, PF; Real fertility, RF; Offspring number, ON; Offspring size, OS.

4.1. Life-history variation in G. holbrooki was mostly attributed to conspecific density

Conspecific density was a prevailing factor in explaining life-history variation in *G. holbrooki*, which is consistent with the assert that the impact of a species on its resources has repercussions on life-history (Ricklefs and Wikelski, 2002). However, an inverse association between male gonad size and *G. holbrooki* density appears to contradict sperm-competition theory (Parker, 1970). Higher competition was expected to be associated with larger testes because higher sperm production should increase the probability of fertilization (Gage et al., 1995). Nevertheless, reproduction success in males can increase in other ways, including changes in courtship behaviour (Smith and Sargent, 2006). If the association between the density and life-history of *G. holbrooki* is primarily influenced by resource availability, effects are likely to have been strong in our study due to the absence of competitors. Most sampling sites did not have other fish species and invasion success in the studied water bodies might be due to the existence of empty niches (Fridley and Sax, 2014). The localities G1, C1 and C2 had the invasive cyprinids *C. carpio* and *Carassius* sp. (*authors pers. observ.*). However, adults of the two species are primary detritivores and are unlikely to be strong competitors for the mostly invertivorous *G. holbrooki* (King, 2005). Amphibians are present in the study region (e.g. *Pelophylax perezi*, *Alytes obstetricans*, AHE, 2016) but direct predation, injuries caused by predation attempts, and intense trophic competition are likely to have made *G. holbrooki* a better competitor than amphibian larvae (Vannini et al., 2018).

Without strong competitors from other species, the strategy of fast-living species, including *G. holbrooki*, might be to exploit available resources and increase population size until intraspecific competition and diseases curb population-growth rate (see Stearns, 1992). Parasites

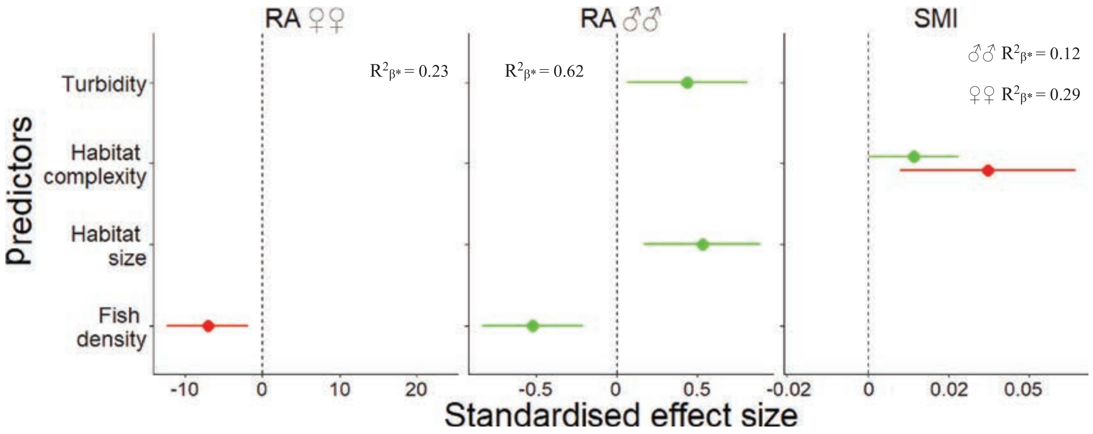


Fig. 4. Standardized regression coefficients (±95% interval coefficients) showing the direction and strength of the associations between significant ecosystem factors and reproductive allotment (RA) and Scaled Mass Index (SMI, as indicator of body condition) in males (green) and females (red) separately. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

are unlikely to much affect *G. holbrooki* life-history because of their low prevalence in the study region (Benejam et al., 2008; Maceda-Veiga et al., 2019 *in press*). However, parasite outbreaks are often abrupt and hard to predict and *G. holbrooki* populations have not yet been screened for bacteria and virus, both of which are other common pathogens (Noga, 2010). Therefore, the most plausible density-dependent factor for *G. holbrooki* is intraspecific competition; a finding which indirectly suggests that predation and interspecific competition, through altering *G. holbrooki* density, may help to manage invasions by this species. Despite this, *G. holbrooki* often feeds upon its newborns (Vargas, 1993), so that the effects of cannibalism cannot be discarded.

4.2. Trade-offs among predation, G. holbrooki density and offspring size and number

There was a negative association between *G. holbrooki* offspring size and bird abundance; used as proxy for bird predation. It is possible that we would have obtained stronger results if we had been able to use direct measures of fish predation, but this information is not provided by the citizen-science project (https://www.ornitho.cat/). Differences in body size among birds might have affected outcomes. However, 46% of the birds were the small cattle egrets (*Bulbucus ibis*, Table S1), which often feed upon *G. holbrooki* (Bredin, 1984). Nevertheless, *G. holbrooki* is unlikely to be the main item for the birds if other prey are available in the region (Ashoori et al., 2017). Our finding that predation reduced *G. holbrooki* offspring size is in broad agreement with extensive experimental work in Trinidadian guppies *Poecilia reticulata* (Reznick and Endler, 1982; Stearns, 1992). However, our result should be interpreted with caution. Even though newborns at sites with many piscivorous birds may have been smaller compared to other sites, fish can grow relatively fast (Bashey, 2008; Livingston et al., 2014). Moreover, the ponds available in the study area did not allow us to account for interactions between bird abundance and habitat conditions and interactions might have had an effect. Predation pressure is often reduced in turbid or complex habitats, both of which make prey more difficult to locate and to catch (Horppila et al., 2004). Moreover, we seldom saw predators other than birds, e.g. the snake *Natrix maura* (Santos et al., 2000), but a very low number of records prevented using them in analyses.

Further support for the effect of the recorded bird piscivores on *G. holbrooki* is the negative but weak association between bird abundance and fish density ($\rho = -0.29$, $P > 0.05$; Table S2). Interestingly, *G. holbrooki* density showed opposed patterns with offspring size and number, which is consistent with the trade-offs reported in life-history theory (Smith and Fretwell, 1974; Stearns, 1992). Experimental work in poeciliids (Reznick and Yang, 1993; Gordon et al., 2017) found that females had more and smaller newborns at lower than at higher densities of conspecifics. Therefore, *G. holbrooki* probably offset

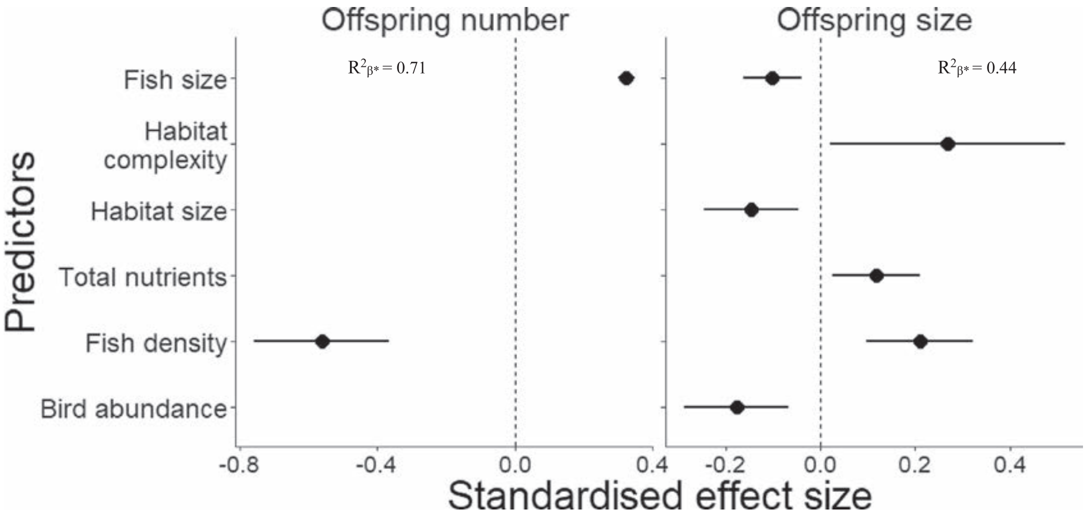


Fig. 5. Offspring number and size. Forest plot indicating the effect sizes of the selected environmental variables in the final model. Weight stands for fish eviscerated weight, as it was included in the full and all nested models.

Table 5
Standardized regression coefficients (S.C.) and standard errors (S.E.) from the final generalized linear mixed models showing the effects of ecosystem factors on specific growth rate (SGR) and back-calculated length (BCL) of *Gambusia holbrooki*. Covariate (fish age) is also shown alongside the explained variance of models ($R^2_{\beta^*}$) as calculated by Jaeger et al. (2016). Significance of ecosystem factors was reached at *P* value ≤0.05 and denoted by *.

	Sex	Predictors and covariates	S.C.	S.E.	X^2	*P*-value		$R^2_{\beta^*}$
SGR	♀♀	Fish age	−0.09	0.01	194.2	<0.001	*	0.71
		Habitat complexity	−0.04	0.03	1.9	0.17		
		Habitat size	−0.03	0.01	3.6	0.06		
		Temperature	−0.05	0.02	10.5	<0.01	*	
	♂♂	Fish age	−0.09	0.01	42.1	<0.001	*	0.26
		Fish density	−0.01	0.01	1.5	0.22		
BCL	♀♀	Fish age	6.60	0.06	11,034	<0.001	*	0.89
		pH	−1.39	0.58	5.8	0.02	*	
		Temperature	1.66	0.60	7.7	0.01	*	
	♂♂	Fish age	4.53	0.13	1214.0	<0.001	*	0.53
		pH	−0.72	0.25	8.3	0.01	*	
		Bird abundance	−0.60	0.26	5.2	0.02	*	

individual loss (either caused by natural predation or culling) by increasing the baseline production of newborns for this species. Such living strategy might increase population size relatively fast if a female (>17 mm, Meffe, 1992; Benejam et al., 2008) can give birth up to 100 newborns every 4 weeks and these can be sexually mature at 3 weeks (Meffe, 1992; Pyke, 2005).

4.3. Acting on water quality probably does not alter too much G. holbrooki life-history

There was an overall limited association between water-quality variables and *G. holbrooki* life-history traits, supporting this fish is relatively tolerant to environmental change (Pyke, 2005). One of the exceptions was water turbidity, which is known to reduce the probability of mating in fish and there are experimental studies in poeciliids showing that males increase reproductive effort in turbid waters (Ehlman et al., 2018). Our correlative study added the invasive *G. holbrooki* to the list and suggests that larger testis might be a frequent strategy among poeciliids to increase fitness at sub-optimal environmental conditions. The fact that conductivity was not retained as having an important effect in our models may be due to differences in the conductivity range tested in ours compared to previous studies (Alcaraz and García-Berthou, 2007; Martin et al., 2009). For instance, our values were much lower (2950 ± 628) than those (up to 35,100 μS/cm) of Alcaraz and García-Berthou (2007). The same rationale may apply to nutrients because their concentrations in most sampling sites were relatively 'safe' for fish (Table 1). However, some sites had values of ammonia and nitrite (>3 mg L^{-1} and 1 mg L^{-1}) that are toxic to fish under chronic exposure (Noga, 2010).

The concentrations of nutrients within the range studied probably mostly acted as subsidies for *G. holbrooki* without major toxic effects, even though man-made eutrophication profoundly alters ecosystems (Wetzel, 2001). The positive associations between high nutrient levels and offspring size, or between potential fertility and the concentration of chlorophyll-a, suggest that breeding success in *G. holbrooki* increases under eutrophication. However, we do caution that identifying the individual effects of environmental variables in correlative studies, such as ours, is difficult because variables are always correlated to some extent, even though collinearity was of little statistical concern (Table S2; Zuur et al., 2009). Habitat complexity naturally increases with ecosystem size, which in turn boosts resource availability (MacArthur and Wilson, 1967; Blakely and Didham, 2010). Therefore, resource availability may partially explain the positive association between habitat complexity and the body condition and reproductive traits of *G. holbrooki*. It is also possible that habitat complexity might moderate agonistic interactions providing shelter and refugee (Sutton et al., 2013), as experimentally demonstrated with artificial refugia in *G. holbrooki* co-occurring with the globally threatened Spanish toothcarp *Aphanius iberus* (Magellan and García-Berthou, 2016).

5. Management conclusions

Our study shows that life-history variation in *G. holbrooki* is mostly explained by conspecific density compared to ecosystem factors, such as changes in water quality and bird predation. Overall, these findings suggest that direct removal probably is a more effective management action for *G. holbrooki* invasion (e.g. Ruiz-Navarro et al., 2013) than changing environmental conditions, i.e. an ecosystem approach, especially in the studied stagnant waters. However, an ecosystem approach that increased refugees and naturalised pond margins may moderate agonistic interactions of *G. holbrooki* with native species and promote co-occurrence (e.g. Hartel et al., 2007; Magellan and García-Berthou, 2016), even though we found that *G. holbrooki* body condition improved with habitat complexity. The social dimension of the management of invasive species should not be overlooked in any ecosystem approach. *Gambusia holbrooki* may have been introduced because stakeholders were not aware that native species with similar diet to *G. holbrooki* naturally occur in the study area (e.g. *A. iberus*, Vargas, 1993). If authorities enforce a reintroduction program of native fish species in waterbodies

Table 6
Standardized regression coefficients (S.C.) and standard errors (S.E.) from the final generalized linear mixed models showing the effects of ecosystem factors on potential fertility (PF, oocyte stage I) and real fertility (RF, oocyte stages *II–VI*) of *Gambusia holbrooki*. Covariates (fish weight) are shown alongside the explained variance of models ($R^2_{\beta^*}$) as calculated by Jaeger et al. (2016). For GLMMs with binomial distribution, area under the ROC curve (AUC, denoted by †) was used instead for accuracy estimation. Significance of ecosystem factors was reached at *P* value ≤0.05 and denoted by *.

	GLMM	Predictors and covariates	S.C.	S.E.	X^2	*P*-value		AUC $R^2_{\beta^*}$
PF	Absence/ Presence	Fish weight	0.07	0.19	0.1	0.72		0.83†
		Fish density	−0.86	0.36	6.5	0.01	*	
		Bird abundance	−0.56	0.34	2.6	0.10		
	>0	Fish weight	0.20	0.07	8.8	<0.01	*	0.73
		Fish density	−0.87	0.16	30.8	<0.001	*	
		Habitat complexity	−0.21	0.29	0.5	0.48		
		Habitat size	0.31	0.14	5.4	0.02	*	
		Total nutrients	0.05	0.13	0.2	0.66		
		Chlorophyll-a	0.40	0.15	7.5	<0.01	*	
RF	Absence/ Presence	Fish weight	0.62	0.21	8.72	<0.01	*	0.86†
		pH	0.52	0.41	1.56	0.21		
		Turbidity	0.86	0.41	4.48	0.03	*	
		Temperature	0.40	0.46	0.75	0.39		
	>0	Fish weight	0.33	0.04	71.3	<0.001	*	0.64
		Fish density	−0.16	0.05	12.6	<0.001	*	
		Habitat complexity	0.11	0.10	1.3	0.26		
		Habitat size	0.08	0.05	3.3	0.07		

adequately naturalised, then they can aid in the conservation of these small fish species while giving enthusiasts a safe alternative to *G. holbrooki* for mosquito control.

Acknowledgements

We thank Clàudia Pastor for assistance in laboratory procedures and the owners of the sampling ponds for giving us access. We are also grateful to Sergi Vargas, Josep Solà, Carles Aranda, Eugeni Navas and Francesc X. Santaeufemia for field assistance. Guillem Pascual, Sergi García, Carmen Maté, Albert Montori, Xavier Ferrer and Toni Arrizabalaga also collaborated with the sampling design. This study was funded by a PRIC grant (3RB 057) awarded by Fundació Barcelona Zoo and an APIF grant by the University of Barcelona.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.02.460.

References

AHE, 2016. Base de datos de anfibios y reptiles de España. Asociación Herpetológica Española. http://siare.herpetologica.es, Accessed date: 12 August 2016.

Alcaraz, C., García-Berthou, E., 2007. Life history variation of invasive mosquitofish (*Gambusia holbrooki*) along a salinity gradient. Biol. Conserv. 139 (1–2), 83–92. https://doi.org/10.1016/j.biocon.2007.06.006.

Alemadi, S.D., Jenkins, D.G., 2007. Behavioral constraints for the spread of the eastern mosquitofish, *Gambusia holbrooki* (Poeciliidae). Biol. Invasions 10 (1), 59–66. https://doi.org/10.1007/s10530-007-9109-x.

Ashoori, A., Moradi, H.V., Rezaiee, H.R., Mahiny, A.S., 2017. Dietary segregation of four Ardeid species breeding in Anzali international wetland, Northern Iran. Waterbirds 40 (4), 377–389. https://doi.org/10.1675/063.040.0409.

Bartoń, K., 2018. MuMIn: multi-model inference, R package version 0.12.0 [software]. http://r-forge.r-project.org/projects/mumin/.

Bashey, F., 2008. Competition as a selective mechanism for larger offspring size in guppies. Oikos 117 (1), 104–113. https://doi.org/10.1111/j.2007.0030-1299.16094.x.

Bassar, R.D., Lopez-Sepulcre, A., Reznick, D.N., Travis, J., 2013. Experimental evidence for density-dependent regulation and selection on Trinidadian guppy life histories. Am. Nat. 181 (1), 25–38. https://doi.org/10.1086/668590.

Benejam, L., Alcaraz, C., Sasal, P., Simon-Levert, G., García-Berthou, E., 2008. Life history and parasites of the invasive mosquitofish (*Gambusia holbrooki*) along a latitudinal gradient. Biol. Invasions 11 (10), 2265–2277. https://doi.org/10.1007/s10530-008-9413-0.

Blakely, T.J., Didham, R.K., 2010. Disentangling the mechanistic drivers of ecosystem-size effects on species diversity. J. Anim. Ecol. 79 (6), 1204–1214. https://doi.org/10.1111/j.1365-2656.2010.01729.x.

Bredin, D., 1984. Regime alimentaire du heron garde-bœuf a la limite de son expansion geographique recente. Revue d'Écologie (Terre Vie) 39, 431–445.

Courtenay Jr., W.R., Meffe, G.K., 1989. Small fishes in strange places: a review of introduced poeciliids. In: Meffe, G.K., Snelson, F.F. (Eds.), Ecology and Evolution of Livebearing Fishes (Poeciliidae). Prentice Hall, pp. 319–331 https://doi.org/10.5860/choice.27-1536.

Cowan, P., Warburton, B., 2011. Animal welfare and ethical issues in island pest eradication. In: Veitch, C.R., Clout, M.N., Towns, D.R. (Eds.), Island Invasives: Eradication and Management. IUCN, Gland, Switzerland, pp. 418–421.

Davies, S.R., Sayer, C.D., Greaves, H., Siriwardena, G.M., Axmacher, J.C., 2016. A new role for pond management in farmland bird conservation. Agric. Ecosyst. Environ. 233, 179–191. https://doi.org/10.1016/j.agee.2016.09.005.

DIISE, 2018. The database of island invasive species eradications, developed by island conservation, coastal conservation action laboratory UCSC, IUCN SSC invasive species specialist group, University of Auckland and Landcare research New Zealand. http://diise.islandconservation.org, Accessed date: 11 August 2018.

Doadrio, I., 2002. Atlas y libro rojo de los peces continentales de España. 2nd ed. Museo Nacional de Ciencias Naturales, Dirección General de Conservación de la Naturaleza, Madrid.

Ehlman, S.M., Martinez, D., Sih, A., 2018. Male guppies compensate for lost time when mating in turbid water. Behav. Ecol. Sociobiol. 72 (3). https://doi.org/10.1007/s00265-018-2468-8.

Essl, F., Bacher, S., Genovesi, P., Hulme, P.E., Jeschke, J.M., Katsanevakis, S., et al., 2018. Which taxa are alien? Criteria, applications, and uncertainties. BioScience 68 (7), 496–509. https://doi.org/10.1093/biosci/biy057.

Fernández-Delgado, C., Rossomanno, S., 1997. Reproductive biology of the mosquitofish in a permanent natural lagoon in south-west Spain: two tactics for one species. J. Fish Biol. 51 (1), 80–92. https://doi.org/10.1111/j.1095-8649.1997.tb02515.x.

Fox, J., Weisberg, S., 2011. An R Companion to Applied Regression. 2nd edition. Sage, Thousand Oaks CA [software]. http://socserv.socsci.mcmaster.ca/jfox/Books/Companion.

Fridley, J.D., Sax, D.F., 2014. The imbalance of nature: revisiting a Darwinian framework for invasion biology. Glob. Ecol. Biogeogr. 23 (11), 1157–1166. https://doi.org/10.1111/geb.12221.

Froese, R., Pauly, D., 2018. Fishbase. www.fishbase.org, Accessed date: 11 November 2018.

Gage, M.J.G., Stockley, P., Parker, G.A., 1995. Effects of alternative male mating strategies on characteristics of sperm production in the Atlantic salmon (*Salmo salar*): theoretical and empirical investigations. Philos. Trans. R. Soc. Lond. B Biol. Sci. 350 (1334), 391–399. https://doi.org/10.1098/rstb.1995.0173.

Genovesi, P., 2011. Are we turning the tide? Eradications in times of crisis: how the global community is responding to biological invasions. In: Veitch, C.R., Clout, M.N., Towns, D.R. (Eds.), Island Invasives: Eradication and Management. IUCN, Gland, Switzerland, pp. 5–8.

Gobierno de España, 2011. Real Decreto 1628/2011, de 14 de noviembre, por el que se regula el listado y catálogo español de especies exóticas invasoras. 2011. BOE, p. 298.

Gordon, S.P., Hendry, A.P., Reznick, D.N., 2017. Predator-induced contemporary evolution, phenotypic plasticity, and the evolution of reaction norms in guppies. Copeia 105 (3), 514–522. https://doi.org/10.1643/ce-16-522.

Hartel, T., Nemes, S., Cogălniceanu, D., Öllerer, K., Schweiger, O., Moga, C.I., et al., 2007. The effect of fish and aquatic habitat complexity on amphibians. Hydrobiologia 583 (1), 173–182. https://doi.org/10.1007/s10750-006-0490-8.

Haynes, J.L., 1995. Standardized classification of Poeciliid development for life-history studies. Copeia 1995 (1), 147–154. https://doi.org/10.2307/1446809.

Horppila, J., Liljendahl-Nurminen, A., Malinen, T., 2004. Effects of clay turbidity and light on the predator–prey interaction between smelts and chaoborids. Can. J. Fish. Aquat. Sci. 61 (10), 1862–1870. https://doi.org/10.1139/f04-123.

Hulme, P.E., 2006. Beyond control: wider implications for the management of biological invasions. J. Appl. Ecol. 43 (5), 835–847. https://doi.org/10.1111/j.1365-2664.2006.01227.x.

Jaeger, B.C., Edwards, L.J., Das, K., Sen, P.K., 2016. An R2 statistic for fixed effects in the generalized linear mixed model. J. Appl. Stat. 44 (6), 1086–1105. https://doi.org/10.1080/02664763.2016.1193725.

King, A.J., 2005. Ontogenetic dietary shifts of fishes in an Australian floodplain river. Mar. Freshw. Res. 56 (2), 215–225. https://doi.org/10.1071/mf04117.

Lea, E., 1910. Contributions to the methodics in herring-investigations. ICES J. Mar. Sci. s1 (53), 7–33. https://doi.org/10.1093/icesjms/s1.53.7.

Lee, F., Simon, K.S., Perry, G.L.W., 2017. Increasing agricultural land use is associated with the spread of an invasive fish (*Gambusia affinis*). Sci. Total Environ. 586, 1113–1123. https://doi.org/10.1016/j.scitotenv.2017.02.101.

Livingston, J.D., Kahn, A.T., Jennions, M.D., 2014. Sex differences in compensatory and catch-up growth in the mosquitofish *Gambusia holbrooki*. Evol. Ecol. 28 (4), 687–706. https://doi.org/10.1007/s10682-014-9691-1.

Lowe, S., Browne, M., Boudjelas, S., De Poorter, M., 2000. 100 of the world's Worst Invasive Alien Species: A Selection from the Global Invasive Species Database (Vol. 12). Invasive Species Specialist Group, Auckland.

MacArthur, R.H., Wilson, E.O., 1967. The Theory of Island Biogeography. Princeton, NJ. https://doi.org/10.1515/9781400881376.

Maceda-Veiga, A., Green, A.J., de Sostoa, A., 2014. Scaled body mass index shows how habitat quality influences the condition of four fish taxa in north-eastern Spain, and provides a novel indicator of ecosystem health. Freshw. Biol. 59 (6), 1145–1160. https://doi.org/10.1111/fwb.12336.

Maceda-Veiga, A., Baselga, A., Sousa, R., Vilà, M., Doadrio, I., de Sostoa, A., 2017. Fine-scale determinants of conservation value of river reaches in a hotspot of native and non-native species diversity. Sci. Total Environ. 574, 455–466. https://doi.org/10.1016/j.scitotenv.2016.09.097.

Maceda-Veiga, A., Mac Nally, R., de Sostoa, A., 2018. Environmental correlates of food-chain length, mean trophic level and trophic level variance in invaded riverine fish assemblages. Sci. Total Environ. 644, 420–429. https://doi.org/10.1016/j.scitotenv.2018.06.304.

Maceda-Veiga, A., Cano-Rocabayera, O., de Sostoa, A., Cable, J., 2019. Low parasite prevalence in the invasive fish *Gambusia holbroki* in riverine and stagnant waters in north-eastern Spain. Bull. Eur. Assoc. Fish Pathol. (In press).

Magellan, K., García-Berthou, E., 2016. Experimental evidence for the use of artificial refugia to mitigate the impacts of invasive *Gambusia holbrooki* on an endangered fish. Biol. Invasions 18 (3), 873–882. https://doi.org/10.1007/s10530-016-1057-x.

Martin, S., Hitch, A., Purcell, K., Klerks, P., Leberg, P., 2009. Life history variation along a salinity gradient in coastal marshes. Aquat. Biol. 8, 15–28. https://doi.org/10.3354/ab00203.

Meffe, G.K., 1992. Plasticity of life-history characters in eastern mosquitofish (*Gambusia holbrooki*: Poeciliidae) in response to thermal stress. Copeia 1992 (1), 94. https://doi.org/10.2307/1446539.

Murphy, C.A., Grenouillet, G., García-Berthou, E., 2015. Natural abiotic factors more than anthropogenic perturbation shape the invasion of Eastern Mosquitofish (*Gambusia holbrooki*). Freshwater Science 34 (3), 965–974. https://doi.org/10.1086/681948.

Nico, L., Fuller, P., 2019. *Gambusia holbrooki* Girard, 1859. U.S. Geological Survey, Nonindigenous Aquatic Species Database, Gainesville, FLhttps://nas.er.usgs.gov/queries/FactSheet.aspx?SpeciesID=849, Accessed date: 1 February 2019.

Noga, E.J., 2010. Fish Disease: Diagnosis and Treatment. 2nd ed. Wiley-Blackwell, Ames.

Paini, D.R., Sheppard, A.W., Cook, D.C., De Barro, P.J., Worner, S.P., Thomas, M.B., 2016. Global threat to agriculture from invasive species. Proc. Natl. Acad. Sci. 113 (27), 7575–7579. https://doi.org/10.1073/pnas.1602205113.

Parker, G.A., 1970. Sperm competition and its evolutionary consequences in the insects. Biol. Rev. 45 (4), 525–567. https://doi.org/10.1111/j.1469-185x.1970.tb01176.x.

Parsons, T.R., Strickland, J.D.H., 1963. Discussion of spectrophotometric determination of marine plant pigments, with revised equations for ascertaining chlorophylls and carotenoids. J. Mar. Res. 21, 155–163.

Peig, J., Green, A.J., 2009. New perspectives for estimating body condition from mass/length data: the scaled mass index as an alternative method. Oikos 118 (12), 1883–1891. https://doi.org/10.1111/j.1600-0706.2009.17643.x.

Perrings, C., Naeem, S., Ahrestani, F., Bunker, D.E., Burkill, P., Canziani, et al., 2010. Ecosystem services for 2020. Science 330 (6002), 323–324. https://doi.org/10.1126/science.1196431.

Pianka, E.R., 1970. On r- and K-selection. Am. Nat. 104 (940), 592–597. https://doi.org/10.1086/282697.

Pilcher, M.W., Copp, G.H., 1997. Winter distribution and habitat use by fish in a regulated lowland river system of south-east England. Fish. Manag. Ecol. 4 (3), 199–215. https://doi.org/10.1046/j.1365-2400.1997.00120.x.

Pimentel, D., Zuniga, R., Morrison, D., 2005. Update on the environmental and economic costs associated with alien-invasive species in the United States. Ecol. Econ. 52 (3), 273–288. https://doi.org/10.1016/j.ecolecon.2004.10.002.

Prior, K.M., Adams, D.C., Klepzig, K.D., Hulcr, J., 2018. When does invasive species removal lead to ecological recovery? Implications for management success. Biol. Invasions 20 (2), 267–283. https://doi.org/10.1007/s10530-017-1542-x.

Pyke, G.H., 2005. A review of the biology of *Gambusia affinis* and *G. holbrooki*. Rev. Fish Biol. Fish. 15 (4), 339–365. https://doi.org/10.1007/s11160-006-6394-x.

Reznick, D., 1981. “Grandfather effects”: the genetics of interpopulation differences in offspring size in the mosquito fish. Evolution 35 (5), 941–953. https://doi.org/10.2307/2407865.

Reznick, D., Endler, J.A., 1982. The impact of predation on life history evolution in Trinidadian guppies (*Poecilia reticulata*). Evolution 36 (1), 160. https://doi.org/10.2307/2407978.

Reznick, D., Yang, A.P., 1993. The influence of fluctuating resources on life history: patterns of allocation and plasticity in female guppies. Ecology 74 (7), 2011–2019. https://doi.org/10.2307/1940844.

Ricklefs, R.E., Wikelski, M., 2002. The physiology/life-history nexus. Trends Ecol. Evol. 17 (10), 462–468. https://doi.org/10.1016/s0169-5347(02)02578-8.

Roy, H.E., Bacher, S., Essl, F., Adriaens, T., Aldridge, D.C., Bishop, J.D.D., et al., 2018. Developing a list of invasive alien species likely to threaten biodiversity and ecosystems in the European Union. Glob. Chang. Biol. https://doi.org/10.1111/gcb.14527.

Ruiz-Navarro, A., Verdiell-Cubedo, D., Torralva, M., Oliva-Paterna, F.J., 2013. Removal control of the highly invasive fish *Gambusia holbrooki* and effects on its population biology: learning by doing. Wildl. Res. 40 (1), 82. https://doi.org/10.1071/wr12172.

Runge, C.A., Martin, T.G., Possingham, H.P., Willis, S.G., Fuller, R.A., 2014. Conserving mobile species. Front. Ecol. Environ. 12 (7), 395–402. https://doi.org/10.1890/130237.

Santos, X., González-Solis, J., Llorente, G.A., 2000. Variation in the diet of the viperine snake *Natrix maura* in relation to prey availability. Ecography 23 (2), 185–192. https://doi.org/10.1111/j.1600-0587.2000.tb00274.x.

Seebens, H., Blackburn, T.M., Dyer, E.E., Genovesi, P., Hulme, P.E., Jeschke, J.M., et al., 2017. No saturation in the accumulation of alien species worldwide. Nat. Commun. 8, 14435. https://doi.org/10.1038/ncomms14435.

Servanty, S., Gaillard, J.-M., Ronchi, F., Focardi, S., Baubet, É., Gimenez, O., 2011. Influence of harvesting pressure on demographic tactics: implications for wildlife management. J. Appl. Ecol. 48 (4), 835–843. https://doi.org/10.1111/j.1365-2664.2011.02017.x.

Smith, C.C., Fretwell, S.D., 1974. The optimal balance between size and number of offspring. Am. Nat. 108 (962), 499–506. https://doi.org/10.1086/282929.

Smith, C.C., Sargent, R.C., 2006. Female fitness declines with increasing female density but not male harassment in the western mosquitofish, *Gambusia affinis*. Anim. Behav. 71 (2), 401–407. https://doi.org/10.1016/j.anbehav.2005.06.003.

Stearns, S.C., 1992. The Evolution of Life Histories. Oxford University Press, Oxford, UK.

Sutton, T.M., Zeiber, R.A., Fisher, B.E., 2013. Agonistic behavioral interactions between introduced western mosquitofish and native topminnows. J. Freshw. Ecol. 28 (1), 1–16. https://doi.org/10.1080/02705060.2012.688492.

Torchin, M.E., Lafferty, K.D., Dobson, A.P., McKenzie, V.J., Kuris, A.M., 2003. Introduced species and their missing parasites. Nature 421 (6923), 628–630. https://doi.org/10.1038/nature01346.

Vannini, A., Bruni, G., Ricciardi, G., Platania, L., Mori, E., Tricarico, E., 2018. *Gambusia holbrooki*, the “tadpolefish”: the impact of its predatory behaviour on four protected species of European amphibians. Aquat. Conserv. Mar. Freshwat. Ecosyst. 28 (2), 476–484. https://doi.org/10.1002/aqc.2880.

Vargas, M.J., 1993. Interacción entre *Aphanius iberus* y *Gambusia holbrooki* en el delta del Ebro: sus ciclos biológicos y ecologías tróficas. PhD thesis. Universitat de Barcelona.

Wetzel, R.G., 2001. Limnology: Lake and River Ecosystems. 3rd ed. Academic Press, London.

Whitney, R.R., Carlander, K.D., 1956. Interpretation of body-scale regression for computing body length of fish. J. Wildl. Manag. 20 (1), 21. https://doi.org/10.2307/3797243.

Winemiller, K.O., Rose, K.A., 1992. Patterns of life-history diversification in north American fishes: implications for population regulation. Can. J. Fish. Aquat. Sci. 49 (10), 2196–2218. https://doi.org/10.1139/f92-242.

Zavaleta, E.S., Hobbs, R.J., Mooney, H.A., 2001. Viewing invasive species removal in a whole-ecosystem context. Trends Ecol. Evol. 16 (8), 454–459. https://doi.org/10.1016/s0169-5347(01)02194-2.

Zuur, A.F., Ieno, E.N., Walker, N., Saveliev, A.A., Smith, G.M., 2009. Mixed Effects Models and Extensions in Ecology with R. Springer, New York https://doi.org/10.1007/978-0-387-87458-6.

Supplementary materials

Table S1. Species of potentially piscivorous birds recorded from 21/05/2013 to 20/05/2016 by users of the citizen science project (www.ornitho.cat) in the study area. The relative abundance of each species in relation to the total abundance of birds is shown as a percentage.

Podicipedidae					
Podiceps nigricollis	0.18	*Podiceps cristatus*	3.24	*Tachybaptus ruficollis*	3.96
Ardeidae					
Ardeola ralloides	0.90	*Ardea purpurea*	1.44	*Ardea cinerea*	10.79
Bubulcus ibis	45.68	*Nycticorax nycticorax*	1.98	*Casmerodius albus*	0.72
Egretta garzetta	13.49	*Ixobrychus minutus*	1.44		
Alcedinidae					
Alcedo atthis	3.60				
Rallidae *					
Gallinula chloropus	10.97	*Porphyrio porphyrio*	1.26	*Rallus aquaticus*	0.36

* *Fulica atra* was present in the study area but not included in analyses because this species is mostly herbivorous (Metna et al., 2015).

Table S2. Spearman rank correlation coefficients among the original set of 15 ecosystem factors measured in the 18 water bodies surveyed in north-eastern Spain. The list of the least collinear predictors according to the threshold of variation inflation factor (VIF) < 3 established in Zuur et al. (2009) is shown at the bottom. This bottom list shows the predictors included in the models to explore their associations with the life history of *Gambusia holbrooki* and was built after deleting highly correlated variables (|ρ|>0.60). Predation refers to the abundance of piscivorous birds and density is G. holbrooki density. Nutrients include ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-) and phosphates (PO_4). Natural water mineralization is general (GH) and carbonate hardness (KH). Habitat size is the surface area of the water bodies surveyed. Chl-a is the concentration of chlorophyll-a.

	Density	**Temperature**	**Conductivity**	**O_2%**	**pH**	**NH_4^+**	**NO_2^-**	**NO_3^-**	**PO_4**	**KH**	**GH**	**Chl-a**	**Predation**	**Turbidity**
Density														
Temperature	0.02													
Conductivity	-0.30*	0.22												
O_2%	0.18	0.24	-0.17											
pH	0.31*	0.20	0.00	0.55*										
NH_4^+	-0.13	-0.10	0.03	-0.20	-0.41*									
NO_2^-	-0.35*	-0.01	-0.11	0.05	-0.26	0.43*								
NO_3^-	-0.43*	0.14	-0.11	0.02	-0.36*	0.29	0.75*							
PO_4	-0.11	0.33*	0.41*	-0.13	0.00	0.28	0.27	0.23						
KH	-0.35*	-0.02	0.61*	-0.37*	-0.31*	0.07	0.03	0.13	0.38*					
GH	-0.28	0.08	0.85*	-0.26	-0.17	0.11	0.00	0.02	0.45*	0.77*				
Chl-a	0.09	-0.11	0.11	0.10	-0.01	-0.33	-0.07	-0.07	-0.13	0.28	0.01			
Predation	-0.29	-0.01	0.47*	-0.17	-0.28	0.10	-0.01	0.03	0.14	0.44*	0.47*	0.28		
Turbidity	-0.06	0.18	0.32*	-0.22	0.14	-0.15	-0.33*	-0.23	0.00	0.24	0.13	0.51*	0.25	
Pond size	-0.13	-0.05	-0.14	0.24	-0.06	-0.09	0.05	0.22	-0.01	0.17	-0.09	0.26	-0.13	0.11

‘*’ $P < 0.05$

Variation Inflation Factors (VIF) of the least collinear set of variables: Density 2.33, Temperature 2.04, pH 2.46, Chlorophyll-a 1.63, Predation 1.41, Turbidity 1.76, Habitat size 1.67, Nutrients 1.65. The new variable ‘Nutrients’ was computed as z-scored $\sum NH_4^+$, NO_2^-, NO_3^- and PO_4.

Table S3. Spearman rank correlation coefficients among life history traits in male *Gambusia holbrooki*.

	Length	**SMI**	**RA**	**SGR**
Length				
SMI	-0.15 *			
RA	0.12	-0.14 *		
SGR	0.14 *	0.12	-0.03	
BCL	0.82 *	-0.22 *	0.10	-0.07

‘*’ P < 0.05. Scaled mass index, SMI; Reproductive allotment, RA; Specific growth rate, SGR; Back-calculated fish length, BCL. Back-calculated lengths correspond to length at age=+1 for all fish.

Table S4. Spearman rank correlation coefficients among life history traits in female *Gambusia holbrooki*.

	Length	**SMI**	**RA**	**SGR**	**BCL**	**ON**	**PF**	**RF**
Length								
SMI	-0.27 *							
RA	0.06	-0.19 *						
SGR	-0.54 *	0.25 *	-0.02					
BCL	0.53 *	-0.30 *	0.09	-0.51 *				
ON	0.74 *	-0.19 *	0.48 *	-0.35 *	0.34 *			
PF	0.37 *	-0.14 *	0.41 *	-0.07	0.11	0.67 *		
RF	0.29 *	0.01	0.08	-0.22 *	0.17 *	0.29 *	-0.36 *	
OS	-0.09	0.16 *	0.08	-0.04	0.07	-0.42 *	-0.39 *	0.03

‘*’ P < 0.05. Scaled mass index, SMI; Reproductive allotment, RA; Specific growth rate, SGR; Back-calculated fish length, BCL; Offspring number, ON; Potential fertility, PF; Real fertility, RF; Offspring size, OS. Back-calculated lengths correspond to length at age=+1 for all fish.

Table S5. Standardized regression coefficients (S.C.) and standard errors (S.E.) from the final generalized linear mixed models showing the effects of ecosystem factors on body condition (Scaled Mass Index, SMI) and reproductive allotment (RA) of *Gambusia holbrooki*. Covariate (fish age) is also shown alongside the explained variance of models ($R^2_{\beta*}$) as calculated by Jaeger et al. (2016). Significance of ecosystem factors was reached at P value ≤ 0.05 (*).

	Sex	**Predictors**	**S.C.**	**S.E.**	**X^2**	**P-value**		**$R^2_{\beta*}$**
SMI	♀♀	Habitat complexity	0.014	0.007	4.0	<0.05	*	0.12
		Habitat size	-0.007	0.004	3.7	0.06		
		Fish density	0.004	0.003	1.8	0.18		
	♂♂	Habitat complexity	0.037	0.014	7.1	<0.01	*	0.29
		Habitat size	-0.006	0.006	0.8	0.38		
		Bird abundance	-0.009	0.006	2.3	0.13		
RA	♀♀	Habitat complexity	11.4	6.3	3.3	0.07		0.23
		Fish density	-7.1	2.7	6.8	<0.01	*	
		Bird abundance	-4.4	3.1	1.9	0.17		
	♂♂	Habitat size	0.53	0.18	8.4	<0.01	*	0.62
		Fish density	-0.52	0.16	10.5	<0.01	*	
		Turbidity	0.44	0.19	5.3	0.02	*	

Table S6. Standardized regression coefficients (S.C.) and standard errors (S.E.) from the final generalized linear mixed models showing the effects of ecosystem factors on offspring number and offspring size of *Gambusia holbrooki*. Covariate (fish weight and brood size) is also shown alongside the explained variance of models ($R^2_{\beta*}$) as calculated by Jaeger et al. (2016). Significance of ecosystem factors was reached at P value ≤ 0.05 and denoted by *.

	Predictors and covariates	**S.C.**	**S.E.**	**X^2**	**P-value**		**$R^2_{\beta*}$**
Offspring number	Fish weight	0.32	0.01	711.1	<0.001	*	0.71
	Fish density	-0.56	0.10	31.4	<0.001	*	
	Bird abundance	-0.19	0.11	3.1	0.08		
Offspring size	Brood size	-0.10	0.03	10.3	<0.01	*	0.44
	Oocyte stage	0.18	0.03	27.5	<0.001	*	
	Oocyte stage^2	-0.012	0.003	17.4	<0.001	*	
	Habitat complexity	0.27	0.13	4.5	0.03	*	
	Habitat size	-0.15	0.05	8.3	<0.01	*	
	Total nutrients	0.12	0.05	6.1	0.01	*	
	Chlorophyll-a	-0.10	0.06	3.2	0.07		
	Fish density	0.21	0.06	13.5	<0.001	*	
	Bird abundance	-0.18	0.06	9.7	<0.01	*	

CAPÍTOL 4

Baixa prevalença de paràsits en el peix invasor *Gambusia holbrooki* en aigües fluvials i estancades al nord-est d’Espanya

Alberto Maceda-Veiga[a,b]; Oriol Cano-Rocabayera[a]; Adolfo de Sostoa[a]; Joe Cable[c]

[a] *Departament de Biologia Evolutiva, Ecologia i Ciències Ambientals – Institut de Recerca de la Biodiversitat (IRBio-UB), Universitat de Barcelona*

[b] *Departamento de Ecología Integrativa, Estación Biológica de Doñana (EBD-CSIC), Sevilla*

[c] *School of Biosciences, Cardiff University, Cardiff*

Bull. Eur. Ass. Fish Pathol., 39(2) 2019

Low parasite prevalence in the invasive fish *Gambusia holbrooki* in riverine and stagnant waters in north-eastern Spain

A. Maceda-Veiga[1,2*], O. Cano-Rocabayera[1],
A. de Sostoa[1], J. Cable[3]

[1]*Department of Evolutionary Biology, Ecology and Environmental Sciences-Institute of Research in Biodiversity, Universitat de Barcelona (IRBio-UB), 08028 Barcelona, Spain;* [2]*Department of Integrative Ecology, Estación Biológica de Doñana (EBD-CSIC), Avda Américo Vespucio, s/n, 41092 Sevilla, Spain;* [3]*School of Biosciences, Cardiff University, CF10 3AX, UK*

Abstract

Many factors can explain the spread of invasive non-native species, including reduced parasite burdens in recipient ecosystems. From multiple parasite surveys of the eastern mosquitofish (*Gambusia holbrooki*) in north-eastern Spain (2002-2015), we detected only two parasite taxa from a total of 2320 fish from 58 sites. From mucus scrapings, dissections and histopathology, a single individual of *Lernaea* sp. and two cysts of unidentified digenean helminths were recovered from three different fish without major pathological signs of disease. In conclusion, this study supports the notion of low vulnerability of wild riverine and lacustrine populations of *G. holbrooki* to parasites and highlights the importance of reporting negative results in parasitology and invasion science.

Introduction

Invasive non-native species (INNS) pose a significant threat to global biodiversity (Kumschick et al., 2014), being responsible for the decline of 42% of endangered species in the U.S. alone (Pimentel et al., 2005). Moreover, INNS have socio-economic impacts, for instance invasion by the ectoparasitic worm *Gyrodactylus salaris* costs the Norwegian salmon industry £30M per annum (Olstad, 2013). Despite significant progress being made on how INNS alter disease dynamics (Kelly et al., 2009; Hatcher et al., 2012; Blackburn and Ewen, 2017), the wide distribution of INNS inevitably results in the impact of associated pathogens being overlooked in many ecosystems. Nonetheless, the success of INNS has been often attributed to low vulnerability to parasites in the recipient invaded ecosystems, often referred to as the Enemy Release Hypothesis (Jeffries and Lawton 1984; Blossey, 2011). Originally from North America, the eastern mosquitofish (*Gambusia holbrooki* Girard, 1859) was introduced into southern Spain, Europe, in 1921 and has since invaded 30 countries due to an intentional introduction for mosquito biocontrol (Krumholz, 1948; Pyke, 2008). The enforcement of legislation in INNS subsequently prohibited such introductions (RD1628/2011; Regulation 1143/2014), but these probably still occur illegally. Moreover, the spread of *G. hol-*

---

* Corresponding author's e-mail: albertomaceda@gmail.com

brooki is likely to be promoted due to its wide tolerance of different water qualities (Pyke, 2008), reaching densities of up to 100 individuals/m^2 in the polluted, downstream Spanish rivers (Maceda-Veiga et al., 2018). Many studies have reported the negative effects of *G. holbrooki* on recipient communities through predation and antagonistic interactions (e.g. Hamer et al., 2002; Caiola and de Sostoa, 2005; Pyke, 2008). Indirect effects of *G. holbrooki* introduction, however, including their role as hosts for infectious agents, are largely unknown. While up to 50 parasite species were recorded on *G. holbrooki* in their native range (Dove, 2000; reviewed by Cable, 2011), the only comparable data in Europe is from river mouths in France and Spain, where just cestode larvae were recorded from introduced *G. holbrooki* (see Benejam et al., 2009). To assess whether the success of *G. holbrooki* in Europe can be attributed to the Enemy Release Hypothesis, there is a need for more parasitological surveys of this fish across different invaded ecosystems.

Here, we inspected invasive populations of *G. holbrooki* for the presence of parasites between 2002 to 2015 in running and stagnant waters of north-eastern Spain, where the spread of *G. holbrooki* continues unabated. If *G. holbrooki* has a low vulnerability to infection in invaded ecosystems, we should not find strong evidence of parasites, tissue alterations or other clinical signs of disease in the invasive populations in north-eastern Spain.

Materials and methods

From 2002 to 2015, freshwater fish communities in north-eastern Spain were surveyed within the framework of several projects, including the development of an index of biotic integrity using fish as bioindicators (e.g. Sostoa et al., 2010; Maceda-Veiga, 2011; Cano-Rocabayera, *in preparation*). We present data on the three surveys in which *G. holbrooki* were inspected for the presence of parasites to inform the potential role of this species as carrier of parasites in an invaded region. Water quality was quantified in each sampling site before fish sampling (Table 1). Fish were captured with dip-nets in ponds and lakes and by electro-fishing in rivers known to contain up to 16 native and other 18 non-native fish species (details in Maceda-Veiga et al., 2018). Mixed-fish stocks were kept in buckets provided with air pumps and anaesthetised (MS222® 0.02%). A sub-sample of *G. holbrooki* (*N* = 40 per site, sex-ratio 1:1) were processed for a detailed health inspection.

The degree of inspection detail varied depending on the number of sampling sites surveyed in each project. The first data-set originated from a large-scale survey from Sènia to Muga rivers in north-eastern Spain, conducted from June to September in 2002-2011. *Gambusia holbrooki* only occurred in 34 of the 530 sampling sites surveyed (N_{Total} = 1360 fish). The entire external surface of *G. holbrooki* was visually inspected on the riverbank for gross clinical signs of disease and macroparasites. Widely distributed fish macroparasites in the study region are the copepod *Lernaea* and the oomycete *Saprolegnia* spp. (see Sostoa, 2003), both of which are easy to detect visually (see Noga, 2011).

The second data-set was derived from a medium-scale survey in which 11 ponds, one river and six irrigation channels were sampled in May 2015 (N_{Total} = 720 fish). Gross external examination and mucus scrapings of skin and fins were conducted on *G. holbrooki*. Fish mucus was distributed using a clean slide on the surface of another slide, air-dried in the field and 30 fields of view per slide per fish were examined using an Olympus CH2 microscope at x400 magnification in the laboratory. Fish were euthanised after mucus sampling, fixed in 70% ethanol and transported to the laboratory for detailed examination of muscle, gonads and gastrointestinal tract under the microscope (x400).

The last data-set is based upon a small-scale survey in the Diagonal Mar urban Lake, the Ripoll River and two agricultural channels close to Barcelona in June 2010, both of which were re-surveyed in November 2012 and May 2015 (N_{Total} = 320 fish). Fish were transported live to the laboratory and euthanised. Half fish (N = 20) in each survey (N = 6) were processed for histology and half for detailed parasitological inspection. The head and all viscera (gill, liver, gonad, kidney), including a sample of dorsal muscle, were fixed separately in 10% neutral-buffered formalin for 24 h, embedded in paraffin wax, sectioned at 5 μm thickness, stained with hematoxylin and eosin and screened for lesions and parasites. Sections of all tissues from each fish individual were observed under the microscope. The remaining half of the sample was dissected using fine forceps to pull apart fish tissues (gills, liver, gonads, brain) under a dissecting microscope. Muscle and skin were crushed between two glass plates and examined by transparency to detect parasites. The digestive tract was cut longitudinally, walls crushed between two glass plates and the content directly screened for parasites under the microscope and dissecting microscope.

Results and discussion

From all information sources combined, we did not observe major external clinical signs of disease from 2320 individuals of *Gambusia holbrooki* (Table 1). Mucus scrapings have been successfully used for detecting ectoparasite in fish, including trichodinids and monogeneans (Lom and Dykova, 1992; Noga, 2011), but the only ectoparasite in the current study was a single individual of the parasitic copepod *Lernaea* sp. directly observed on the skin of a 44 mm-female in an irrigation channel (41º16’N-2º02’E) from the small-scale survey. Higher infection rates were expected in stagnant rather than in running waters because low water flows facilitate the attachment of free-swimming parasites (e.g. Samsing et al., 2015). However, water pollution may have altered host-parasite dynamics (e.g. Marcogliese, 2005; Maceda-Veiga and Cable, 2016). Most fish were captured in polluted waters (Table 1) because clean waters in the study area should have levels of ammonium and nitrite below detection limits because of high toxicity (Noga, 2011) and conductivity values <600 μS/cm (Maceda-Veiga, 2011). Poor preservation of the parasite prevented species level identification, but *L. cyprinacea* infects other fish species in the study area (Maceda-Veiga et al., 2019) and was recorded on mosquitofish in other regions (Innal et al., 2012).

Internal overt clinical signs of disease were rare in the dissected *G. holbrooki* (Table 1). The only endoparasites observed were cysts of an unidentified digenean in the gonad of two 20 mm-males and of a 40 mm-female fixed in 70% ethanol from the medium-scale survey (Figure 1). The lack of fresh material impeded identification, but authorities eradicated *G. holbrooki* at the collection site, preventing further col-

Table 1. Mean (±Standard Error) and minimum-maximum range of body size of eastern mosquitofish (*Gambusia holbrooki*). Fish were captured in waterbodies with a wide range of water-quality conditions as part of three projects (large-, medium- and small-scale surveys) in north-eastern Spain (see methods). The total number of fish examined is shown (N_{Total})

	Large-scale survey (N = 34 sites) N_{Total} **= 1360 fish**			**Medium-scale survey (N = 18 sites)** N_{Total} **= 720 fish**			**Small-scale survey (N = 6 sites)** N_{Total} **= 320 fish**		
	Mean±SE	**Minimum**	**Maximum**	**Mean±SE**	**Minimum**	**Maximum**	**Mean±SE**	**Minimum**	**Maximum**
Fish variables									
Body length (mm)	29.91±11.99	10	48	34.18±0.29	20	57	29.21±0.35	18	51
Water variables									
Water velocity (m/s)	0.65±0.07	0	1.4	<0.01±0.01	0	0.03	0.01±0.001	0	0.35
Temperature (ºC)	19.72±0.84	14.5	27.7	19.39±0.80	6.42	34.56	22.46±0.74	20.9	24.3
pH	8±0.1	7.3	9	8.2±0.1	7.0	9.5	7.8±0.2	7.2	8.5
Conductivity (µS/cm)	1091±88.53	223	1968	2950±628.2	128	30970	1201±355.2	800	3520
Ammonium (mg/l)	0.42±0.14	<0.1	3	0.54±0.13	<0.1	5.0	0.1±0.0	<0.1	0.2
Nitrite (mg/l)	0.14±0.04	<0.02	0.6	0.34±0.08	<0.05	3.72	0.23±0.07	<0.02	0.8
Nitrate (mg/l)	3±0.81	<1	15	10±2.5	<1	129	14±1.5	5	20
Phosphate (mg/l)	0.36±0.08	<0.1	1.5	0.47±0.06	<0.1	2.0	0.27±0.13	<0.1	1.0

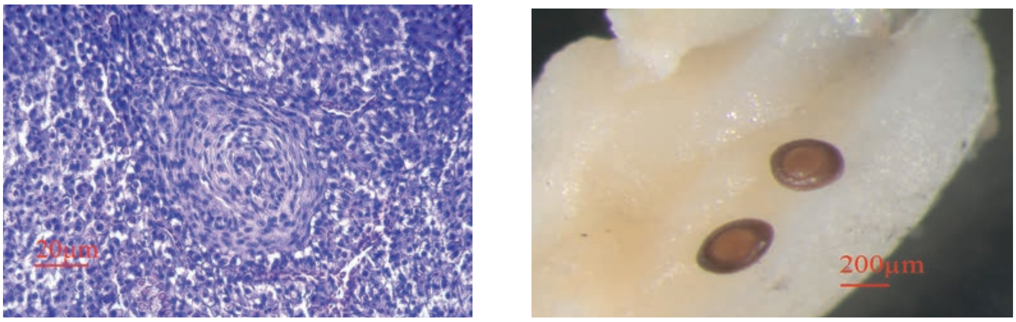


Figure 1. Eastern mosquitofish (*Gambusia holbrooki*). Left, haematoxylin and eosin stained liver showing a granuloma. Right, ethanol-fixed testis showing cysts of unidentified digenean helminths.

lections. All tissues were normal in appearance, even though some artefacts typical of fish processing, such as slight epithelial lifting and telangiectasia, were present. One fish in the small-scale survey presented a granuloma, but the cause is unknown; parasites can induce such an inflammatory response (Ferguson, 2006) even though none were detected on this fish (Figure 1). Most individuals of *G. holbrooki* examined appeared healthy despite being collected across a wide range of water quality conditions and being inspected over a wide range of fish sizes (Table 1). Fish parasites are present at our study sites because the Asian tapeworm *Bothriocephalus acheilognathi* was recovered from native *Squalius laietanus* in the Ripoll River (41º34’N-2º06’E, Maceda-Veiga, 2011). At this site, monogenean infections of *Dactylogyrus* and *Gyrodactylus* spp. were also observed on native *Barbus meridionalis* (Maceda-Veiga, 2011).

In conclusion, this study supports that *G. holbrooki* is a poor carrier of parasites in north-eastern Spain, which is consistent with the river mouth surveys conducted by Benejam et al. (2009). Reporting negative findings for infections in INNS is essential for a balanced perspective of how the introduction of INNS alters parasite dynamics in recipient ecosystems.

Acknowledgments

We are grateful to Francesc Padrós for assistance with histopathology and to Humbert Salvadó and Robert Poulin for advice on parasite ID. An APIF grant from the Autonomous Government of Catalonia supported the PhD of O.C.R. and “la Fundació Barcelona Zoo” financed the sampling.

References

Benejam L, Alcaraz C, Sasal P, Simon-Levert G, and García-Berthou E (2009). Life history and parasites of the invasive mosquitofish (*Gambusia holbrooki*) along a latitudinal gradient. *Biological Invasions* **11**, 2265-2277.

Blackburn TM and Ewen JG (2017). Parasites as drivers and passengers of human-mediated biological invasions. *EcoHealth* **14**, 61-73.

Blossey B (2011). Enemy release hypothesis. *Encyclopedia of Biological Invasions* 3, 193.

Cable J (2011) Poeciliid Parasites. In: Ecology & Evolution of Poeciliid Fishes. (Eds) J.P. Evans, A. Pilastro and I. Schlupp. Chicago University Press, pp. 82-94.

Caiola N, and De Sostoa A (2005). Possible reasons for the decline of two native toothcarps in the Iberian Peninsula: evidence of competition with the introduced Eastern mosquitofish. *Journal of Applied Ichthyology* **21**, 358-363.

Cano-Rocabayera O (*in progress*). Impact of nitrate pollution on the invasion success of *Gambusia holbrooki* and the associated multi-trophic impacts in experimental lagoons. PhD thesis. Universitat de Barcelona.

Dove ADM (2000) Richness patterns in the parasite communities of exotic poeciliid fishes. *Parasitology*, 120, 609–623.

Ferguson HW (2006). Systemic pathology of fish. A text and atlas of comparative tissue responses in diseases of teleosts. Iowa State University Press.

Jeffries MJ, Lawton JH (1984). Enemy free space and the structure of ecological communities. *Biological Journal of the Linnaean Society* **23**, 269–286.

Kelly DW, Paterson RA, Townsend CR, Poulin R, and Tompkins DM (2009). Parasite spillback: a neglected concept in invasion ecology?. *Ecology* **90**, 2047-2056.

Krumholz LA. (1948). Reproduction in the western mosquitofish, *Gambusia affinis affinis* (Baird & Girard), and its use in mosquito control. *Ecological Monographs* **18**, 1-43.

Kumschick S, Gaertner M, Vilà M, Essl F, Jeschke JM, Pyšek P. et al. (2014). Ecological impacts of alien species: quantification, scope, caveats, and recommendations. *Bioscience* **65**, 55-63.

Hamer A, Lane S, and Mahony M (2002). The role of introduced mosquitofish (*Gambusia holbrooki*) in excluding the native green and golden bell frog (*Litoria aurea*) from original habitats in south-eastern Australia. *Oecologia* **132**, 445-452.

Hatcher MJ, Dick JT and Dunn AM (2012). Disease emergence and invasions. *Functional Ecology* **26**, 1275-1287.

Innal D and Avenant-Oldewage A (2012). Occurrence of *Lernaea cyprinacea* on mosquito fish (*Gambusia affinis*) from Kundu Estuary (Antalya-Turkey). *Bulletin of the European Association of Fish Pathologists* **32**, 140-147.

Lom J and Dyková I (1992). Protozoan parasites of fishes. Developments in Aquaculture and Fisheries Science, vol. 26, Elsevier, pp 315.

Maceda-Veiga A (2011). Estado de conservación de los peces continentales catalanes: análisis poblacional, ictiopatología y bioacumulación de metales pesados como herramientas de diagnóstico (In Spanish). PhD thesis. Universitat de Barcelona.

Maceda-Veiga A and Cable J (2016). Nitrate pollution, but not fin clipping, reduces ecto-symbiont abundance on three-spined sticklebacks. *Bulletin of the European Association of Fish Pathologists* **36**, 253-259.

Maceda-Veiga A, Mac Nally R and de Sostoa A (2018). Environmental correlates of food-chain length, mean trophic level and trophic level variance in invaded riverine fish assemblages. *Science of the Total Environment* **644**, 420-429.

Maceda-Veiga A, Mac Nally R, Green AJ, Poulin R and de Sostoa A (2019). Major determinants of the occurrence of a globally invasive parasite in riverine fish over large-scale environmental gradients. *International Journal for Parasitology* (Accepted).

Marcogliese DJ (2005). Parasites of the superorganism: are they indicators of ecosystem health?. *International Journal for Parasitology* **35**, 705-716.

Noga EJ (2011). Fish disease: diagnosis and treatment. John Wiley & Sons. 2st Edition. pp. 130 pp.

Olstad K. (2013): NOBANIS – Invasive Alien Species Fact Sheet – *Gyrodactylus salaris* – From: Online Database of the European Network on Invasive Alien Species – NOBANIS www.nobanis.org, Date of access 02/02/2016.

Pimentel D, Zuniga R and Morrison D. (2005). Update on the environmental and economic costs associated with alien-invasive species in the United States. *Ecological Economics* **52**, 273-288.

Pyke GH (2008). Plague minnow or mosquito fish? A review of the biology and impacts of introduced *Gambusia* species. *Annual Review of Ecology, Evolution, and Systematics* **39**, 171-191.

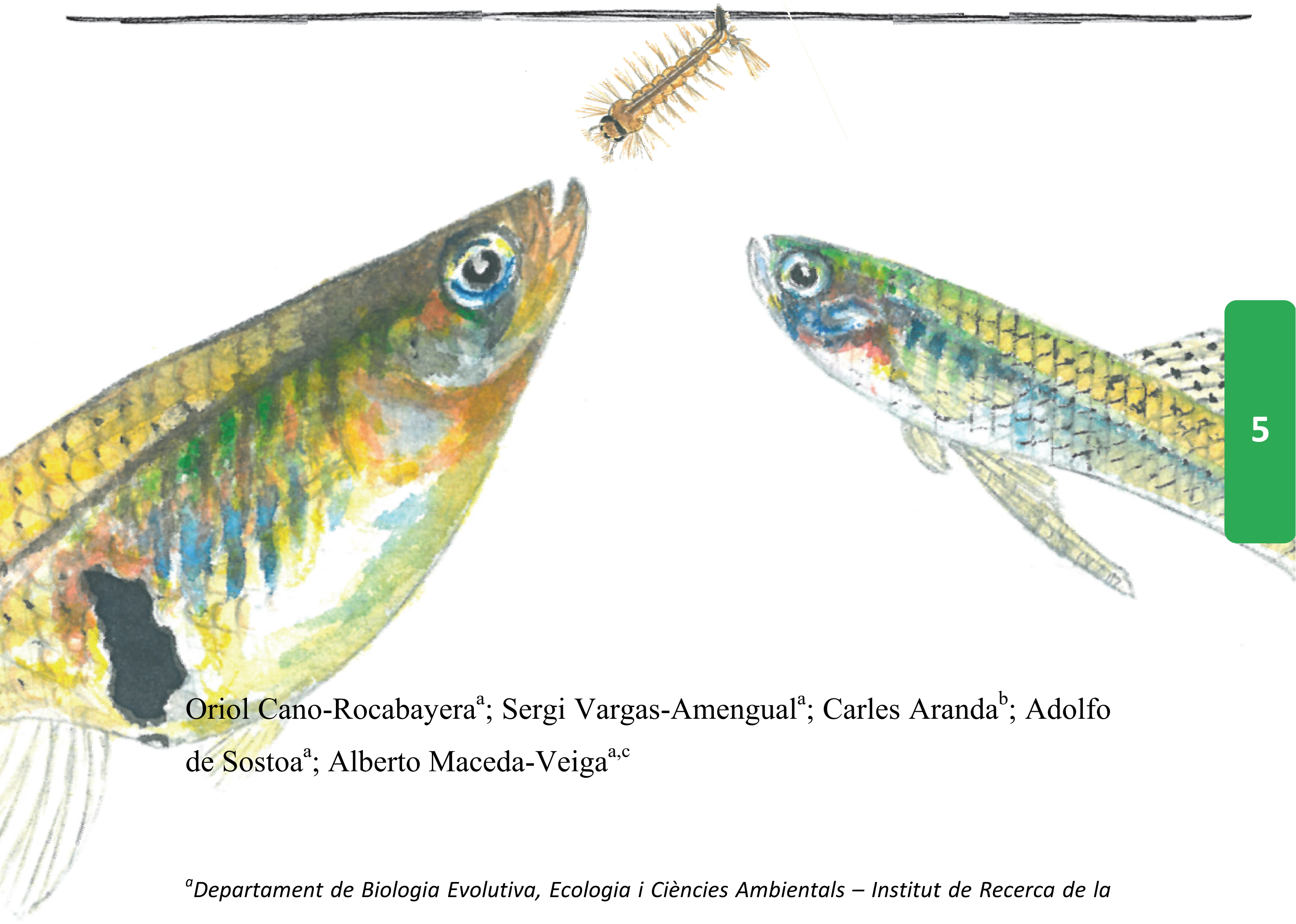
Samsing F, Solstorm D, Oppedal F, Solstorm F and Dempster T. (2015). Gone with the flow: current velocities mediate parasitic infestation of an aquatic host. *International Journal for Parasitology* **45**, 559-565.

Sostoa AD (Coordinator) (2003). Desenvolupament d'un índex d'integritat biòtica (IBICAT) basat en l'ús dels peixos com a indicadors de la qualitat ambientals dels rius de Catalunya (In Catalan). Technical report.

Sostoa AD, Caiola N, Casals F, García-Berthou E, Alcaraz Cazorla C, Benejam LV, Maceda-Veiga A, Solà C and Munné A. (2010). Ajust de l'índex d'Integritat Biòtica (IBICAT) basat en l'ús dels peixos com a indicadors de la qualitat ambiental als rius de Catalunya. © Agència Catalana de l'Aigua. 2010. Ajust de l'Índex d'Integritat Biòtica (IBICAT) basat en l'ús dels peixos com a indicadors de la qualitat ambiental als rius de Catalunya. Departament de Medi Ambient i Habitatge, Generalitat de Catalunya. 187 p.

CAPÍTOL 5

Quan la introducció d’un peix exòtic per al control biològic de mosquits amenaça innecessàriament aquest servei ecosistèmic natural



Oriol Cano-Rocabayera[a]; Sergi Vargas-Amengual[a]; Carles Aranda[b]; Adolfo de Sostoa[a]; Alberto Maceda-Veiga[a,c]

[a]*Departament de Biologia Evolutiva, Ecologia i Ciències Ambientals – Institut de Recerca de la Biodiversitat (IRBio-UB), Universitat de Barcelona*

[b]*Servei de Control de Mosquits del Consell comarcal del Baix Llobregat, El Prat de Llobregat*

[c] *Departamento de Ecología Integrativa, Estación Biológica de Doñana (EBD-CSIC), Sevilla*

When the introduction of alien fish for mosquito biocontrol unnecessarily threatens the natural ecosystem service

ABSTRACT

The conservation of species interactions is central to maintain the services ecosystems provide to society. The management of mosquito-borne diseases has long attracted the attention of natural resource managers and has been the cause of pernicious piscine invasions. This study examines whether the introduction of *Gambusia holbrooki* was justified for mosquito biocontrol at the expense of the service provided by the native *Aphanius iberus*. We found that the presence of *G. holbrooki* reduced *A. iberus* predation on *Culex pipiens*, a mosquito species frequent in eutrophic waters. Both fish species reduced mosquito abundance in aquaria and when interacted to an invertebrate assemblage within mesocosms. However, *G. holbrooki* reacted more rapidly to the presence of mosquitoes and consumed more big mosquitoes than *A. iberus*. The efficacy of the two fish species in mosquito control was reduced under eutrophication. Our findings illustrate the effects that excessive fertilization has on the conservation of species interactions valuable in pest management. Moreover, we report how ignoring the recommendations from ichthyologists in the early twentieth century was the gateway to *G. holbrooki* invasion in Europe. We revisited their work to prevent more invasions by showing that native species provide the same ecosystem service, albeit more slowly.

Keywords: species interactions; pest control; water turbidity; threatened fish; *Gambusia*; *Aphanius*

1. Introduction

The conservation of species interactions is on the agenda of natural resource managers because of their role in maintaining important ecosystem services, including pollination and pest control (Tylianakis et al., 2010; Valiente-Banuet et al., 2015). However, most research has been conducted in terrestrial taxa (e.g. Moleón et al., 2014; Manning et al., 2018; Herrera, 2019), which may result in the importance of preserving ecosystem services provided by aquatic taxa being overlooked (WWF, 2018). Freshwater taxa account for the highest rate of extirpations on vertebrates, with a decline in freshwater populations of 83% from 1970 to 2014 (WWF, 2018). Global threats to freshwater ecosystems include water abstractions, land-use changes, chemical pollution and the establishment of alien species (Ormerod et al., 2010). Improved understanding of how human impacts alter ecosystem services provided by interactions between freshwater species may increase public conservation awareness and promote the restoration of degraded freshwater assemblages.

Wetlands have long been under intensive human pressure, including desiccation to use the fertile lands for agriculture and to reduce the spread of mosquito-borne diseases (Davidson, 2014). These diseases are emerging at unprecedented rates and scales, with recent outbreaks of malaria, dengue, chikungunya and Zika in developing countries (Becker, 2010; Hotez, 2016; WHO, 2018). Although the causes of the emergence of diseases are often uncertain, the degradation of freshwater ecosystems probably contributes substantially to cause many epidemics (Garchitorena et al., 2017). The situation is expected to worsen with global change because the activity of mosquitoes in temperate regions will last longer throughout the year (Paaijmans et al., 2010). Improved means of transport also facilitate the movement of goods and people and with them alien species, as occurred with the Asian tiger mosquito (*Aedes albopictus*) established in all continents except Antarctica (Eritja et al., 2017). However, the effects of global change on mosquito-borne diseases are difficult to predict, given the many host and pathogen factors involved, including environmental and socio-ecological drivers (Garchitorena et al., 2017). Therefore, control methods integrated into natural systems are probably among the most effective and ecologically sound treatments to regulate mosquito pests in the long term.

The introduction of fish and the spray of spores and crystalline insecticidal proteins produced by *Bacillus thuringiensis* are widely used for mosquito biocontrol (Becker, 2010). However, fish are the most cost-effective method because *B. thuringiensis* needs to be applied continuously to water bodies throughout all the mosquito season (Lacey, 2007). Many administrations recommended for decades the release of *Gambusia holbrooki* and *G. affinis* (hereafter mosquitofish) as the most effective method to control mosquitoes (Pyke, 2008). The high fecundity and tolerance of mosquitofish to poor water quality may explain why was the preferred species for mosquito biocontrol (Homski et al., 1994; Pyke, 2008). *Gambusia holbrooki* was established in Spain in 1921 from where it was distributed to other countries (Pena and Domínguez, 1985; Walton et al., 2012). However, the need of introducing mosquitofish was soon questioned by ichthyologists (Lemasson, 1937), including the Spanish ones who demonstrated that *G. holbrooki* kills endemic fish such as *Aphanius iberus* (De Buén, 1929).

Studies in clean water have shown that mosquitofish and *Aphanius* killifish prey upon different larvae stages of mosquitoes (Homski et al., 1994), and that mosquitofish presence reduces the foraging rate of *Aphanius iberus* (Caiola and de Sostoa, 2005). However, studies in more realistic environmental conditions are needed to demonstrate the efficacy of native fish in mosquito control because of the different breeding habitats used by the different mosquito species. The common house mosquito *Culex pipiens* often breeds in permanent eutrophic waters, where it feeds on suspended organic matter and algae (Becker et al., 2010). These nutrient enriched sites are turbid due to the proliferation of algae, but well-preserved wetlands often are also turbid because of run-off and the resuspension of sediment caused by animal movement (Kadlec. 2009; Casas et al., 2011). The type and intensity of turbidity affect prey detection in visual predators (Utne-Palm, 2002; Shoup and Wahl, 2009), so that turbidity may make prey other than mosquitoes easier to detect and to catch. Thus, the comparison of the feeding behaviour of mosquitofish and native fish on mosquito larvae under variable turbid conditions may promote restoration actions and the reintroduction of native fish for mosquito biocontrol.

Here, we explore the hypothesis that the introduction of *G. holbrooki* was not justified for mosquito control because of the presence of equally effective native fish, such as the IUCN endangered *Aphanius iberus* in Spanish wetlands. Both fish species are small and feed on invertebrates, including mosquitoes (Doadrio, 2002). *Aphanius iberus* is in marked regression due to habitat degradation and the injuries and trophic competition caused by *G. holbrooki* (Doadrio, 2002). We compared the efficiency of the two fish species to reduce the abundance of live larvae of *Culex pipiens* under variable turbidity and when interacting with each other and with a planktonic community. Our ultimate goal is to provide quantitative data to avoid more misguided strategies for mosquito biocontrol at the expense of the conservation of native fish given that mosquitofish introductions are still occurring (Figure 1).

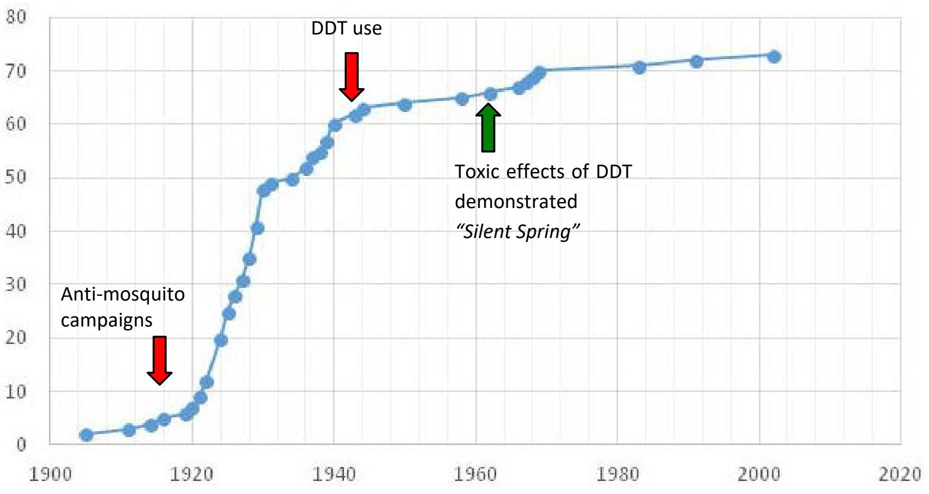


Figure 1. Accumulated number of introductions (*Gambusia holbrooki* and *Gambusia affinis* combined) in new countries in the 20th century. Note that mosquitofish introductions declined when the DDT insecticide began to be used and increased again when the pernicious effects of DDT were reported by Carson (1962). Adapted from: Walton et al. (2012). A handbook of global freshwater invasive species.

2. Material and methods

2.1. General experimental design and fish and mosquito origins

The performance of the IUCN endangered *A. iberus* (*N* = 170) and the invasive *G. holbrooki* (*N* = 170) as mosquito control agents was investigated in both aquaria and mesocosms in the field. *Aphanius iberus* were collected in November 2014 with nets in three artificial ponds kept by the Mosquito Control Service of the Baix Llobregat Council (MCS) and the Natural Areas of the Llobregat Delta. This fish population originated from a wild population in the Llobregat Delta, where *A. iberus* is almost extirpated (Ford, 2016). *Gambusia holbrooki* were captured in November 2014 with dip nets in three channels from the Llobregat Delta. All fish were brought to the aquatic facility at the University of Barcelona (UB) in opaque plastic tanks provided with air-pumps. Each species was acclimatized in a 500 L tank provided with an external filter, artificial plants and flowerpots for refugee. Fish were kept under 20±1ºC and 12h light:12h dark cycle and fed daily with Sera Vipan® flakes and weekly with bloodworms. Fish from these tanks were used in the experiments in aquaria and in mesocosms. All fish were anaesthetized (0.02% MS-222®) and measured (mm) before each assay (see below). Sex ratio was 1:1 in all the experiments apart from the *A. iberus*-*G.*

holbrooki interactive assays in which only females were used. Females often dominate in number in mosquitofish populations (Fryxell et al., 2015). Fish were placed, and left fastened, in the experimental tank 24 h before the assay.

A local wild-type culture of the common house mosquito *C. pipiens* maintained at the MCS for ca. 20 years was brought to the UB to facilitate logistics when feeding fish with live larvae for: (i) examining the preference of the two species for the different larval stages (*Experiment 1*); (ii) determining whether turbidity equally alter the efficacy of the two fish species in mosquito control (*Experiment 2*); and (iii) testing whether the performance of the two fish species as mosquito control agents is reduced when interacting with fish competitors and other prey (*Experiment 3*). Trays with 2 cm of water were introduced into the insectarium to collect mosquito eggs, which hatched in 48h at 25±1 ºC. Mosquito larvae were fed with a mixture of tropical goldfish food.

2.2. Experiment 1 – The mosquito larval stage preferred by the two fish species and the total number consumed in clean water

Individual assays were conducted for *A. iberus* and *G. holbrooki* (N = 60 fish each) following the methods of Homski et al. (1994) and Billman et al. (2007). Fish were placed for 15 min in a 2.5 L jar with 15 individuals from II – IV instar larvae and 10 pupae (N_{total} = 45). Fish were then gently removed by hand and the number of uneaten mosquitoes was recorded. The most preferred larval stage in this experiment was the one used in Experiments 2 and 3.

2.3. Experiment 2 – The effects of turbidity on the consumption of mosquito larvae

Experimental trials were conducted in a 10-L aquarium in the same room of the tanks used for fish acclimation. The aquarium (18 cm wide × 30 cm long × 15 cm water depth) was placed on an opaque sheet to provide fish with a dark substrate like in nature. All sides of the aquarium apart from the front side were covered. Light was provided by a 60 W fluorescent at 70 cm on the top of the aquarium. Turbid conditions were hand-made dissolving the freeze dried green algae *Chlorella vulgaris* (Low: 83 mg/L; High: 314.3 mg/L) or red clay (Low: 10 mg/L; High: 54.3 mg/L) in 1 L of dechlorinated laboratory tap water.

For each trial, we gently poured one of the turbid solutions to an aquarium with an individual of *A. iberus* (N = 110) or *G. holbrooki* (N = 109). The controls were filled with dechlorinated laboratory tap water. Turbidity (mean ± SD) in the three treatments was 0, 11±2 and 39±4 Formazin Turbidity Units (FTUs) using the Turbidimeter Orbeco-Hellige® MP-975 at 420 nm on the termination of the trials. There were 42 trials for *C. vulgaris* and 44 for clay, and one set for each fish species (i.e. 10 trials per sex, species and turbid condition). Experimental turbidity values are within the range we measured in the study area (Table S1). Fish and the turbid solutions were allowed to settle for 20 min before releasing 50 mosquitos of IV instar larvae into the tank. Different fish individuals were used in each trial and the tank was fully cleaned in-between. An observer sat in front of the tank recorded for each fish the time spent to catch the first larvae (latency time) and the time spent to capture first four larvae (voracity). The trial ended at 10 min because it is when both fish species reached satiety in pilot assays. Fish were then gently removed and the number of uneaten larvae recorded after sieving the water through a dip net. At the highest turbid treatment (40 FTU), the lack of visibility prevented the determination of latency time and voracity.

2.4. Experiment 3 – Does the presence of competitors or alternative prey alter the efficacy of the two fish species in mosquito biocontrol?

The effects of the presence of fish competitors on the efficacy of *A. iberus* and *G. holbrooki* in mosquito control were determined in aquaria and mesocosms, where fish also interacted with a natural invertebrate community.

The competition experiment in aquaria consisted of introducing simultaneously a female of the two species in a tank and recording the number of larvae consumed by each fish in each minute over a total of 10 minutes. The experimental setting and the turbidity conditions were like Experiment 2 but using 100 IV-instar larvae and 20-L aquaria.

The competition experiment in mesocosms was conducted in 16 outdoor 500-L tanks filled with water, aquatic vegetation and sediment from a nearby pond. The fertilizer Biocanna® was applied at 11 ml per tank to promote algae and increase water turbidity up to 10 FTU. The tanks were left from September 2015 to November 2015 for the establishment of a natural planktonic community. Mosquito larvae were added at a density of ~ 1.1 ind/L to each mesocosm 24 h before introducing *A. iberus* and *G. holbrooki* at four ratios (10:0, 0:10, 5:5, 0:0; N = 4 mesocosms/ratio), which were the densities reported in nature (Table S2). The single-species mesocosms (10:0 or 0:10) were used to determine the sole effects of the presence of prey other than mosquitoes on fish mosquito predation. To determine the effects of fish foraging on the zooplankton assemblage in general and on mosquitoes in particular, we surveyed the water column of each mesocosm before and at 24h of having released the fish, by using a 23 cm Ø PVC tube following Turner and Trexler (1997). Samples were fixed in 70% ethanol and invertebrates identified to order or family level and the number of individuals counted under a dissecting microscope.

2.5. Statistical analyses

General and generalized linear models (GLMs) were conducted in R v. 3.4.3 (R Core Team, 2016) to test the hypothesis that *A. iberus* is as effective as *G. holbrooki* in mosquito control. The preference of fish for instar-larva was calculated using the Strauss' linear food selection index, $L = r_i - p_i$ (Strauss, 1979), which is widely in studies of fish prey electivity (e.g. Shelton et al., 2016). r_i and p_i are the proportion of prey item i in the gut and environment, respectively (Strauss, 1979).

Visual inspection of the distribution of variables suggested the most appropriate error distribution for GLMs. Poisson errors with log link were assumed for the number of prey and Gaussian errors with identity for L and latency and voracity times. We examined the effects of species on response variables accounting for the main and interactive effects of fish sex and the log-transformed fish weight using the function *glm* within the package *stats*. Sexual dimorphism in both species implies changes in size but differences in the foraging behaviour of mosquitofish between sexes were observed regardless of their differences in body size in Cano-Rocabayera et al. (2019a). Differences in the number of prey eaten by female *A. iberus* and *G. holbrooki* in the competition experiment in aquaria were examined using the function *glmer* within the package *lme4* (Bates et al., 2015). Model included 'Fish ID' as random effect to account for repeated measures on the same fish, species as fixed effect and fish weight as covariate. Differences in the abundance of mosquito larvae in mesocosms were examined using the function *glm*. Fish ratio was included in model as a categorical factor and the abundance of mosquito larvae before releasing the fish was a covariate. Differences in the overall composition of invertebrate assemblages among tanks with different fish ratios were determined using a permutational analysis of variance (PERMANOVA) throughout the function *adonis* within the package *vegan* (Oksanen et al., 2019).

Significance of fish species and covariates in GLMs were assessed using likelihood-ratio χ^2 tests and the function *Anova* within the package *car* (Fox and Weisberg, 2011). Non-significant covariates were omitted in the final GLMs. Final models were validated using diagnostic plots of model residuals to check for the assumptions of normality, homogeneity of variances and detect unduly observations following the methods of Zuur et al. (2009). The explanatory power of models was calculated using the function *r2beta* within the package *r2glmm* (Jaeger, 2017). The statistical threshold in all analyses was at $P \leq 0.05$.

3. Results

3.1. Experiment 1 – The mosquito larval stage preferred by the two fish species and the total number consumed in clean water

IV-instar larvae were the most positively selected by the two fish species while accounting for differences in fish size and sex (Table S3, Fig. 2). The least preferred mosquito stages for the two fish species were II-instar larvae and pupae. The bigger fish captured the bigger prey, but larger *G. holbrooki* tended to capture bigger mosquito larvae than similarly sized *A. iberus* (Table S3). In regard to the total number of mosquitoes consumed, the smaller individuals of *A. iberus* captured significantly more larvae than those of *G. holbrooki* (Table S4, Fig. 3). However, the larger individuals of *G. holbrooki* ate more larvae than *A. iberus* (Fig. 3). Sex did not have a significant effect in this experiment.

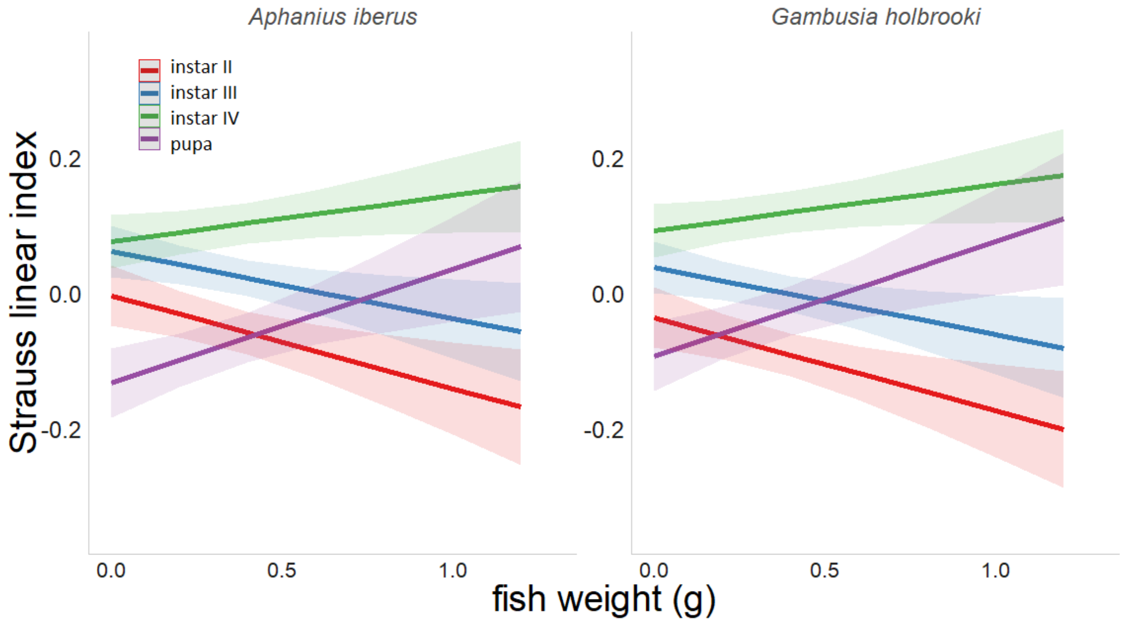


Figure 2. Preference of the IUCN endangered *Aphanius iberus* and the invasive *Gambusia holbrooki* for the different mosquito larvae stages while accounting for the effects of differences in body weight among fish. Each colour represents fish preference as indicated by the Strauss electivity index with 95% confidence intervals, such that: < 0, avoidance, ~0, no preference, and > 0, positive selection.

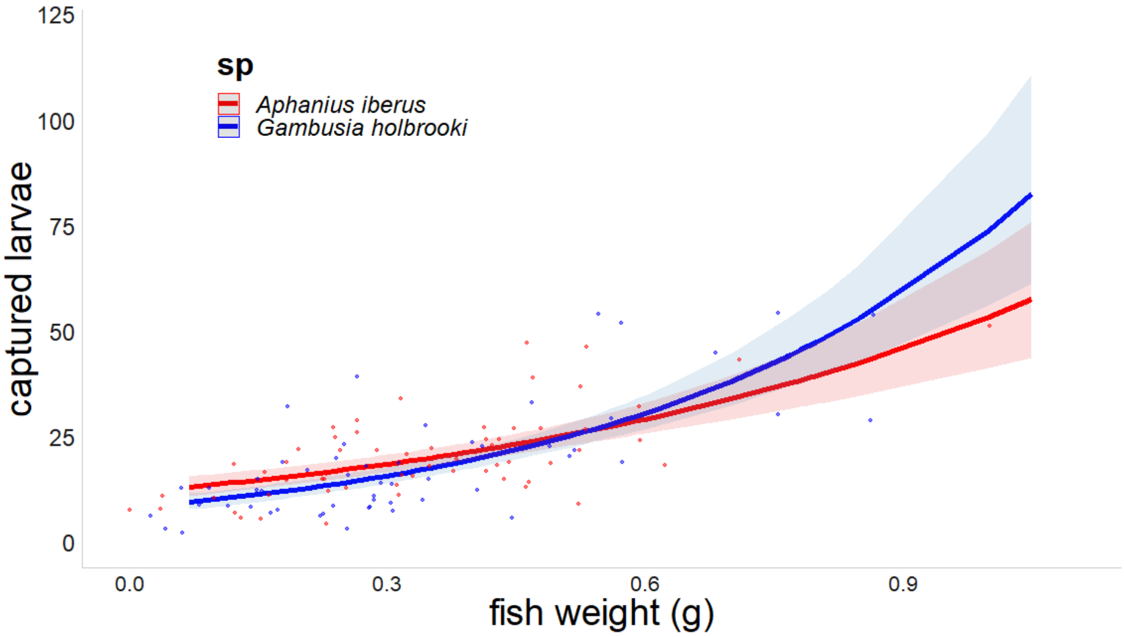
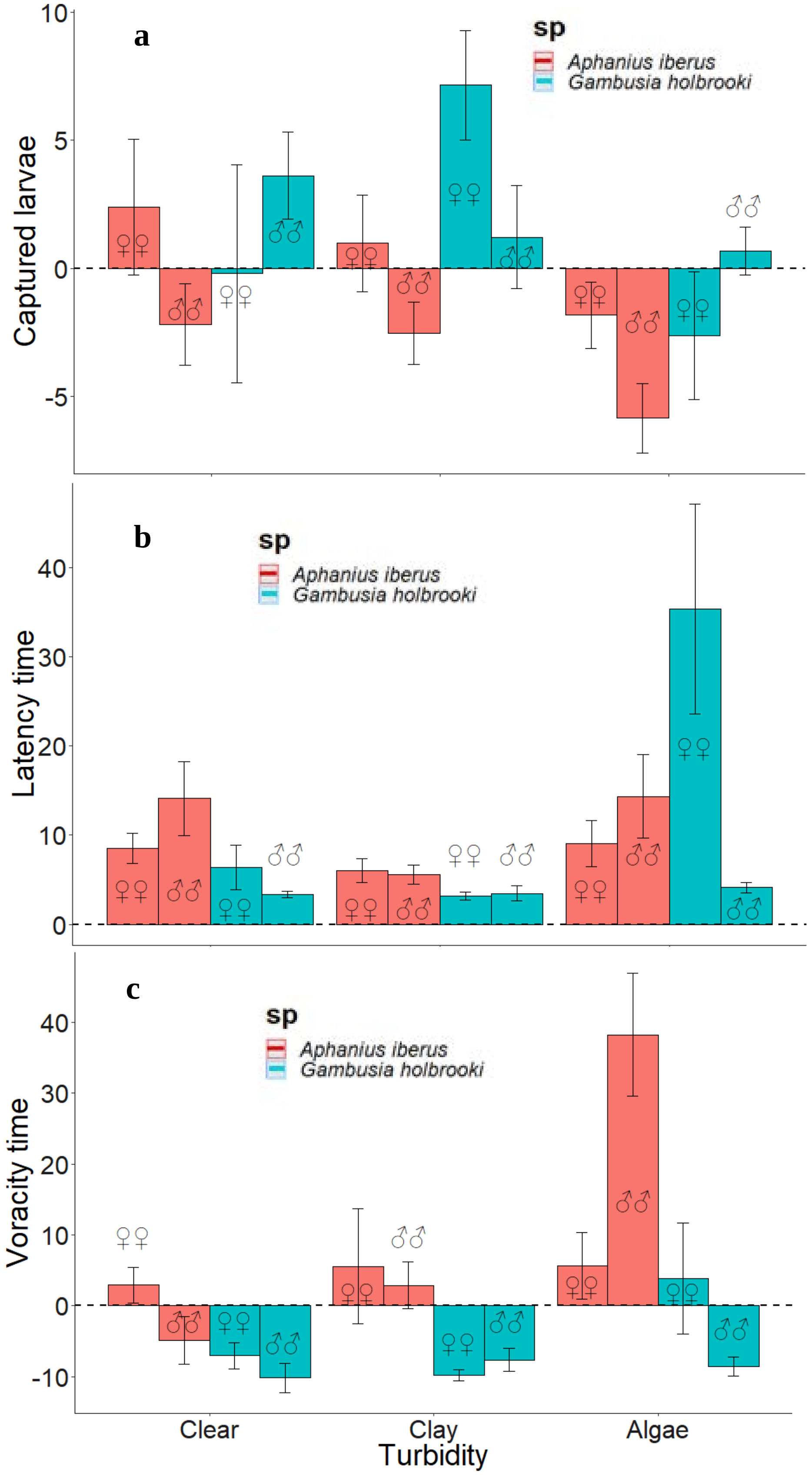


Figure 3. Total mosquito larvae captured by the native *Aphanius iberus* and the invasive *Gambusia holbrooki* as function of the differences in body weight among individuals. Fitted functions include 95% confidence intervals and account for the effects of all predictors highlighted in the final models (from Table S4) as important factors to explain variation in the number of IV-instar larvae eaten.

3.2. Experiment 2 – The effects of turbidity on the consumption of mosquito larvae

IV-instar larvae were preferentially consumed in Experiment 1, and so, was the one selected to determine the effects turbidity on mosquito predation by *A. iberus* and *G. holbrooki*. *Gambusia holbrooki* consumed slightly higher number of larvae than *A. iberus* in all treatments (Table S5, Fig. 4). There was an overall reduction in the number of larvae eaten depending on the type of turbidity (Table S5, Fig. 4a). While algal-induced turbidity reduced the number of larvae eaten in both fish species compared to the controls, clay did not have a significant effect on any fish species (Fig. 4a). The different intensities of turbidity did not have a statistically clear effect on mosquito consumption in any treatment (Table S5).

The time spent to capture the first prey item (latency) generally did not differ between *A. iberus* and *G. holbrooki* in clean and turbid waters (Fig. 4b). However, the algal suspension increased latency in *G. holbrooki* females compared to other treatment combinations (Fig. 4b). *Gambusia holbrooki* generally ate the first four larvae more quickly (voracity) than *A. iberus*. However, there was a marked reduction in the voracity of *A. iberus* males and *G. holbrooki* females exposed to algae compared to clay and controls (Fig. 4c).


a
b
c
sp
Aphanius iberus
Gambusia holbrooki
Captured larvae
Latency time
Voracity time
10
5
0
-5
40
30
20
10
0
-10
♀♀
♂♂
Clear
Clay
Algae
Turbidity

◀**Figure 4.** Mean (±SE) number of larvae consumed, time spent to capture the first larvae (latency) and time spent to capture four larvae (voracity) in the native *Aphanius iberus* and the invasive *Gambusia holbrooki* in clear and turbid waters. Models indicated statistically clear associations with the type of turbidity, species identity and fish sex, but not with turbidity intensity (from Table S5). The effects of the covariate fish weight were accounted for by plotting the residuals of linear regressions between fish weight and dependent variables instead of using raw voracity and latency times.

3.3. Experiment 3 – Does the presence of competitors or alternative prey alter the efficacy of the two fish species in mosquito biocontrol?

Results from interspecific fish feeding assays were almost concordant with those from aquaria with one fish of each species. *Gambusia holbrooki* detected prey faster and was more voracious in clear and turbid waters (Table S6). However, no significant interactive effects were found between fish species and size, implying that *G. holbrooki* captured more larvae than *A. iberus* regardless of their differences in body size (Tables 1 and S6). Interestingly, *G. holbrooki* consumed initially a larger number of mosquito larvae and reached satiety faster than *A. iberus* (Fig. 5). However, on the termination of the assay (10 min), *A. iberus* almost had eaten the same number of larvae than *G. holbrooki* (Fig. 5). Predicted number of larvae consumed by similarly sized *G. holbrooki* and *A. iberus* (0.5 g) at 10 min was only slightly higher for *G. holbrooki* than for *A. iberus* in all treatments (i.e. 1.1-fold in controls, 1.2-fold in *Chlorella* and 1.1-fold in clay).

Table 1. Statistics for the general linear mixed model ($R^2_{\beta*}$ = 0.90) examining the cumulative number of mosquito larvae consumed by *Aphanius iberus* and *Gambusia holbrooki* when there was interspecific competition. Statistical significance was reached using likelihood-ratio χ^2 tests at $P \leq 0.05$.

		Effect size		**χ^2**	**P**
Larvae captured	Intercept	-0.52±1.60		0.1	0.74
	Fish weight	7.79±2.84	*	7.5	<0.01
	Time	1.19±0.25	*	22.6	<0.001
	Time^2	-0.09±0.01	*	216.3	<0.001
	G. holbrooki	7.66±0.91	*	71.1	<0.001
	Time:Fish weight	1.92±0.42	*	20.5	<0.001
	Time:*G.holbrooki*	-0.40±0.12	*	11.7	<0.001

The % biomass of invertebrates in mesocosms before releasing the fish ranked as follows: 71 ± 5 % of Odonata, 13 ± 4% of Ephemeroptera, 7 ± 1 of Cladocera, 4 ± 1% of *C. pipiens,* 4 ± 1% of Chironomidae, 1 ± 0.2% of Copepoda, 0.5 ± 0.1% of Oligochaeta and 0.3 ± 0.1% of Ostracoda. There were not marked differences in the overall taxonomic composition of invertebrates attributed to fish (PERMANOVA, P > 0.05, Table S7). However, the reduction in the number of *C. pipiens* was evident when the two fish species occurred, in single or combination (P < 0.01, Tables 2 and S8). Tanks with *G. holbrooki* alone had less *C. pipiens* at 24 h than those with *A. iberus* or both fish species (Table 2).

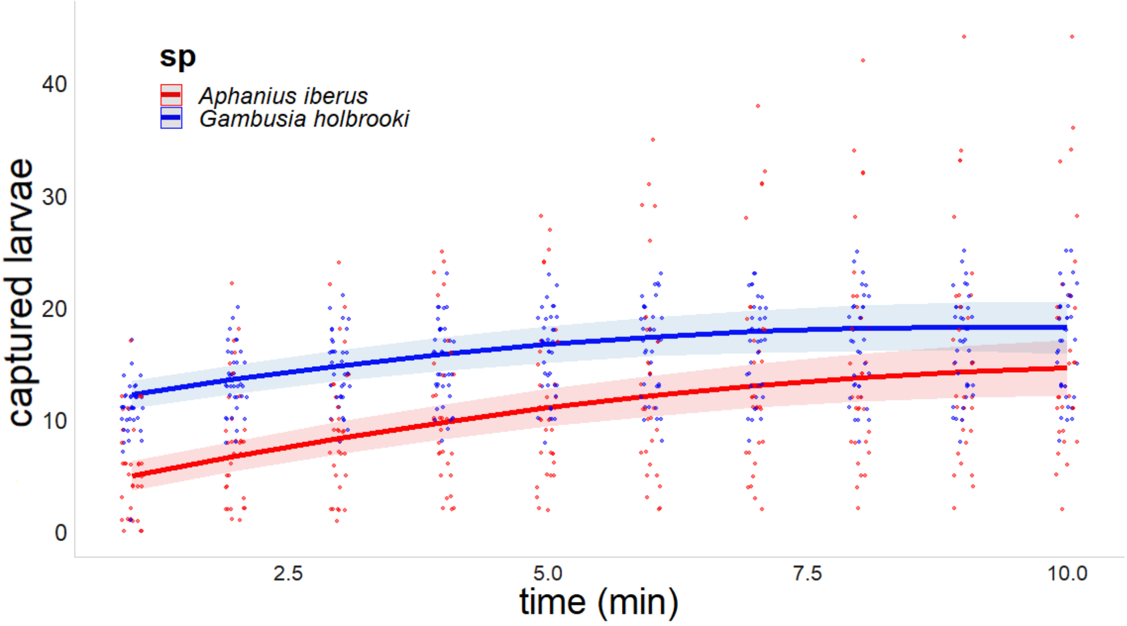


Figure 5. Mosquito larvae cumulative consumption by the native *Aphanius iberus* and the invasive *Gambusia holbrooki* over 10 minutes in the experiment assessing trophic competition among them. Fitted functions accounted for their differences in body weight and other predictors highlighted in the final model (from Table 1) as important factors to explain variation in the number of mosquitoes eaten.

4. Discussion

Our study shows that the invasive eastern mosquitofish *G. holbrooki* and the IUCN endangered Spanish toothcarp *A. iberus* prey on mosquito larvae, so that both species provide the ecosystem service of mosquito biocontrol. However, the poor knowledge of the biology and ecology of native fish at the first half of the XX century might explain why *G. holbrooki* was the preferred species (Pyke, 2008). The foraging efficacy of the two fish species was reduced in algal turbidity compared to clay turbidity, which often occur in waterbodies because of eutrophication and sediment resuspension by wind, fish, mammals and birds (Kadlec, 2009). Our findings therefore are discussed in relation to how water turbidity, one of the most profound forms of ecological change (Utne-Palm, 2002), might affect the conservation of endangered native fish and the proliferation of alien fish and mosquitoes.

Table 2. Mean (± SE) densities (individuals/L) of the different taxonomic groups occurring in the mesocosms to explore the effects of fish predation on mosquito larvae when interacting with a natural invertebrate community. The reduction in mosquito larvae abundance at 24h of having released the fish is specifically shown in bold at $P \leq 0.05$.

Treatment	**% reduction mosquito**	Non-biting midges	Odonata	Mayflies	Ostracoda	Copepoda	Cladocera	Oligochaetes
Fishless	**-9.1±47.0**	3.6±1.1	3.9±0.7	0.2±0.1	1.9±0.4	10.4±4.1	93.5±18.3	2.1±1.7
A. iberus	**-38.8±11.1**	4.1±1.2	2.9±0.9	0.1±0.0	0.9±0.9	10.7±2.7	78.9±17.1	2.9±1.5
G.holbrooki	**-70.7±2.9**	3.2±1.1	4.4±2.2	0.3±0.1	0.4±0.4	13.5±4.8	129.1±29.5	3.4±1.9
Both species	**-62.4±8.2**	4.2±1.8	3.1±0.7	1.1±0.5	1.6±0.7	6.6±1.5	83.2±31.5	3.0±1.2

The Spanish government bred *G. holbrooki* in 1921 and distributed juveniles for free among citizens alongside the leaflet ‘instructions for the use of *Gambusia* against malaria’ (Clavero del Campo, 1961). The contribution of *G. holbrooki* to human malaria eradication in Spain in 1964 is uncertain due to attendant circumstances, such as the use of insecticides and improvements in human living standards and antimalarial drugs (Clavero del Campo, 1961). However, our study suggests that the use of *G. holbrooki* was not justified because *A. iberus* provided the same ecosystem service, albeit more slowly. This probably is the case of other small native species (see Caiola et al., 2001), including endangered fish in Spain, such as the Valencia toothcarp (*Valencia hispanica*) and the three-spined stickleback (*Gasterosteus aculeatus*), all of which eat mosquito larvae (Offill and Walton, 1999). Therefore, the recommendation of mosquitofish for the control of malaria mosquitoes (*Anopheles*) and other mosquito species, including *C. pipiens*, was unnecessary (Lemasson, 1937). Public education, improved sewage treatment, and the promotion of natural enemies such as bats, swallows and native aquatic competitors in natural or man-made waterbodies are the most effective ways to alleviate mosquito issues (DuRant and Hopkins, 2008; Becker, 2010).

At 24h of having released fish into mesocosms, the abundance of mosquito larvae was reduced by 9% in fishless tanks, by 71% in *G. holbrooki* tanks and by 39% in *A. iberus* tanks. Even though fishless tanks had invertebrates that eat mosquito larvae (e.g. dragonflies, Becker, 2010), fish predation had a much stronger effect, probably because fish have a higher voracity and individual biomass and swim closer to the surface, where mosquito larvae are hanging (Clements, 1992). However, this is not to say that fish should be introduced in all waterbodies to prevent mosquitoes. Fish can prey on valuable taxa (e.g. amphibians, Pyke, 2008; but see Maceda-Veiga et al., 2019) and there are mosquito species that breed in habitats were fish cannot live (e.g. tree holes; Clements, 1992). Nonetheless, fish did not cause changes in the overall composition of invertebrates in our experimental tanks, probably because we just conducted a 24h assay to compare the predation of the fish upon mosquitoes while they were interacting with natural plankton. Major alterations in invertebrate assemblages are reported in *G. holbrooki* enclosures at our stocking density (10 ind/m^2) in the long-term (Blanco et al., 2004; Cardona, 2006). Mesocosms had invertebrates consumed by *A. iberus* and *G. holbrooki* (e.g. Chironomidae, Cladocera; Blanco et al., 2004; Alcaraz and García-Berthou, 2007), so that fish probably preferred mosquito larvae than other prey. Differences in the composition of aquatic assemblages among tanks probably would have altered mosquito egg-laying (Clements, 1992), but this question needs to be explored in further studies because we released live mosquito larvae into mesocosms.

Among human alterations affecting aquatic ecosystems, eutrophication is a prominent stressor (WWF, 2018). Our study adds to the list of impacts in literature (Utne-Palm, 2002; Shoup and Wahl, 2009; Nieman et al., 2018) that the efficacy of *A. iberus* and *G. holbrooki* in mosquito control was reduced ~ 6 – 9 %, on average, because of algal turbidity. Many studies reported that turbidity reduces foraging efficiency in fish (Horppila et al., 2004; Nieman et al., 2018), including on mosquito larvae (Asimeng and Mutinga, 1992). However, our study is a first in showing, within comparable experimental conditions, how turbidity alters the potential of two species in mosquito control by the scattering effect of suspended particles on light (Utne-Palm, 2002). Both *G. holbrooki* and *A. iberus* captured more mosquito larvae in clay than in algal suspensions, possibly because the colour of prey (dark brown) contrasted better in the pale background of red clay than in the dark green of *C. vulgaris* (see Nieman et al., 2018). Fish probably were adapted to clay turbidity because the sampling sites of fish are not eutrophied, but run-off and sediment resuspension are likely to occur (authors *pers. observ.*). Despite this, our work supports the idea that *G. holbrooki* performs well in a wide range of environmental conditions (Walton et al., 2012; Cano-Rocabayera et al., 2019b). It consumed a higher rate of mosquito larvae than *A. iberus* within a range of turbid conditions reported in the study area and in other regions around the world (Table S1; Nieman et al., 2018).

Differences in time to detect and to capture prey can be used to compare competitive abilities between native and alien species, but we dismiss the possibility of using this kind of information to justify the introduction of alien species. The establishment of alien species is a major threat to native diversity around the world (Lowe, 2000), so that the precautionary principle must be applied to any introduction (Lodge and Schrader-Frechette, 2003). *Gambusia holbrooki* had a higher voracity than *A. iberus*, in single and interacting assays, which supports that *G. holbrooki* outcompetes species of *Aphanius,* including *A. iberus* (Caiola and de Sostoa, 2005; Alcaraz et al., 2008; Ruiz-Navarro et al., 2013). Lessons learned from this misguided strategy for mosquito control should prevent repeating the same mistake (see also Lodge and Schrader-Frechette, 2003), including in some countries where health agencies still recommend the introduction of mosquitofish (El Sabaawi, 2016; NVBDCP, 2019). If the biology and ecology of native fish is not described in scientific literature, authorities could seek the expert advice of local aquarist associations because they are get used to maintain fish species from around the world (Maceda-Veiga et al., 2016).

The efficacy of *G. holbrooki* and *A. iberus* in mosquito control differed depending on the size and sex of fish individuals, which illustrates the importance of detailed studies of trophic ecology in wildlife management (Simberloff et al., 2013). While the two fish species mostly preyed on IV-instar larvae, the interaction between fish size and species identity revealed that *A. iberus* juveniles consumed more larvae than those of *G. holbrooki*. This is explained by the preference of small *A. iberus* for II- and III-instar larvae, which results in a more effective control of mosquitoes than when fish eat a few larger IV-instar. Differences in prey electivity are explained by the optimal foraging theory (Wanzenböck, 1995). Studies in conspecific species reported that *A. dispar* has a low prey size/mouth size ratio (PS/MS) compared to *G. affinis,* which affects its ability to handle bigger prey (Homski et al., 1994). Despite this limitation, our 10 min assay did not show a saturation curve for *A. iberus* in the number of mosquitoes eaten as did for *G. holbrooki* after accounting for their differences in fish size and sex. Therefore, the efficacy of *A. iberus* in mosquito control is probably higher than the values we report here.

5. Take home message: restoring the natural ecosystem service for mosquito control

Mosquito-borne diseases are the result of a complex interplay between biological, environmental and socioeconomic factors (Garchitorena et al., 2017). Alien fish introductions not only may not be the solution to mosquito issues (Lardeux, 1992; Walton et al., 2012), but can increase the list of biological invasions. *Gambusia holbrooki* is now listed as among the worst piscine invaders, and authorities should enforce eradication campaigns (Walton et al., 2012). We suggest authorities to develop a reintroduction plan for endangered invertivorous fish, such as *A. iberus*, *V. hispanica* and *G. aculeatus* in restored waterbodies, as suggested by ichthyologists in the 1930s (De Buén, 1929; Lemasson, 1937). Authorities could breed native species as did with *G. holbrooki* during the anti-malaria campaigns in Spain (Clavero del Campo, 1961) and aid in the conservation of native species while reducing the magnitude of mosquito issues in natural waters. Our study illustrates the pernicious consequences of ignoring the biology and ecology of native species by assuming that exotic peers are better candidates (de Buén, 1929; Lodge and Schrader-Frechette, 2003). Restoring aquatic ecosystems, including or not native fish, should prevail over the use of alien species in mosquito biocontrol.

Acknowledgements

We are grateful to F. X. Santaeufemia for providing *A. iberus* from the Espais Nanturals del Delta del Llobregat conservation programme. We also thank to all the staff from the aquatic animal facilities at UB - Faculty of Biology. This study was funded by the Fundació Barcelona Zoo - Ajuntament de Barcelona. SVA was financed by a Fundació Bosch i Gimpera grant and OCR by an APIF grant from the University of Barcelona.

Ethics statement

The experimental procedure was authorised by the Natural Environment and Biodiversity Division at the Catalan Department of Agriculture and Fisheries (Num. DAAM 8289). Fish capture and maintenance were approved by the Committee for an Ethical use of Experimental Animals at the University of Barcelona (Num. 193/15). Lab experimentation with endangered *A. iberus* intended for *ex situ* conservation purposes was compensated by returning to wildlife the experimental individuals and the newborn hatchlings born in the lab.

6. References

Alcaraz, C., García-Berthou, E., 2007. Food of an endangered cyprinodont (Aphanius iberus): ontogenetic diet shift and prey electivity. Environmental Biology of Fishes 78(3), 193–207. doi:10.1007/s10641-006-0018-0

Alcaraz, C., Bisazza, A., García-Berthou, E., 2007. Salinity mediates the competitive interactions between invasive mosquitofish and an endangered fish. Oecologia 155(1), 205–213. doi:10.1007/s00442-007-0899-4

Asimeng, E. J., Mutinga, M. J., 1992. Field evaluation of *Tilapia zilli* (Gervais) as a biological control agent for mosquito control. Biological Control 2(4), 317–320. doi:10.1016/1049-9644(92)90025-9

Bates, D., Maechler, M., Bolker, B., Walker, S., 2015. Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software 67(1), 1–48. doi:10.18637/jss.v067.i01

Becker, N., Petrić, D., Boase, C., Lane, J., Zgomba, M., Dahl, C., Kaiser, A., 2010. Mosquitoes and their control (2nd ed.). Heidelberg: Springer. doi:10.1007/978-3-540-92874-4

Billman, E. J., Wagner, E. J., Arndt, R. E., 2007. A comparison of mosquito consumption and prey selection between least chub (*Iotichthys phlegethontis*) and western mosquitofish (*Gambusia affinis*). Western North American Naturalist 67(1), 71–79. doi:10.3398/1527-0904(2007)67[71:acomca]2.0.co;2

Blanco, S., Romo, S., Villena, M. J., 2004. Experimental Study on the Diet of Mosquitofish (*Gambusia holbrooki*) under Different Ecological Conditions in a Shallow Lake. International Review of Hydrobiology 89(3), 250–262. doi:10.1002/iroh.200310684

Caiola, N., Vargas, M. J., de Sostoa, A., 2001. Feeding ecology of the endangered Valencia toothcarp, *Valencia hispanica* (Actinopterygii: Valenciidae). Hydrobiologia 448(1-3), 97-105. doi:10.1023/A:1017584903305

Caiola, N., de Sostoa, A., 2005. Possible reasons for the decline of two native toothcarps in the Iberian Peninsula: evidence of competition with the introduced Eastern mosquitofish. Journal of Applied Ichthyology 21(4), 358–363. doi:10.1111/j.1439-0426.2005.00684.x

Cano-Rocabayera, O., de Sostoa, A., Padrós, F., Cárdenas, L., Maceda-Veiga, A., 2019a. Ecologically relevant biomarkers reveal that chronic effects of nitrate depend on sex and life stage in the invasive fish *Gambusia holbrooki*. PLOS ONE 14(1), e0211389. doi:10.1371/journal.pone.0211389

Cano-Rocabayera, O., de Sostoa, A., Coll, L., Maceda-Veiga, A., 2019b. Managing small, highly prolific invasive aquatic species: Exploring an ecosystem approach for the eastern mosquitofish (*Gambusia holbrooki*). Science of the Total Environment 673, 594–604. doi:10.1016/j.scitotenv.2019.02.460

Cardona, L., 2006. Trophic Cascades Uncoupled in a Coastal Marsh Ecosystem. Biological Invasions 8(4), 835–842. doi:10.1007/s10530-005-0420-0

Carson, R., 1962. Silent spring. Boston, MA: Houghton Mifflin Co., pp. 400.

Casas, J. J., Sánchez-Oliver, J. S., Sanz, A., Furné, M., Trenzado, C., Juan, M., Paracuellos, M., Suárez, M. D., Fuentes, F., Gallego, I., Gil, C., Ramos-Miras, J. J., 2011. The paradox of the conservation of an endangered fish species in a Mediterranean region under agricultural intensification. Biological Conservation 144(1), 253–262. doi:10.1016/j.biocon.2010.08.023

Clavero del Campo, G., 1961. La erradicación del paludismo en España. Revista de Sanidad e Higiene Pública 35, 265–284.

Clements, A. N., 1992. The biology of mosquitoes. Volume 1: development, nutrition and reproduction. London: Chapman & Hall. pp. 509.

Davidson, N. C., 2014. How much wetland has the world lost? Long-term and recent trends in global wetland area. Marine and Freshwater Research 65(10), 934. doi:10.1071/mf14173

de Buen, F., 1929. La invasión de nuestras aguas dulces por las Gambusias (*Gambusia holbrooki* Grd.). Revista de Biología forestal y Limnología 1, 49–53.

Doadrio, I., 2002. Atlas y libro de los peces continentales de España. 2nd ed. Museo Nacional de Ciencias Naturales, Dirección General de Conservación de la Naturaleza. Madrid.

DuRant, S. E., Hopkins, W. A., 2008. Amphibian predation on larval mosquitoes. Canadian Journal of Zoology 86(10), 1159–1164. doi:10.1139/z08-097

El-Sabaawi, R. W., Frauendorf, T. C., Marques, P. S., Mackenzie, R. A., Manna, L. R., Mazzoni, R., Phillip, … Zandonà, E. (2016). Biodiversity and ecosystem risks arising from using guppies to control mosquitoes. Biology Letters, 12(10), 20160590. doi:10.1098/rsbl.2016.0590

Eritja, R., Palmer, J. R. B., Roiz, D., Sanpera-Calbet, I., Bartumeus, F., 2017. Direct Evidence of Adult *Aedes albopictus* Dispersal by Car. Scientific Reports 7(1). doi:10.1038/s41598-017-12652-5

Ford, M., 2016. Project Fartet, Barcelona (2007-2013): a case of conflicting interests. Saving Freshwater fishes and habitats. Newsletter of the IUCN SSC/WI Freshwater Fish Specialist Group. Issue 11, May 2016.

Fox, J., Weisberg, S., 2011. An R Companion to Applied Regression. 2nd edition. Sage, Thousand Oaks CA [software]. http://socserv.socsci.mcmaster.ca/jfox/Books/Companion

Fryxell, D. C., Arnett, H. A., Apgar, T. M., Kinnison, M. T., Palkovacs, E. P., 2015. Sex ratio variation shapes the ecological effects of a globally introduced freshwater fish. Proceedings of the Royal Society B: Biological Sciences 282(1817), 20151970. doi:10.1098/rspb.2015.1970

Garchitorena, A., Sokolow, S. H., Roche, B., Ngonghala, C. N., Jocque, M., Lund, A., Barry, M., Mordecai, E. A., Daily, G. C., Jones, J. H., Andrews, J. R., Bendavid, E., Luby, S. P., LaBeaud, A. D., Seetah, K., Guégan, J. F., Bonds, M. H., De Leo, G. A., 2017. Disease ecology, health and the environment: a framework to account for ecological and socio-economic drivers in the control of neglected tropical diseases. Philosophical Transactions of the Royal Society B: Biological Sciences 372(1722), 20160128. doi:10.1098/rstb.2016.0128

Herrera, C. M., 2018. Complex long-term dynamics of pollinator abundance in undisturbed Mediterranean montane habitats over two decades. Ecological Monographs 89(1), e01338. doi:10.1002/ecm.1338

Homski, D., Goren, M., Gasith, A. 1994. Comparative evaluation of the larvivorous fish *Gambusia affinis* and *Aphanius dispar* as mosquito control agents. Hydrobiologia 284(2), 137–146. doi:10.1007/bf00006885

Horppila, J., Liljendahl-Nurminen, A., Malinen, T., 2004. Effects of clay turbidity and light on the predator–prey interaction between smelts and chaoborids. Canadian Journal of Fisheries and Aquatic Sciences 61(10), 1862–1870. doi:10.1139/f04-123

Hotez, P. J., 2016. Neglected Tropical Diseases in the Anthropocene: The Cases of Zika, Ebola, and Other Infections. PLOS Neglected Tropical Diseases 10(4), e0004648. doi:10.1371/journal.pntd.0004648

Jaeger, B.C., Edwards, L.J., Das, K., Sen, P.K., 2016. An R2 statistic for fixed effects in the generalized linear mixed model. Journal of Applied Statistics 44(6), 1086–1105. https://doi.org/10.1080/02664763.2016.1193725

Kadlec, R. H., 2009. Wetlands for contaminant and wastewater treatment. In: Maltby, E., Barker, T. (Eds.), The Wetlands Handbook. Wiley-Blackwell, pp. 440–463. doi:10.1002/9781444315813.ch20

Lacey, L. A., 2007. *Bacillus thuringiensis* serovariety *israelensis* and *Bacillus sphaericus* for mosquito control. Journal of the American Mosquito Control Association 23(sp2), 133-164.

Lardeux, F. J. R., 1992. Biological control of Culicidae with the copepod *Mesocyclops aspericornis* and larvivorous fish (Poeciliidae) in a village of French Polynesia. Medical and Veterinary Entomology 6(1), 9–15. doi:10.1111/j.1365-2915.1992.tb00028.x

Lemasson, J., 1937. L’utilisation de “Gambusia affinis” et “Girardinus guppyi” pour la lutte antimalarienne. Bulletin économique de l’Indo-Chine 40(2), 328–330.

Lodge, D. M., Shrader-Frechette, K., 2003. Nonindigenous Species: Ecological Explanation, Environmental Ethics, and Public Policy. Conservation Biology 17(1), 31–37. doi:10.1046/j.1523-1739.2003.02366.x

Lowe, S., Browne, M., Boudjelas, S., De Poorter, M., 2000. 100 of the world's Worst Invasive Alien Species: A Selection from the Global Invasive Species Database (Vol. 12). Invasive Species Specialist Group, Auckland.

Maceda-Veiga, A., Domínguez-Domínguez, O., Escribano-Alacid, J., Lyons, J., 2014. The aquarium hobby: can sinners become saints in freshwater fish conservation? Fish and Fisheries 17(3), 860–874. doi:10.1111/faf.12097

Maceda-Veiga, A., Mac Nally, R., de Sostoa, A., 2019. Congruence in riverine conditions and associations between native fish and several species of amphibians in a region prone to fish invasions. Hydrobiologia. doi:10.1007/s10750-019-3945-4

Manning, P., van der Plas, F., Soliveres, S., Allan, E., Maestre, F. T., Mace, G., Whittingham, M. J., Fischer, M., 2018. Redefining ecosystem multifunctionality. Nature Ecology & Evolution 2(3), 427–436. doi:10.1038/s41559-017-0461-7

Moleón, M., Sánchez-Zapata, J. A., Selva, N., Donázar, J. A., Owen-Smith, N. (2014). Inter-specific interactions linking predation and scavenging in terrestrial vertebrate assemblages. Biological Reviews 89(4), 1042–1054. doi:10.1111/brv.12097

Nieman, C. L., Oppliger, A. L., McElwain, C. C., Gray, S. M., 2018. Visual detection thresholds in two trophically distinct fishes are compromised in algal compared to sedimentary turbidity. Conservation Physiology 6(1), coy044. doi:10.1093/conphys/coy044

NVBDCP, 2019. Guidelines on the use of larvivorous fish for vector control. National Vector Borne Disease Control Programme, Ministry oh Health & Welfare, Governement of India. Available from www.nvbdcp.gov.in (accessed February 2019).

Offill, Y. A., Walton, W. E., 1999. Comparative efficacy of the threespine stickleback (*Gasterosteus aculeatus*) and the mosquitofish (*Gambusia affinis*) for mosquito control. Journal of the American mosquito control association 15(3), 380–390.

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P. R., O’Hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., Szoecs, E., Wagner, H., 2019. Community Ecology package, R package version 2.5-4 [software]. https://CRAN.R-project.org/package=vegan

Ormerod, S. J., Dobson, M., Hildrew, A. G., Townsend, C. R., 2010. Multiple stressors in freshwater ecosystems. Freshwater Biology 55, 1–4. doi:10.1111/j.1365-2427.2009.02395.x

Paaijmans, K. P., Blanford, S., Bell, A. S., Blanford, J. I., Read, A. F., Thomas, M. B., 2010. Influence of climate on malaria transmission depends on daily temperature variation. Proceedings of the National Academy of Sciences 107(34), 15135–15139. doi:10.1073/pnas.1006422107

Pena, J. C., Domínguez, J., 1985. Contribución al conocimiento de la ictiofauna leonesa: La Gambusia (*Gambusia affinis holbrooki* Girard 1859). Tierras de León 61, 103–114.

Pyke, G. H., 2008. Plague Minnow or Mosquito Fish? A Review of the Biology and Impacts of IntroducedGambusiaSpecies. Annual Review of Ecology, Evolution, and Systematics 39(1), 171–191. doi:10.1146/annurev.ecolsys.39.110707.173451

R Core Team. 2018 Sep 20. In: R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria [software]. Available from: http://www.R-project.org/.

Ruiz-Navarro, A., Torralva, M., Oliva-Paterna, F., 2013. Trophic overlap between cohabiting populations of invasive mosquitofish and an endangered toothcarp at changing salinity conditions. Aquatic Biology 19(1), 1–11. doi:10.3354/ab00512

Shelton, J. M., Bird, M. S., Samways, M. J., Day, J. A., 2016. Non-native rainbow trout (*Oncorhynchus mykiss*) occupy a different trophic niche to native Breede River redfin (*Pseudobarbus burchelli*) which they replace in South African headwater streams. Ecology of Freshwater Fish 26(3), 484–496. doi:10.1111/eff.12293

Shoup, D. E., Wahl, D. H., 2009. The Effects of Turbidity on Prey Selection by Piscivorous Largemouth Bass. Transactions of the American Fisheries Society 138(5), 1018–1027. doi:10.1577/t09-015.1

Simberloff, D., Martin, J. L., Genovesi, P., Maris, V., Wardle, D. A., Aronson, J., Courchamp, F., Galil, B., García-Berthou, E., Pascal, M., Pyšek, P., Sousa, R., Tabacchi, E., Vilà, M., 2013. Impacts of biological invasions: what's what and the way forward. Trends in ecology & evolution 28(1), 58-66. doi:10.1016/j.tree.2012.07.013

Strauss, R. E., 1979. Reliability Estimates for Ivlev's Electivity Index, the Forage Ratio, and a Proposed Linear Index of Food Selection. Transactions of the American Fisheries Society 108(4), 344–352. doi:10.1577/1548-8659(1979)108<344:refiei>2.0.co;2

Turner, A. M., Trexler, J. C., 1997. Sampling Aquatic Invertebrates from Marshes: Evaluating the Options. Journal of the North American Benthological Society 16(3), 694–709. doi:10.2307/1468154

Tylianakis, J. M., Laliberté, E., Nielsen, A., Bascompte, J., 2010. Conservation of species interaction networks. Biological Conservation 143(10), 2270–2279. doi:10.1016/j.biocon.2009.12.004

Utne-Palm, A. C., 2002. Visual feeding of fish in a turbid environment: Physical and behavioural aspects. Marine and Freshwater Behaviour and Physiology 35(1-2), 111–128. doi:10.1080/10236240290025644

Valiente-Banuet, A., Aizen, M. A., Alcántara, J. M., Arroyo, J., Cocucci, A., Galetti, M., García, M. B., García, D., Gómez, J. M., Jordano, P., Medel, R., Navarro, L., Obeso, J. R., Oviedo, R., Ramírez, N., Rey, P. J., Traveset, A., Verdú, M., Zamora, R., 2014. Beyond species loss: the extinction of ecological interactions in a changing world. Functional Ecology 29(3), 299–307. doi:10.1111/1365-2435.12356

Walton, W. E., Henke, J. A., Why, A. M., 2012. *Gambusia affinis* (Baird & Girard) and *Gambusia holbrooki* Girard (mosquitofish). In: Francis, R. A. (Ed.), A handbook of global freshwater invasive species. Washington: Easthscan, pp. 261–273.

Wanzenböck, J., 1995. Changing handling times during feeding and consequences for prey size selection of 0+ zooplanktivorous fish. Oecologia 104(3), 372–378. doi:10.1007/bf00328373

WHO, 2018. World malaria report 2018. Geneva: World Health Organization.

WWF, 2018. Living planet report - 2018: aiming higher. Grooten, M., Almond, R. E. A. (Eds.). WWF, Gland, Switzerland.

Zuur, A.F., Ieno, E.N., Walker, N., Saveliev, A.A., Smith, G.M., 2009. Mixed Effects Models and Extensions in Ecology with R. New York: Springer, pp. 574. doi:10.1007/978-0-387-87458-6

Supplementary materials

Table S1. Turbidity values occurring in waterbodies within the distributional area of *Aphanius iberus* and *Gambusia holbrooki* in Natural Areas of Llobregat Delta. FTU: Formazin Turbidity Units.

Locality	**Turbidity (FTU)**
Can Comas pond	38.1
Cal Tet pond	38.8
Ca l'Arana pond	3.62
Bitxot pond	44.9
Can Camins marshes	22.7

Table S2. Range of densities (ind/m^2, minimum-maximum) of *Aphanius* killifish (*A. iberus* and *A. fasciatus*) and mosquitofish (*Gambusia holbrooki* and *G. affinis*) occurring in nature. Note that the size of *Aphanius* populations can be as high as those of mosquitofish, and so, their predation pressure on mosquito larvae may be similar. Median (± SE) is 18.1 ± 23.6 for *Gambusia* and 6.9 ± 18.6 for *Aphanius*.

Species	**Locality**	**Individuals / m^2**	**Reference**
Gambusia holbrooki	Australia (n=2)	0.1 – 2.45	Pen et al. 1991
	Italy (n=1)	6	Zulian et al. 1993
	Florida, USA (n=1)	60	Schaefer 1994
	Spain (n=1)	113.47	Pena y Domínguez 1985
	Italy (n=7)	1.46 – 57.66	Zulian et al. 1995
	Delta Llobregat (n=12)	0.01 – 596.9	de Sostoa et al. 2009
Gambusia affinis	worldwide? (n=1)	46	Rafatjah et al. 1975
	India (n=1)	0.05	Tabibzadeh et al.1971
	Central America (n=1)	3	Lloyd et al. 1986
	India (n=6)	28.6 – 164.2	Ravikumar et al. 2016
	USA (n=2)	0.02 – 0.099	Stefferud et al. 2011
Aphanius iberus	Murcia	108	Oliva-Paterna et al., 2009
	Catalunya (n=3)	15 – 235	Alcaraz et al. 2008
	Catalunya (n=5)	2.33 – 32.38	Badosa et al. 2007
Aphanius fasciatus	Italia (n=4)	0.11 – 1.03	Cavraro et al. 2013

Table S3. Statistics for the general linear mixed model ($R^2_{\beta*} = 0.20$) examining differences in values of the Strauss linear index, which shows the preference of *Aphanius iberus* and *Gambusia holbrooki* for the four mosquito larvae stages. Statistical significance was reached using likelihood-ratio χ^2 tests at $P \leq 0.05$.

	Effect size			χ^2	P
Strauss linear index	Intercept	-0.14±0.04	*	15.7	<0.001
	Fish weight	-0.19±0.06	*	10.6	0.001
	Larvae weight	0.26±0.03	*	66.2	<0.001
	Larvae weight^2	-0.07±0.01	*	94.7	<0.001
	Fish sp. *G. holbrooki*	-0.05±0.03		3.1	0.080
	Fish W:Larvae W	0.09±0.02	*	15.1	<0.001
	Larvae W:*G.holbrooki*	0.02±0.01	*	4.4	0.037

Table S4. Statistics for the general linear mixed model ($R^2_{\beta*} = 0.51$) examining differences in the number of mosquito larvae consumed between *Aphanius iberus* and *Gambusia holbrooki* in preference trials. Statistical significance was reached using likelihood-ratio χ^2 tests at $P \leq 0.05$.

	Effect size			χ^2	P
Total captured larvae	Intercept	2.47±0.10		-	-
	Fish sp. *G. holbrooki*	-0.37±0.15	*	6.0	0.015
	Fish weight	1.51±0.21	*	45.3	<0.001
	Fish W:*G.holbrooki*	0.70±0.31	*	5.1	0.024

Table S5. Statistics for the general linear mixed models examining differences in the number of mosquito larvae consumed ($R^2_{\beta*}$ = 0.49), latency time ($R^2_{\beta*}$ = 0.36) and voracity time ($R^2_{\beta*}$ = 0.61) between *Aphanius iberus* and *Gambusia holbrooki* in monospecific fish assays under variable turbid conditions. Statistical significance was reached using likelihood-ratio χ^2 tests at $P \leq 0.05$. Dependent variables were log-transformed.

	Effect size			χ^2	**P**
Total captured larvae	Intercept	1.71±0.15		-	-
	Fish sp. *G. holbrooki*	0.62±0.18	*	12.2	<0.001
	Males	-0.23±0.11	*	4.6	0.03
	Turbidity_clay	-0.03±0.10		11.7	<0.01
	Turbidity_*Chlorella*	-0.28±0.10	*	“	“
	Fish weight	2.81±0.29	*	96.0	<0.001
	G.holbrooki:Males	0.27±0.16		2.9	0.09
	G.holbrooki:Fish W.	-1.40±0.36	*	15.1	<0.001
Latency time	Intercept	1.92±0.22		-	-
	Fish sp. *G. holbrooki*	-0.46±0.35		1.7	0.19
	Males	0.48±0.35		1.9	0.16
	Turbidity_clay	-0.32±0.32		1.1	0.57
	Turbidity_*Chlorella*	-0.06±0.33		“	“
	Turbidity_clay:Male	-0.54±0.50		1.5	0.48
	Turbidity_*Chl*:Male	-0.04±0.50		“	“
	Turb_clay:*G.holbrooki*	-0.11±0.46		12.8	<0.01
	Turb_*Chl*:*G.holbrooki*	1.43±0.49	*	“	“
	G.holbrooki:Males	-0.80±0.50		2.6	0.11
	G.holbr:M:Turb_clay	0.92±0.69		8.7	0.01
	G.holbr:M:Turb_*Chl*	-1.15±0.72	*	“	“
Voracity time	Intercept	3.49±0.22		-	-
	Fish sp. *G. holbrooki*	-0.87±0.28	*	9.6	<0.01
	Males	-0.56±0.37		2.2	0.13
	Turbidity_Clay	-0.23±0.27		0.7	0.70
	Turbidity_*Chlorella*	-0.09±0.25		“	“
	Fish Weight	-1.40±0.29	*	23.8	<0.001
	Turbidity_clay:Males	0.68±0.49		11.7	<0.01
	Turbidity_*Chl*:Males	1.55±0.46	*	“	“
	Turb_clay:*G.holbrooki*	-0.15±0.38		5.2	0.08
	Turb_*Chl*:*G.holbrooki*	0.68±0.40		“	“
	G.holbrooki:Males	0.20±0.46		0.2	0.67
	G.holbr:M:Turb_clay	0.02±0.62		13.6	<0.01
	G.holbr:M:Turb_*Chl*	-1.87±0.62	*	“	“

Table S6. Statistics for the general linear mixed models examining differences in the number of mosquito larvae consumed ($R^2_{\beta*} = 0.38$), latency time ($R^2_{\beta*} = 0.38$) and voracity time ($R^2_{\beta*} = 0.56$) between *Aphanius iberus* and *Gambusia holbrooki* when there was interspecific interactions under variable turbid conditions. Statistical significance was reached using likelihood-ratio χ^2 tests at $P \leq 0.05$. Variables were log-transformed in **.

	Effect size			χ^2	P
Total captured larvae	Intercept	2.00±2.98		0.5	0.50
	Fish sp. *G. holbrooki*	13.11±5.33	*	6.1	0.01
	Clay turbidity	-1.24±1.82		3.8	0.15
	Chlorella turbidity	-3.73±1.96		3.8	0.15
	Fish weight	31.95±5.18	*	38.1	<0.001
	Fish W:*G.holbrooki*	-23.50±12.40		3.6	0.06
Latency time **	Intercept	2.63±0.16		-	-
	Fish sp. *G. holbrooki*	-1.29±0.21	*	38.3	<0.001
Voracity time **	Intercept	3.35±0.21		-	-
	Fish sp. *G. holbrooki*	-1.67±0.23	*	52.6	<0.001

Table S7. Permutational analysis of variance using the Bray-Curtis distance to examine the effects of the different types of fish predation on the relative abundance of all invertebrate taxa in mesocosms at 24h of having released the fish. Statistical significance was reached using *F* tests at $P \leq 0.05$.

		F Model	R^2	P
Bray-Curtis distance	*A. iberus*	0.52	0.04	0.64
	G. holbrooki	0.15	0.01	0.89
	A. iberus:*G. holbrooki*	1.26	0.09	0.34
	Residuals		0.86	
	Total		1.00	

Table S8. Statistics for the general linear model ($R^2_{\beta*} = 0.85$) showing the specific effect that the introduction of fish had on the abundance of mosquito larvae in mesocosms. Statistical significance was reached using likelihood-ratio χ^2 tests at $P \leq 0.05$.

	Effect size			χ^2	P
Larvae abundance	Intercept	0.92±0.08		-	-
	A. iberus	-0.44±0.11	*	16.3	<0.001
	G. holbrooki	-0.63±0.11	*	33.1	<0.001
	Log (t_0)	0.24±0.04	*	29.9	<0.001
	A.iberus:*G.holbrooki*	0.49±0.15	*	10.5	<0.01

DISCUSSIÓ GENERAL

"Yo soy yo y mis circunstancias"
José Ortega y Gasset

Aquesta tesi doctoral pretén profunditzar sobre el coneixement dels efectes directes i indirectes de l'eutrofització per nitrat en *G. holbrooki*, una de les espècies amb més poblacions invasores arreu del món (Meffe i Snelson, 1989; Lowe, 2000). Els resultats més rellevants dels seus cinc capítols s'exposen de forma integrada a continuació com a recordatori pel lector abans de la discussió final.

Gambusia holbrooki és una espècie amb un rang de tolerància ampli a condicions ambientals diverses (Homski et al., 1994; Pyke, 2005), i els capítols I, II, i III confirmen que la contaminació per nitrat, en el rang de concentracions testat aquí (< 10 – 425 mg/l), no li suposa cap problema greu. No obstant, la reducció en condició física i biomassa de *G. holbrooki* del capítol I, probablement deguda a la pèrdua d'abundància de preses en els tancs amb nitrat podria tenir altres causes afegides, com un efecte tòxic directe del nitrat sobre el peix (capítol II), canvis en els trets d'història de vida de *G. holbrooki* a conseqüència de l'eutrofització (capítol III) o canvis en la terbolesa de l'aigua que dificultarien la captura de preses (capítol V). En els experiments de toxicitat directa les afectacions només van aparèixer en mascles i juvenils, i de caràcter lleu (capítol II), fet que si es troba generalitzat en altres condicions ambientals, podria explicar la proporció de sexes esbiaixada cap a les femelles tant en poblacions invasores i natives (Hildebrand, 1927; Vargas, 1993). En canvi, sí que es trobà una variabilitat interpoblacional en els trets d'història de vida, però no degut a la disponibilitat de nutrients sinó regulada sobretot per la densitat de *G. holbrooki* en cada localitat (capítol III), patró vist també en altres pecílids (Bassar et al., 2012). Això, juntament amb la ràtio de sexes dominada per les femelles i la baixa prevalença de paràsits externs (capítol IV), fa difícil el control d'una espècie tan prolífica, més enllà de l'eliminació directa de poblacions senceres (ex. Ruiz-Navarro et al., 2013b). En cas que algunes d'aquestes actuacions fossin viables localment, caldria assegurar-se que amb la desaparició de *G. holbrooki,* la pròpia comunitat nativa mantindria el servei ecosistèmic de control de mosquits. Les condicions tèrboles d'eutrofització dificultaren la captura de mosquits a *G. holbrooki* i *A. iberus*, i aquest, si bé mostrà una inferioritat competitiva, assolí junt amb la comunitat d'invertebrats per si sola una reducció progressiva de l'abundància de mosquits.

Integrant la representativitat ecològica i nivells d'organització biològica creixents

És important destacar l'èmfasi que s'ha intentat establir en aquest treball per incloure tant estudis de camp observacionals, els resultats dels quals són el més propers possibles a la realitat que es vol estudiar, però a la vegada complementat amb estudis experimentals en mesocosmos i en laboratori. Per altra banda, també s'ha intentat integrar diferents nivells d'organització biològica, una aproximació recomanable seguint la premissa que les pertorbacions afecten des de l'individu fins a l'ecosistema, amb un temps de resposta creixent en efectes crònics (Colin et al., 2016). L'especialització creixent en la ciència tendeix a aïllar el focus d'estudi, és a dir, la histopatologia i la fisiologia situen l'individu com a unitat d'estudi, mentre l'ecologia tendeix a obviar els canvis individuals i es fixa més en els fluxos de matèria i energia. Amb l'aproximació aïllada d'un histopatòleg es diria que les altes concentracions de nitrat han tingut un efecte irrellevant, contradient les conclusions d'un ecòleg, que diria que el nitrat ha fet davallar la condició física, la biomassa total de *G. holbrooki* i la seva efectivitat en la depredació de mosquits, derivats d'un efecte indirecte.

Els experiments de laboratori permeten una màxima replicabilitat i aïllament d'efectes concrets que permeten una explicació mecanicista de l'efecte testat. Podem inferir que les relacions vistes i validades estadísticament es deuen a una relació de causa-efecte, és a dir, tenen una validesa interna, però desconeixem el grau de validesa externa, que és el grau de confiança en els resultats obtinguts per poder generalitzar-ho a altres animals, poblacions o àrees geogràfiques (Campbell i Stanley, 1963). Concretament, les inferències de la depredació sobre mosquits en condicions de laboratori podrien capgirar-se en condicions naturals on les larves de mosquit són un component més de la comunitat d'invertebrats. Els resultats de laboratori i mesocosmos, però, foren coincidents. Possibles factors que amenaçarien la validesa externa en els experiments de laboratori descrits aquí serien per exemple la manca d'interaccions mascle – femella perquè estaven separats per sexes, que representarien un cost energètic extra, o la impossibilitat per als individus experimentals d'escapar-se de condicions ambientals desfavorables, reacció que en determinades circumstàncies sí que poden fer els animals en condicions naturals. És per això que la realització de tests experimentals i estudis observacionals amb escales temporals i espacials diferents permet reduir lleugerament la incertesa sobre la validesa externa a la vegada que obtenir un màxim coneixement mecanicista.

La contaminació per nutrients: un problema persistent 200 anys després

Malgrat hi ha un creixent interès els últims anys en estudiar els efectes produïts per nous contaminants emergents com els microplàstics i els productes farmacèutics o de cosmètica, el vell problema de la contaminació per nutrients és un dels principals problemes de degradació dels ecosistemes aquàtics en l'actualitat (MEA, 2005). Els primers problemes de contaminació orgànica greus aparegueren amb la Revolució Industrial a la Gran Bretanya, quan el creixement poblacional, la creixent urbanització i l'emissió directa dels efluents de rebuig sobrepassaren la capacitat natural de depuració dels rius. El cas més cèlebre és la històrica contaminació del Tàmesi al voltant de Londres, el qual perdé la població de peixos durant bona part dels segles XIX i XX, i fins que no es va millorar el tractament d'aigües residuals amb la inclusió del tractament secundari no el pogueren recolonitzar (Mason, 1996). L'entrada excessiva de nutrients segueix sent un problema actual en àrees on no hi ha un tractament d'aigües residuals, per exemple certes badies del llac Titicaca (Fontúrbel, 2008) o en àrees d'alta activitat agrícola com Osona i la Bretanya (Grup de Defensa del Ter, 2019; Ratmaya et al., 2019).

En concret, aquesta tesi ha examinat la toxicitat directa d'un dels nutrients més poc estudiats, el nitrat, i a més pretén ser un dels estudis més complets sobre les conseqüències de l'exposició crònica del nitrat sobre un peix: la gambúsia *G. holbrooki*.

La interacció de la gambúsia amb els nitrats

Els primers dos capítols de la tesi han tractat sobre l'efecte directe dels nitrats sobre la fisiologia de *G. holbrooki* i la interacció d'aquesta amb l'ecosistema recipient. La bateria de biomarcadors a diferents nivells de complexitat biològica suggereixen una ràpida aclimatació de *G. holbrooki* a una pujada de la concentració de nitrats, però aquesta és més efectiva en femelles. Aquesta variabilitat entre sexes i estadis de vida tenen una rellevància ecològica no gens menyspreable. Moltes poblacions silvestres de pecílids tenen una distribució de sexes esbiaixada cap a les femelles (Snelson, 1989), incloent-hi *G. holbrooki* (Hildebrand, 1927; Vargas, 1993). Tenint en compte que en néixer, la ràtio de sexes en pecílids rarament es desvia de 1:1 (Snelson, 1989), aquest biaix seria atribuïble a una predisposició a patir una major mortalitat. Hi ha un grapat d'estudis en gambúsia on els mascles sucumbiren més ràpid a diferents estressos ambientals, incloent-hi temperatures altes, superpoblació, inanició i hipòxia (Krumholz, 1948; Winkler, 1975; Cech et al., 1985). No obstant, la manca de mortalitat en l'experiment del capítol II, juntament amb el que s'exposarà a continuació suggereixen que la toxicitat del nitrat tindria unes conseqüències gairebé nul·les a nivell poblacional. L'experiment de laboratori no va estudiar els efectes sobre la reproducció perquè és un dels aspectes de la toxicitat del nitrat més ben documentats (Edwards et al, 2006; Edwards i Guillette, 2007). No obstant, en cas d'aparèixer alteracions en la població invasora estudiada, hom creu que tindrien poc efecte ja que un sol mascle dominant pot fertilitzar diverses femelles (Constantz, 1989). A més, les femelles emmagatzemen esperma viable durant mesos, per la qual cosa una mortalitat sobtada de la majoria de mascles per una exposició aguda a amoni o nitrat podria ser contrarestada amb el naixement de nous mascles a la següent cohort. Per últim, una alimentació alterada en mascles a conseqüència del nitrat tindria un efecte menor a nivell de l'ecosistema. Experimentalment, Fryxell et al. (2015) demostraren que les poblacions de *G. holbrooki* amb major proporció de femelles produïen fortes cascades tròfiques en comparació amb poblacions amb major proporció de mascles, causant en el primer cas reduccions substancials en l'abundància de zooplàncton i l'augment del fitoplàncton, la productivitat, el pH i la temperatura. Aquesta major susceptibilitat en mascles es troba estesa en molts animals, inclús en els propis humans, on l'esperança de vida és major en dones. Les femelles guanyarien aptitud incrementant la seva longevitat, que dóna la possibilitat de tenir més camades, mentre els mascles guanyarien aptitud incrementant les possibilitats de reproduir-se (Bateman, 1948; Rolff, 2002). Per tal de complir-se això les femelles haurien d'invertir més recursos en immunitat (Rolff, 2002; Zuk, 2009) que proporciona, entre altres, resistència a la pol·lució ambiental.

L'experiment de laboratori, a més d'ajudar a comprendre millor perquè les poblacions de *G. holbrooki* poden estar esbiaixades cap a les femelles, també permeté estudiar els mecanismes fisiològics de la tolerància al nitrat de *G. holbrooki*. En concret, s'esmenta breument en la discussió del capítol II, que els juvenils reduïren l'acumulació de reserves energètiques, sense implicacions en el seu creixement, cosa que apunta a una prioritat en assignar l'energia assimilada en créixer ràpidament (Fig. 1). L'etapa de juvenil és la que té més risc de depredació, i donat que les xarxes tròfiques estan fortament estructurades per mida (fitoplàncton < zooplàncton < zooplanctívors < depredadors), el fet de prioritzar el creixement i assolir una determinada talla pot ser una mesura de defensa per escapar de depredadors (Martin et al., 2017). Aquesta plasticitat per assignar l'energia assimilada a un o altre compartiment segons les condicions ambientals suposa un avantatge en la colonització d'un ambient nou (Handelsman et al., 2013).

Els resultats dels capítols I i III contradiuen, en part, les conclusions d'un treball sobre els efectes del nitrat en el rang natiu de l'espècie (Edwards et al, 2006; Edwards i Guillette, 2007). Aquests autors trobaren una correlació entre les concentracions creixents de nitrat (1 – 22 mg/l) i un augment en el nombre de femelles no reproductores, un descens en el pes mitjà embrionari, així com un descens en el nombre d'espermatozous i un augment en l'índex gonadosomàtic masculí. En el capítol I el nombre de juvenils augmentà de forma marginal en els tancs amb nitrat, mentre en el capítol III els nutrients totals, incloent el nitrat, en feu pujar el pes mitjà embrionari. Els valors màxims de nitrat en les llacunes de Florida eren 2 i 19 vegades inferiors als de les localitats del present treball i de l'experiment en mesocosmos, respectivament, amb la qual cosa és possible que hi hagi una variabilitat interpoblacional en quant a la tolerància als nitrats, accentuada a més per la condició de població nativa o introduïda tal com s'explicarà a continuació.

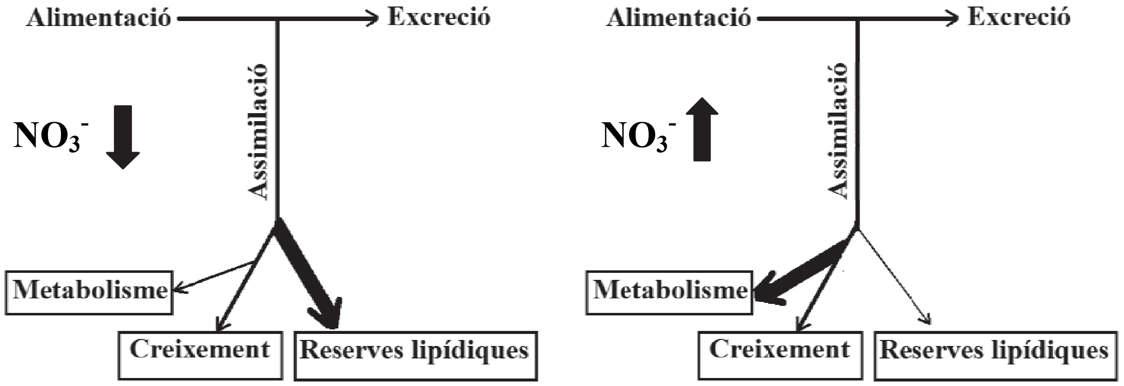


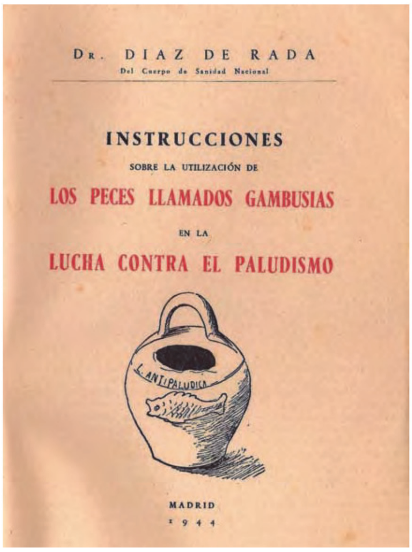
Figura 1. Esquema representatiu de la hipòtesi sobre la distribució d'energia assimilada entre els costos de metabolisme, l'energia destinada al creixement i la destinada a la reserva de lípids. A l'esquerra es mostra la situació d'un hipotètic individu en un entorn baix en nitrats, mentre la situació de la dreta representa el mateix individu en un entorn contaminat.

Les translocacions de peixos com a “filtres” de selecció artificial

Els primers enviaments de peixos per a control biològic, sobretot *G. holbrooki*, *G. affinis* i *Poecilia reticulata* (Peters, 1859) es feren a principis de segle XX, sovint en viatges transoceànics que duraven setmanes, com el del primer enviament de *G. holbrooki* a Europa des dels Estats Units el 1921. Sembla que l’enviament anava dirigit a Itàlia, però davant l’alta mortalitat soferta es va decidir aturar el viatge i aclimatar-les prèviament a Espanya (Nájera, 1944; Navarro-García, 2013). Del lot inicial de 200 exemplars només en van arribar 40, és a dir, patiren una mortalitat del 80%, i abans de l’alliberament al medi natural s’aclimataren en els aquaris de l’Institut Espanyol d’Oceanografia a Madrid (Oliver, 2017). El transport es feu mitjançant bidons de llauna, amb oxigenació insuficient i sense filtració, amb canvis d’aigua parcial per evitar el deteriorament excessiu de l’aigua. Tot i això, i tenint en compte que els peixos segurament estaven confinats en grans densitats, és probable que s’acumulessin grans quantitats de compostos nitrogenats i la concentració d’oxigen fos baixa, cosa que explica la gran mortalitat, també expressada per altres autors en transports més curts (36 – 41 % en un transport de 48 h, Geiser, 1921). Actualment les condicions de transport en el comerç de peixos ornamentals han millorat però se segueixen donant mortalitats que seleccionarien certs trets que proferirien una major resistència (Wabnitz et al., 2003; Deacon et al., 2011).

És probable que les últimes 12 gambúsies supervivents alliberades a Talayuela (Cáceres) fossin les més resistents, o amb més capacitat d’aclimatació a condicions d’anòxia o eutrofització. Aquest efecte fundador, que efectivament reduí la variabilitat genètica de les poblacions introduïdes (Vera et al., 2016; Díez-del-Molino, 2018), podria no tenir efectes deleteris sobre la seva capacitat d’invasió i d’adaptació a noves condicions ambientals si reté una certa variabilitat genètica. La mort de peixos durant el transport hauria actuat com una mena de selecció artificial inicial d’individus més resistents a la toxicitat de compostos nitrogenats, reforçada per les posteriors translocacions entre localitats, realitzades en recipients petits (Calero et al., 2014; Fig. 2) on l’acumulació d’amoníac també seria altament probable. Una exposició repetida a condicions tòxiques pot induir una aclimatació i processos microevolutius que ajudarien a sobreviure en aquestes condicions poc favorables (Reid et al., 2016).

Aquesta selecció artificial ocorreguda durant el transport podria haver afectat també la resistència a infeccions per paràsits. Tal com explica Stephenson (2014), en un experiment amb *P. reticulata* de Trinidad es van introduir quatre noves poblacions des d'una zona amb forta depredació i parasitisme cap a una altra amb baixa depredació i lliure de paràsits. Els resultats no foren els esperats, és a dir, no s'observà una resistència als ectoparàsits monogenis menor en les poblacions introduïdes, sinó que foren més resistents, fet atribuït a la selecció artificial durant el transport i aclimatació dels individus translocats. No se sap si la baixa prevalença de paràsits externs observat en el capítol IV es deu a la falta de paràsits específics en àrees fora del seu rang natiu o per una major resistència de les poblacions invasores, però el fet és que sembla que els proporciona un avantatge en el seu èxit invasor.


DR. DIAZ DE RADA
Del Cuerpo de Sanidad Nacional

INSTRUCCIONES
SOBRE LA UTILIZACIÓN DE
LOS PECES LLAMADOS GAMBUSIAS
EN LA
LUCHA CONTRA EL PALUDISMO

MADRID
1944

Figura 2. Fulletó informatiu distribuït per la Direcció General de Sanitat del Ministeri de Governació per difondre l'ús de *G. holbrooki* com a agents de control biològic.

Possibles interaccions amb altres compostos iònics

Un altre aspecte que podria haver afectat els resultats sobre la toxicitat del nitrat en els capítols I i II és que la font de nitrat fou la sal nitrat de sodi ($NaNO_3$). Tot i que un dels fertilitzants més utilitzats en agroecosistemes actualment és el nitrat d'amoni (NH_4NO_3), aquest hauria pogut emmascarar els resultats obtinguts experimentalment, ja que estaríem abocant amoni a la vegada que nitrat. Tradicionalment, la mescla de nitrat de potassi (KNO_3) i nitrat de sodi era el principal fertilitzant nitrogenat mineral utilitzat, el salnitre o nitrat de Xile (Fig. 3). No obstant, per facilitar els càlculs estequiomètrics en tots els experiments aquí descrits s'ha utilitzat nitrat de sodi exclusivament. La presència de sodi, però, té l'efecte col·lateral d'augmentar la salinitat del medi, fet que no és neutre a nivell fisiològic ni ecològic i mereix ser discutit.

Figura 3. Característic anunci del nitrat de Xile a Espanya.

Certament, en alguns estudis de toxicologia on s'avalua l'efecte d'algun ió, a més dels controls amb aigua de xarxa sense clor, també inclogueren un control avaluant l'efecte de l'ió amb càrrega oposada que forma l'enllaç iònic, el sodi en aquest cas. L'estudi de Pereira et al. (2017) amb peix zebra *Danio rerio* (Hamilton, 1822) inclogué aquest control de sodi, afegint-hi NaCl per obtenir una conductivitat similar al seu tractament més alt amb nitrat, la qual cosa donà una salinitat equivalent a ~2 ‰. En aquestes rèpliques l'avaluació histopatològica de brànquia, pell, ronyó i intestí no fou diferent que el d'aigua control. L'únic òrgan amb una afectació moderada fou el fetge, però no afectà ni la supervivència ni el creixement tal com passà al capítol II. *Gambusia holbrooki* és un peix amb una major tolerància a la salinitat que *D. rerio* (Uliano et al., 2010), de fet, individus de *G. holbrooki* exposats a salinitats de fins el 20 ‰ tingueren variacions mínimes en la taxa de metabolisme en repòs (Akin i Neill, 2003). A més, l'estudi d'aclimatació a salinitats creixents de Nordlie i Mirandi (1996) tingué una supervivència del 95 % a 15 ‰ de salinitat. Amb aquests estudis previs podem descartar un efecte significatiu del sodi sobre *G. holbrooki* en les concentracions utilitzades en el present treball: al final de l'experiment en mesocosmos, els dipòsits tenien una salinitat estimada a partir de la mesura de conductivitat de 0,80 ‰ per als controls i 1,18 ‰ per als de nitrat. En el laboratori la salinitat dels aquaris a 250 mg/l de nitrat s'estimà inferior a 0,85 ‰.

Respecte a l'experiment en mesocosmos, la pujada de salinitat per influència del sodi sí que pogué haver influït moderadament en la composició d'invertebrats. Molts gèneres d'oligoquets, els pòlips d'*Hydra* i certs grups de crustacis com cladòcers, copèpodes i ostracodes d'aigua dolça són molt sensibles a salinitats superiors a 1 ‰ (Tesh et al., 1990; Hart et al., 1991). És possible doncs que el sodi hagi tingut un paper determinant en la pèrdua de biomassa d'invertebrats en el capítol I, tot i que els nitrats són també particularment tòxics per a *Hydra* spp. (Tesh et al., 1990). Una altra de les diferències entre les condicions experimentals del capítol I i II és que en el de laboratori hi havia instal·lat a cada aquari un sistema de filtració biològica que reduïa la concentració d'amoni i nitrits al mínim. En els mesocosmos del capítol I es va voler avaluar l'efecte del nitrat en condicions més naturals on el fitoplàncton, els macròfits i la desnitrificació fossin els responsables del manteniment de la qualitat de l'aigua. Les anàlisis en cromatografia líquida indicaren una concentració alta de nitrits (7,4 mg/l NO_2^- en les rèpliques de nitrat, <0,1 mg/l en els controls, de mitjana), amb la qual cosa hi va haver una reducció de nitrat a nitrit que no fou compensada per la nitrificació. Anàlogament, en sediments d'estuari s'ha mesurat una major taxa de reducció de NO_3^- a NH_4^+ que taxa de desnitrificació a N_2, i un increment notable d'aquest patró després d'enriquir el medi amb nitrat (An i Gardner, 2002). Aquestes concentracions de nitrit poden ser efectivament tòxiques, tot i que les concentracions de clorur superiors a 300 mg/l presents als tancs actuen com a agent protector (Lewis i Morris, 1986). A més, l'aclimatació a la metahemoglobinèmia a llarg termini fa que les diferències entre les toxicitats cròniques i agudes siguin mínimes (Lewis i Morris, 1986). La toxicitat de nitrit en invertebrats pot ser altament variable, des de grups més sensibles com els efemeròpters i gammàrids fins als més tolerants, oligoquets i mol·luscs (Soucek i Dickinson, 2012). La presència de nitrits evidentment aporta soroll en l'avaluació de l'efecte net dels nitrats sobre la comunitat, i no es descarta que minvés la condició física i creixement dels peixos exposats, però es guanyà en versemblança dels resultats. De fet en l'estudi de camp del capítol III, en les mostres on el nitrat sortí alt, gran part de vegades aquest anà acompanyat de valors de nitrit per sobre la mitjana (fins a quatre vegades > 2 mg/l NO_2^-). En qualsevol cas, aquests processos d'oxidació i reducció de compostos de nitrogen podrien ocórrer de manera natural si es produís un vessament d'aigües amb una elevada concentració de nitrogen, tal i com succeeix després d'un vessament d'aigua de piscifactoria on practiquin la recirculació (ex. 1036 mg/l N_{total}, Summerfelt et al., 1999), després d'una recàrrega d'aigua superficial utilitzant aigua de pou contaminada per nitrat (ex. 400 mg/l NO_3^-, García-Galán, 2010) o, també, l'escorrentia de zones amb intensificació agrícola (ex. > 50 mg/l NO_3^-, Comissió Europea, 2018). De fet, aquestes entrades de nutrients porten de retruc d'altres ions; així doncs es creu que el disseny experimental utilitzat té un realisme ecològic raonable.

L'efecte del nitrat sobre els determinants biòtics de la invasió de *G. holbrooki*

Aquesta tesi demostra que *G. holbrooki* té una elevada tolerància al nitrat i a les condicions d'eutrofització, si bé sembla que la *fitness* dels individus exposats a eutrofització per excés de nitrat seria menor (Capítol I – menys biomassa i condició física). A més, es constatà que el nitrat produí un empobriment de les comunitats de productors secundaris. No obstant, la pregunta que deixa oberta la present tesi és com l'eutrofització pot afectar la resistència biòtica a la invasió de *G. holbrooki* exercida per la presència d'espècies natives. En els experiments de mesocosmos no es va considerar l'exposició d'*A. iberus*, potser l'espècie més estudiada on es demostri el desplaçament degut a *G. holbrooki*, per dos motius principals. Un, l'ètic, *A. iberus* és una espècie catalogada en perill d'extinció segons la IUCN i en el Catàleg Nacional d'Espècies Amenaçades (Real Decreto 139/2011; Crivelli, 2006). En segon lloc, la disponibilitat de rèpliques, ja que es va preferir que hi haguessin almenys cinc rèpliques per condició experimental que no pas tres, augmentant així la potència estadística. Tot i això, els experiments de depredació de mosquits al capítol V, juntament amb els resultats dels capítols I i II, permeten fer certes inferències al respecte. No es disposen de comparacions directes de toxicitat a nitrat en espècies desplaçades per *G. holbrooki*, ni la tolerància a la metahemoglobina, però en l'estudi de Homski et al. (1994) compararen l'alimentació i mortalitat en condicions d'hipòxia del ciprinodòntid *Aphanius dispar* (Rüppell, 1829) i el pecílid *G. affinis*. Les dues espècies toleraren concentracions tan baixes com 1 mg/l, però fins i tot *A. dispar* fou més resistent a < 1 mg/l. Quan els exposaren dues setmanes a les aigües eutròfiques del riu Alexander (Israel), la mortalitat d'*A. dispar* fou lleugerament menor. En el nostre estudi *G. holbrooki* ha mostrat gran capacitat d'aclimatació a l'entrada excessiva de nitrogen, però a partir dels resultats del present treball i els de Homski et al. (1994) tenim indicis per pensar que el fet de tenir aigües eutrofitzades amb condicions d'hipòxia i amb una terbolesa major no haurien de suposar un avantatge directe per a *G. holbrooki*. Possiblement sigui més important la dessalinització de les llacunes del delta del Llobregat degut als règims hídrics dependents del regadiu, per exemple la Ricarda i la Murtra, que perjudiquen clarament *A. iberus*, més eurihalí. No obstant, en experiments en mesocosmos de competència tròfica entre *A. iberus*, el samaruc *Valencia hispanica* (Valenciennes, 1846) i *G. holbrooki* les dues espècies natives presentaren un percentatge major de digestius buits, i *G. holbrooki* a més tingué un consum absolut de preses i una voracitat majors (Caiola i de Sostoa, 2005). Ruiz-Navarro et al. (2013a) trobaren resultats similars en poblacions naturals de la zona del mar Menor (Múrcia). El percentatge de preses capturades en aquari fou també major en *G. holbrooki* respecte a *Aphanius fasciatus* (Valenciennes, 1821), *A. iberus* i *V. hispanica*, així com l'agressivitat interespecífica fou sempre major en l'espècie no nativa, fins i tot en aigües de salinitat moderada (Rincón et al., 2002; Alcaraz et al., 2008). De fet, hi ha observacions sobre la mortalitat soferta per l'espècie nativa quan es posa en un mateix aquari amb *G. holbrooki* (De Buén, 1929; Moreno i Morán, 1981). Hi ha per tant un clar avantatge competitiu de l'espècie nord-americana força generalitzat en un rang ampli de condicions ambientals.

A més dels peixos, hom no pot oblidar altres vertebrats, com els amfibis, els rèptils o les aus que podrien exercir resistència biòtica. En relació a les últimes, al capítol III s'observà una reducció en la mida dels embrions i dels mascles a abundàncies creixents de depredadors potencials, correlació demostrada experimentalment en *P. reticulata* de Trinidad, un pecílid molt utilitzat com a model evolutiu (Reznick i Endler, 1982; Stearns, 1992). En relació als rèptils, si bé hi ha *Natrix maura* (*Linnaeus*,1758) per la zona, no es va disposar de prous dades quantitatives per incloure-ho als models. I en relació als amfibis, poden exercir competència tròfica o en el cas dels anurs depredació directa sobre juvenils de *G. holbrooki* (McCoid i Fritts, 1980). Tots aquests organismes, però, patirien les conseqüències de la toxicitat directa del nitrat com així ho demostren els treballs de Camargo et al. (2005) i Hickey i Martin (2009). A més, alguns d'aquests depredadors potencials depreden visualment (Barlow, 1998), per tant, la terbolesa de l'aigua dificultaria la captura de preses. Tanmateix, conèixer com afavorir el control *top-down* en les poblacions invasores de *G. holbrooki* en els ambients degradats, on normalment habita, seria de gran interès en la lluita biològica.

Per acabar, convé destacar en aquest apartat de resistència biòtica la competència intraespecífica, que com es veié al capítol III és un dels factors més importants que afecten el cicle reproductor de *G. holbrooki*. Està força demostrat l'efecte negatiu de la densitat sobre el creixement, el nombre de descendents i fins i tot els costos energètics (Marchand i Boisclair, 1998; Smith i Sargent, 2006); tot i així, si *G. holbrooki* manté una capacitat competitiva major que les espècies de l'ecosistema receptor el seu potencial invasor es veuria poc afectat.

Altres possibles mecanismes favorables a la invasió de *G. holbrooki*

Nordlie (2014) apunta la possibilitat que les poblacions de distribució reduïda, per exemple en illes petites o estanys i maresmes aïllades, tinguin unes taxes metabòliques inferiors que les d'illes grans o continentals, fenomen vist també en ocells i quiròpters (McNab, 2012). Resta com a incògnita si *G. holbrooki*, d'àmplia distribució, té una taxa metabòlica superior a les espècies endèmiques ibèriques amb una àrea de distribució més reduïda i disseminada. Però amb les dades de captura de mosquits del capítol V i la resta de treballs bibliogràfics podem intuir que així és. Com a norma general, els individus amb una taxa metabòlica superior tindran un requeriment més gran d'aliments i una agressivitat incrementada (Lahti et al., 2002; Killen et al., 2011). Com s'ha vist al capítol I, l'excés de nutrients i productivitat primària ha portat a una pèrdua de biomassa i riquesa d'invertebrats, fet també descrit en rius i llacs somers quan se supera un cert llindar (Dodson et al., 2000; Romo et al., 2004; Pereira et al., 2016). Una menor disponibilitat de preses facilitaria la invasió d'una espècie amb uns requeriments metabòlics superiors i una superioritat competitiva evident? L'autor suggereix que espècies amb una superioritat competitiva, en condicions d'escassetat d'aliments obtindran majoritàriament els pocs recursos que queden, excloent les menys competitives.

Les espècies que tinguin també una major capacitat de convertir els recursos en reproducció assoliran una densitat major en l'ecosistema, atresorant la majoria de recursos energètics i espacials en detriment dels competidors (Fig. 4). És difícil, però, de mesurar el potencial reproductor, ja que requereix mesurar la productivitat de la població. Una estimació grollera en seria l'índex gonadosomàtic: en un estudi fet al delta de l'Ebre, es comparà l'índex gonadosomàtic d'individus d'*A. iberus* i *G. holbrooki* capturats a la mateixa llacuna i per tant amb una disponibilitat de recursos semblant (Vargas, 1993). Tant els mascles com femelles de *G. holbrooki* presentaren índexs gonadosomàtics superiors a *A. iberus*, de gairebé el doble en el mes de màxima activitat reproductora. No obstant, el fet de ser ovovivípara li dóna a *G. holbrooki* una protecció a les cries que podria explicar en part el seu èxit reproductor. En el treball de Homski et al. (1994) 50 exemplars de *G. affinis* i *A. dispar* cadascun es van escapar de la gàbia experimental per una pujada del nivell de l'aigua i al cap de sis mesos la població de *G. affinis* s'havia reproduït satisfactòriament fins a atènyer grans densitats, mentre *A. dispar* fracassà en reproduir-se. No són les mateixes espècies que les de la península ibèrica, però serveixen per inferir els potencials mecanismes de superioritat competitiva. El fet és que tal com es mostra a la taula S2 del capítol V, les densitats de *Gambusia* spp. cercades en la bibliografia foren lleugerament majors que les d'*Aphanius* spp, donant suport al patró d'abundàncies d'espècies natives – introduïdes a nivell global (Hansen et al., 2013). En aquesta metanàlisi la diferència entre les mitjanes d'abundància fou de 2,7 vegades superior per a les poblacions introduïdes, una diferència molt semblant a l'exposada a la taula S2.

Tot i que la biologia d'invasions ha girat en gran part entorn de la proposta d'ecosistemes desequilibrats d'Elton, la teoria de selecció natural de Darwin ja havia establert un marc teòric per explicar certs patrons d'invasions biològiques en les quals la superioritat competitiva es feia evident:

> *La selecció natural actua per competència, adapta els habitants de cada regió només en relació amb el grau de perfeccionament dels seus associats. [...] No ens hauríem de sorprendre que els habitants de qualsevol regió, encara que suposéssim que estan especialment creats i adaptats per a aquest hàbitat, es trobessin batuts i suplantats per altres organismes produïts naturalment en un altre territori.*
>
> *C. Darwin, 1859*

Zones amb una alta diversitat genètica serien per tant més propenses a ser fonts d'organismes invasors, mentre zones amb menor diversitat filogenètica serien més propenses a ser envaïdes, el que es coneix com a hipòtesi del desequilibri evolutiu (Fridley i Sax, 2014). Aquest patró s'ha comprovat per exemple en l'asimetria entre el nombre d'espècies intercanviades entre el mar Roig i el mar Mediterrani, sent aquest últim el major receptor.

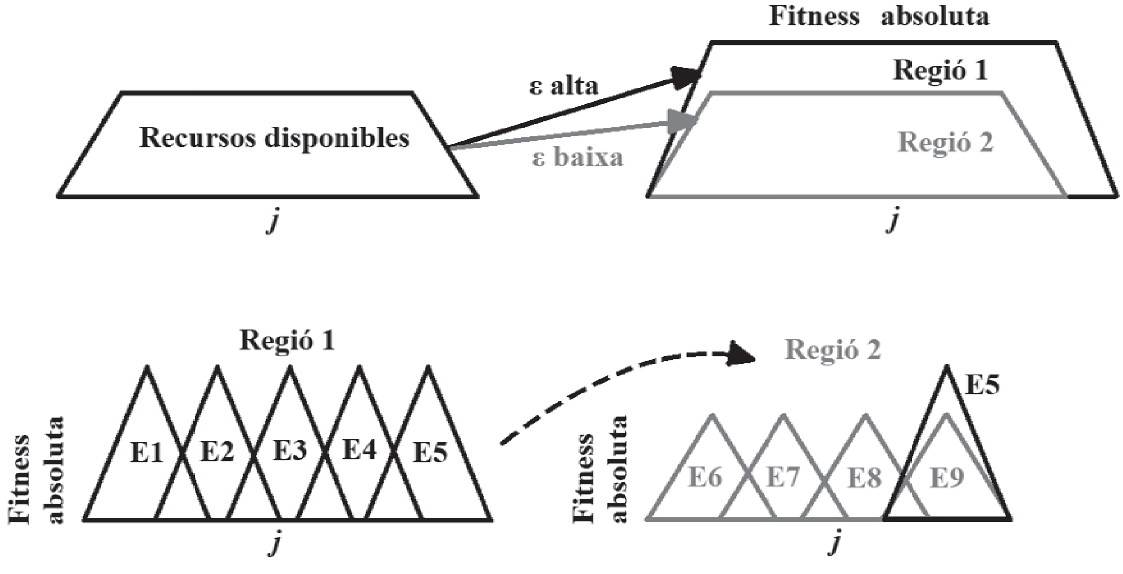


Figura 4. El potencial invasor varia entre regions, com a resultat d'aptituds o *fitness* absolutes diferents. La variació genètica (mida de població, àrea) i la intensitat competitiva (nombre de competidors) podrien provocar desequilibris evolutius, manifestats en el seu potencial reproductor (ε) variable entre regions. Així, les espècies (E) de la regió 1 tenen un ε major que les de la regió 2, és a dir, en un gradient ambiental (*j*), la capacitat de convertir els recursos en descendència és major, i n'augmenta la *fitness* absoluta. Quan per acció humana s'introdueix una espècie de la regió 1 (E5) a la regió 2, aquesta és competitivament superior i pot suplantar espècies de la regió 2 que utilitzin un ventall de recursos semblant. Adaptat de Fridley i Sax (2014).

La gestió de les poblacions de *G. holbrooki* invasores a l'àrea d'estudi

L'autor creu que aquesta tesi ha contribuït al coneixement mecanicista dels efectes de la contaminació per nitrats i la invasió per *G. holbrooki*, però també pretén contribuir a la conservació de l'àrea d'estudi. Tot sovint aquests missatges de gestió regional queden difuminats als articles internacionals i la tesi és un bon fórum per a fer-ne esment. La gestió de les invasions requereix un coneixement de les característiques biològiques amb l'objectiu de predir-ne el potencial invasor (Simberloff, 2003). El fet que la pròpia densitat de *G. holbrooki* sigui el factor més determinant en regular la seva estratègia reproductiva en fa molt difícil la reducció efectiva de les poblacions, ja que poden recuperar en poc temps les seves abundàncies prèvies si l'extirpació no és completa, fenomen conegut com a "efecte rebot" i que s'ha descrit també en espècies subjectes a caça com els senglars i castors (Servanty et al., 2011; Parker i Rosell, 2014). Aquesta influència tan alta de la densitat intraespecífica sobre els trets d'història de vida s'ha descrit també en *P. reticulata* de Trinidad. Es comprovà que modificant experimentalment les densitats de *P. reticulata* en cadascun dels pous naturals i en mesocosmos els peixos responien incrementant o disminuint la supervivència, el creixement, el nombre d'embrions i l'activitat reproductora per adequar la taxa de creixement poblacional a les noves condicions imposades (Bassar et al., 2012).

Per tant, les accions més efectives serien les actuacions repetides per tal d'eradicar la població (Ruiz-Navarro et al., 2013b). Ara bé, en un hàbitat degradat i en presència de vàries espècies invasores com la tortuga de Florida *Trachemys scripta elegans* (Wied, 1839) i el cranc americà *Procambarus clarkii* (Girard, 1852) en el delta del Llobregat, la simple eradicació de *G. holbrooki*, sense afrontar de forma integrada la resta de problemes, no portarà a una recuperació ecològica de l'ecosistema (Prior et al., 2018). En determinades situacions la recuperació ecològica és molt ràpida, com en el cas de l'eliminació de la carpa europea *Cyprinus carpio* (Linnaeus, 1758) de les llacunes de Medina i Zóñar a Andalusia. En pocs mesos es restaurà la cobertura de macròfits i la comunitat d'aus aquàtiques original (Maceda-Veiga et al., 2017). Cal tenir en compte, però, les diferències entre intentar eliminar la població en un espai petit i confinat, on un simple assecatge de la bassa podria ser suficient, o gran i amb múltiples metapoblacions. Fins i tot, en basses aïllades es podria valorar la possibilitat de no intervenir en la població si no suposa una font de nous propàguls, és a dir, si no és d'accés públic.

En el cas de la zona mostrejada del delta del Llobregat, hi ha un nombre gran de poblacions estables i d'altres temporals que colonitzen nous hàbitats i desapareixen seguint el cicle estacional hidrològic, en el cas per exemple de la localitat L1 a la Ricarda, o poblacions situades en canals que es troben subjectes als calendaris de rec. Per tal de comprovar l'estat de les 18 localitats del capítol III es tornaren a visitar el maig de 2019 ex professo, quatre anys després de fer el mostreig. Donades les limitacions de format exigides en una tesi per publicacions, hom ha decidit incloure aquests resultats en aquest apartat per ser considerats d'interès per millorar la gestió d'aquesta espècie exòtica. El fet que en vuit de les divuit localitats els peixos hagin desaparegut definitiva o de forma intermitent en el període 2015 – 2019 (Taula A3), ja sigui per acció humana o per dinàmiques hidrològiques, indica que la viabilitat d'algunes poblacions és altament estocàstica. En aquests casos seria recomanable fer un inventari previ de les poblacions estables i les que tenen una major capacitat de dispersió, per tal d'evitar accions de control innecessàries en localitats on possiblement desapareixerien a curt termini. Per exemple, si s'agrupen en clústers les diferents localitats del capítol III segons trets d'història de vida es poden diferenciar aquelles en què els peixos tingueren un esforç més gran en reproducció i en la producció de propàguls (clúster 1), de les localitats on els peixos invertiren més recursos en el manteniment somàtic i el creixement en femelles (clúster 2). En canvi, el clúster 3 es caracteritzà per un creixement menor en l'època de mostreig, segurament perquè ja havien assolit l'asímptota de creixement (Fig. A1 i Taula A4). Aquest agrupament segons tipologies, suggerit com a simple exemple que caldria perfeccionar i desenvolupar a major escala, permetria al gestor localitzar les basses o canals que continguin individus amb un fenotip que afavoreixi altes fecunditats, fet que alertaria sobre una possible major taxa de dispersió en cas que es fes alguna translocació amb individus originaris d'aquestes basses. Això permetria focalitzar les mesures de control en localitats concretes, alhora que optimitzar la gestió de recursos en la lluita biològica de les invasions.

Tot i que en aquesta tesi no s'han pogut trobar factors ambientals que permetin controlar el creixement poblacional de *G. holbrooki*, la gestió de les invasions mitjançant aquesta aproximació ecosistèmica pot tenir resultats satisfactoris en casos concrets, sobretot en distribucions a gran escala en què l'eradicació és impossible, molt cara i potencialment contraproduent (Zavaleta et al., 2001; Carroll, 2011). Per exemple, en espais oberts o si la vegetació és abundant l'ús de rotenona ha suposat un fracàs en l'eliminació de pecílids (Rayner i Creese, 2006). En canvi, si es tenen en compte altres accions, com la restauració de les espècies natives per incrementar la competència amb la invasora i afavorir-ne la coexistència, o restaurar un balanç de nutrients desequilibrat que estigués afavorint una determinada espècie en detriment de les natives, la recuperació pot ser gradual i en alguns casos reeixida (Hulme, 2006; Carroll, 2011).

Aphanius iberus, el fartet atlàntic *Aphanius baeticus* (Doadrio, Carmona & Fernández-Delgado, 2002) i altres peixos de la mateixa posició tròfica com *V. hispanica*, i l'espinós, *Gasterosteus aculeatus* (Linnaeus, 1758), han patit una regressió paulatina en les seves distribucions naturals. *Aphanius iberus* fou descrit com a molt abundant en àrees com el delta del Llobregat (Maluquer, 1916) i l'Albufera de València (Steindachner, 1865), zones on actualment *G. holbrooki* es troba àmpliament distribuïda. Realment, discernir a posteriori si *G. holbrooki* fou un oportunista que aprofità la degradació dels hàbitats originals o fou un agent actiu de canvi en les comunitats biòtiques és difícil de dir, inclús amb els resultats d'aquesta tesi, ja que les dos pertorbacions es van donar generalment al mateix temps. La gran transformació de la zona humida del Llobregat s'esdevingué durant el primer terç del segle XX, amb l'augment de la població (entre 1920 i 1936 es passà de prop de seixanta mil habitants a més de noranta-cinc mil, amb unes instal·lacions de clavegueram i depuració inexistents), el canvi a un règim agrícola de regadiu, la dessecació de zones humides i la construcció de l'aeroport el 1916 (Montijano i Lucena, 2013). Paral·lelament, el 1921 *G. holbrooki* s'introduí a Europa, concretament a la província de Cáceres i s'anà distribuint sobretot en les províncies amb major incidència del paludisme, i el 1942 ja tenim constància d'una distribució semblant a l'actual (Lozano, 1946; Fig. 5). La creació de vivers en diverses províncies facilità la dispersió gratuïta promoguda per diferents institucions administratives de Sanitat, per les confederacions hidrogràfiques i posteriorment per l'Institut Nacional de Colonització franquista (Navarro-García, 2013). Aquesta evidència clara de l'estreta relació entre la distribució endèmica històrica del paludisme i la de *G. holbrooki* actual és potser la prova fefaent que per sobre dels factors abiòtics com la temperatura, la salinitat, la contaminació per nitrats o la degradació de l'hàbitat, la dispersió per acció humana és el principal factor facilitador de la invasió de *G. holbrooki*. El flux genètic entre diferents poblacions no connectades, descrit en diferents poblacions empordaneses i neozelandeses és una evidència que les translocacions per acció humana són freqüents i contribueixen en gran mesura a la seva expansió (Purcell i Stockwell, 2014; Díez-del-Molino, 2016). Seguint aquestes polítiques de distribució gratuïta de *G. holbrooki* per part dels Instituts Provincials d'Higiene i els Dispensaris Antipalúdics al segle passat (Calero et al., 2014), l'autor proposa la creació d'un viver d'*A. iberus*, com el que gestionava el Consorci per a la Protecció dels Espais Naturals del delta del Llobregat, i que ara continua per mitjà de la Fundació Barcelona Zoo, per promocionar-ne la seva reintroducció en basses de ciutadans o entitats interessades en participar de la conservació d'una espècie amenaçada. La remesa d'*A. iberus* s'hauria de fer sempre amb un control oficial per saber el destí de les reintroduccions i evitar la introgressió a d'altres poblacions que en farien perdre la diversitat de genotips locals.

Per acabar, i sense restar-li importància, cal recordar que la prevenció de noves introduccions o translocacions i una bona campanya informativa ciutadana sobre l'alliberament d'animals domèstics són elements essencials. En un moment de màxim volum de passatgers i mercaderies i amb la progressiva pujada de temperatures és possible que certes malalties transmeses per mosquits ampliïn el seu rang de distribució, apareguin brots epidèmics com els dels virus Zika i Chikungunya a Amèrica del Sud i el Carib, o reapareguin casos endèmics de malalties que ja havien estat eradicades localment (Costello et al., 2009; Azevedo-Santos et al., 2016). Aquesta situació pot comportar noves introduccions de peixos com a arma de control biològic en nous països, sovint impulsada per les mateixes administracions. Actualment s'estan realitzant noves introduccions de *P. reticulata* per combatre l'expansió del dengue al Brasil i Pakistan (El-Sabaawi et al., 2016), o *G. affinis* a l'Índia (NVBDCP, 2019). Fins i tot en una recerca ràpida en una pàgina de compravenda per Internet a Espanya apareixen particulars que venen i envien individus de *G. holbrooki* en línia. No obstant, la millora de les condicions sanitàries, de la salubritat de les aigües circumdants, evitar l'acumulació d'aigua en petits volums d'aigua estancats i la millora dels tractaments són mesures més efectives per evitar la proliferació de mosquits (Azevedo-Santos et al., 2016). Tot i que no se sap encara del cert si la presència de peixos larvífags contribueixen en gran mesura a la reducció de la incidència d'aquestes malalties (Walshe et al., 2017), si es vol utilitzar peixos com a agents de control biològic hauria de ser sempre mitjançant les espècies natives de cada regió (Courtenay i Meffe, 1989; Rupp, 1996).

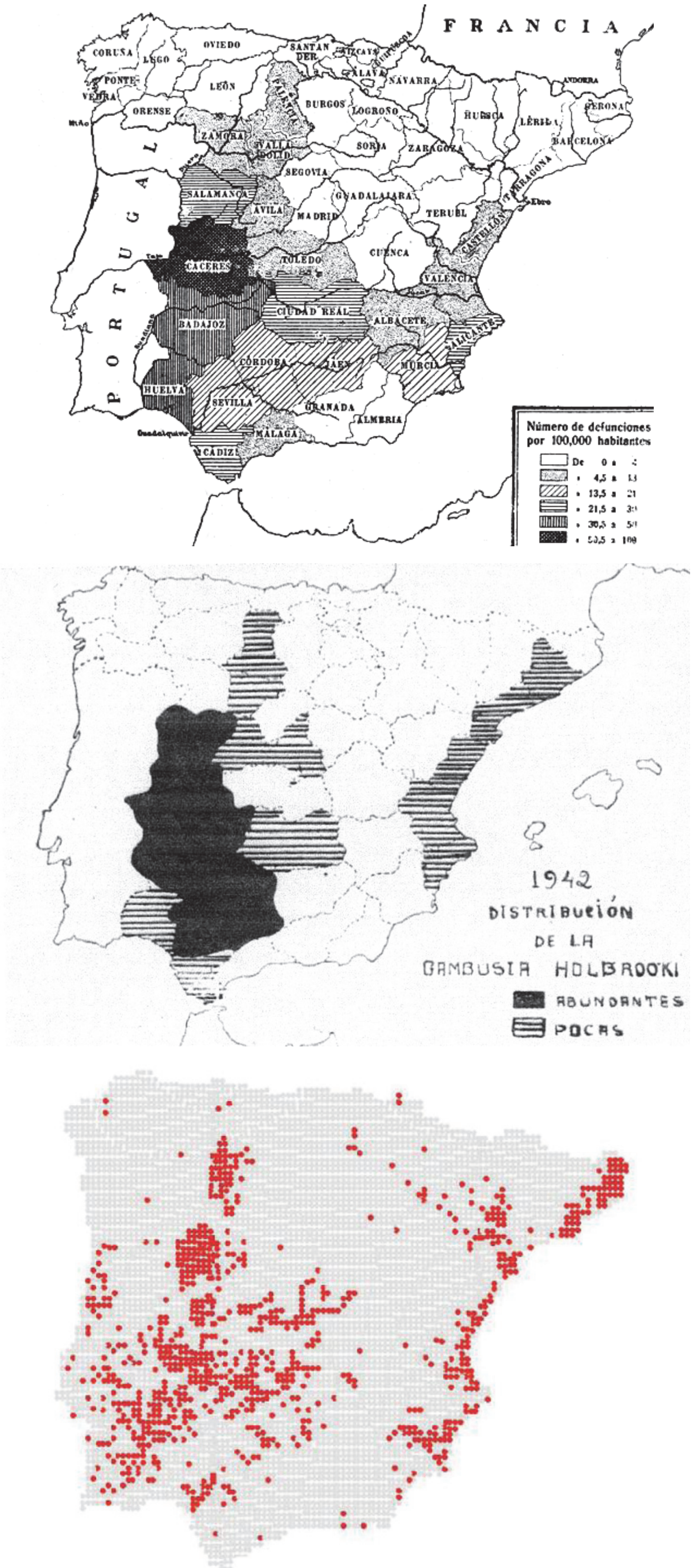


Figura 5. a) Defuncions causades per les febres palúdiques a l'Espanya peninsular durant l'any 1921. b) Distribució de *Gambusia holbrooki* a Espanya l'any 1942. c) Mapa de registres de distribució actual de *G. holbrooki* a data de 2015. Fonts: Pittaluga, 1923; Lozano, 1946; Murphy et al., 2015.

CONCLUSIONS

"A love of nature keeps no factories busy"
Aldous Huxley, Brave New World

1. L'experiment en mesocosmos mostrà que els efectes de la introducció de *G. holbrooki* sobre la comunitat biòtica i els processos ecològics foren comparables en presència o absència de la fertilització per nitrats, produint canvis en l'estequiometria del material en descomposició i la producció primària, però d'una magnitud menor en comparació als canvis provocats pels nitrats i la successió temporal.

2. El nitrat reduí la biomassa però no l'abundància de *G. holbrooki*, probablement degut a la menor disponibilitat de preses, un efecte tòxic directe del nitrat o a la plasticitat en la reproducció que faci variar el nombre i mida de la descendència.

3. L'experiment de laboratori confirmà que l'exposició crònica a nitrat afecta a la capacitat de *G. holbrooki* de capturar preses i d'emmagatzemar energia, sense canviar la condició física. Aquests efectes s'observaren en juvenils i mascles, però no en femelles.

4. Aquesta tolerància podria explicar que la proporció de sexes de les poblacions de *G. holbrooki* estiguin, en general, esbiaixades a favor de les femelles, tot i que aquesta tesi no estudià els efectes directes de la contaminació sobre la reproducció de *G. holbrooki*.

5. L'estudi dels condicionants ambientals que determinen l'èxit invasor de *G. holbrooki* oferiren poques esperances a una estratègia de gestió basada en canvis en la qualitat de l'aigua o de l'hàbitat físic en basses. No obstant, afavorir la presència de depredadors natius podria ajudar al control quan l'eradicació directa no fos possible.

6. La pròpia densitat de *G. holbrooki* pot limitar el seu creixement poblacional, però s'albira una gran plasticitat en els trets d'història de vida. Les infeccions parasitàries semblen jugar un paper poc important en les poblacions introduïdes, tot i que la presència de molts individus típicament s'associï a una major possibilitat de contagis.

7. La introducció de *G. holbrooki* no sembla justificada per al control de mosquits donat que una espècie de mida similar i amenaçada, el fartet (*Aphanius iberus*), mostrà una

efectivitat semblant, però més lenta, en condicions d'eutrofització o d'aigües netes. De fet, una comunitat d'invertebrats natius per si sola pot exercir aquest control biològic.

8. La terbolesa derivada de l'eutrofització no donà superioritat competitiva a *A. iberus*, qui, en totes les condicions experimentals, fou superat en velocitat de captura de preses per *G. holbrooki*, considerada la principal espècie responsable de la seva regressió.

9. Aquesta tesi confirma, proveint d'evidència experimental en eutrofització, que la *G. holbrooki* és una espècie amb una àmplia tolerància a les condicions ambientals.

10. Coneguda la dificultat de gestionar les poblacions invasores de *G. holbrooki*, és prioritari desenvolupar campanyes d'educació ambiental per evitar-ne més introduccions i que posin en valor serveis ecosistèmics com el control de mosquits que subministren les espècies natives.

BIBLIOGRAFIA

Adelman, I. R., Kusilek, L. I., Koehle, J., & Hess, J. (2009). Acute and chronic toxicity of ammonia, nitrite, and nitrate to the endangered Topeka shiner (*Notropis topeka*) and fathead minnows (*Pimephales promelas*). Environmental Toxicology and Chemistry, 28(10), 2216.

Agència Catalana de l'Aigua. (2014). In: Catalan Water Agency. Interactive applications [Internet]. Disponible a: http://aca-web.gencat.cat/sdim21/seleccioXarxes.do.

Akin, S., & Neill, W. H. (2003). Routine metabolism of mosquitofish (*Gambusia affinis*) at three different salinities. Texas Journal of Science, 55(3), 255-262.

Alcaraz, C., & García-Berthou, E. (2007). Life history variation of invasive mosquitofish (*Gambusia holbrooki*) along a salinity gradient. Biological Conservation, 139(1-2), 83–92.

Alcaraz, C., Bisazza, A., & García-Berthou, E. (2007). Salinity mediates the competitive interactions between invasive mosquitofish and an endangered fish. Oecologia, 155(1), 205–213.

An, S., & Gardner, W. (2002). Dissimilatory nitrate reduction to ammonium (DNRA) as a nitrogen link, versus denitrification as a sink in a shallow estuary (Laguna Madre/Baffin Bay, Texas). Marine Ecology Progress Series, 237, 41–50.

Aranda, C., Eritja, R., & Roiz, D. (2006). First record and establishment of the mosquito *Aedes albopictus* in Spain. Medical and Veterinary Entomology, 20(1), 150–152.

Arthington, A. H., & Lloyd, L. N. (1989). Introduced poeciliids in Australia and New Zealand. In: Meffe, G. K., & Snelson, F. F. (eds.). Ecology and evolution of livebearing fishes (Poeciliidae), p. 333-348.

Azevedo-Santos, V. M., Vitule, J. R. S., Pelicice, F. M., García-Berthou, E., & Simberloff, D. (2016). Nonnative Fish to Control *Aedes* Mosquitoes: A Controversial, Harmful Tool. BioScience, 67(1), 84–90.

Baker, J., Waights, V., 1993. The effect of sodium nitrate on the growth and survival of toad tadpoles (*Bufo bufo*) in the laboratory. Herpetological Journal, 3, 147–148

Barlow, L. A. (1998). The biology of amphibian taste. In: Heatwole, H., Dawley, E. (eds.). Amphibian biology, Volume 3: Sensory perception, pp. 743-782.

Bassar, R. D., Lopez-Sepulcre, A., Reznick, D. N., & Travis, J. (2013). Experimental Evidence for Density-Dependent Regulation and Selection on Trinidadian Guppy Life Histories. The American Naturalist, 181(1), 25–38.

Bateman, A. J. (1948). Intra-sexual selection in *Drosophila*. Heredity, 2(3), 349–368.

Benejam, L., Alcaraz, C., Sasal, P., Simon-Levert, G., & García-Berthou, E. (2008). Life history and parasites of the invasive mosquitofish (*Gambusia holbrooki*) along a latitudinal gradient. Biological Invasions, 11(10), 2265–2277.

Bonada, N., Prat, N., Resh, V. H., & Statzner, B. (2006). Developments in aquatic insect biomonitoring: a comparative analysis of recent approaches. Annual Review of Entomology, 51(1), 495–523.

Caiola, N., & Sostoa, A. (2005). Possible reasons for the decline of two native toothcarps in the Iberian Peninsula: evidence of competition with the introduced Eastern mosquitofish. Journal of Applied Ichthyology, 21(4), 358–363.

Calero Bernal, R., Sánchez Murillo, J. M., Alarcón-Elbal, P. M., Madeira de Carvalho, L. M., Crespo Martín, J. M., Peral Pacheco, D., & Calero-Carretero, R. (2014). In: El caso de Extremadura y la introducción de *Gambusia holbrooki* (Girard, 1859) como agente de control biológico [Internet]. Disponible a: http://www.colvet.es/node/389

Camargo, J. A., Alonso, A., & Salamanca, A. (2005). Nitrate toxicity to aquatic animals: a review with new data for freshwater invertebrates. Chemosphere, 58(9), 1255–1267.

Camargo, J. A., & Alonso, Á. (2006). Ecological and toxicological effects of inorganic nitrogen pollution in aquatic ecosystems: A global assessment. Environment International, 32(6), 831–849.

Campbell, D. T., & Stanley, J. C. (1963). Experimental and quasi-experimental designs for research. In: Gage, N. L. (ed.). Handbook of research on teaching, Houghton Mifflin Co, Boston.

Carroll, S. P. (2011). Conciliation biology: the eco-evolutionary management of permanently invaded biotic systems. Evolutionary Applications, 4(2), 184–199.

Caut, S., Angulo, E., & Courchamp, F. (2008). Avoiding surprise effects on Surprise Island: alien species control in a multitrophic level perspective. Biological Invasions, 11(7), 1689–1703.

Cech, J. J., Massingill, M. J., Vondracek, B., & Linden, A. L. (1985). Respiratory metabolism of mosquitofish, *Gambusia affinis*: effects of temperature, dissolved oxygen, and sex difference. Environmental Biology of Fishes, 13(4), 297–307.

Chen, X. (2012). Distribution patterns of invasive alien species in Alabama, USA. Management of Biological Invasions, 3(1), 25–36.

Chervinski, J. (1983). Salinity tolerance of the mosquitofish, *Gambusia affinis* (Baird & Girard). Journal of Fish Biology, 22, 9–11.

Chiron, F., Shirley, S., & Kark, S. (2009). Human-related processes drive the richness of exotic birds in Europe. Proceedings of the Royal Society B: Biological Sciences, 276(1654), 47–53.

Colin, N., Porte, C., Fernandes, D., Barata, C., Padrós, F., Carrassón, M., … & Maceda-Veiga, A. (2016). Ecological relevance of biomarkers in monitoring studies of macro-invertebrates and fish in Mediterranean rivers. Science of The Total Environment, 540, 307–323.

Comissió Europea. (2018). Report from the Commission to the Council and the European Parliament on the implementation of Council Directive 91/676/EEC concerning the protection of waters against pollution caused by nitrates from agricultural sources based on Member State reports for the period 2012-2015. 1-102.

Constantz, G. D. (1989). Reproductive biology of poeciliid fishes. In: Meffe, G. K., & Snelson, F. F. (eds.). Ecology and evolution of livebearing fishes (Poeciliidae), p. 33-50.

Costello, A., Abbas, M., Allen, A., Ball, S., Bell, S., Bellamy, R., ... & Lee, M. (2009). Managing the health effects of climate change: lancet and University College London Institute for Global Health Commission. The Lancet, 373(9676), 1693-1733.

Côté, I. M., Vinyoles, D., Reynolds, J. D., Doadrio, I., & Perdices, A. (1999). Potential impacts of gravel extraction on Spanish populations of river blennies *Salaria fluviatilis* (Pisces, Blenniidae). Biological Conservation, 87(3), 359–367.

Courtenay, W. R., & Meffe, G. K. (1989). Small fishes in strange places: a review of introduced poeciliids. In: Meffe, G. K., & Snelson, F. F. (eds.). Ecology and evolution of livebearing fishes (Poeciliidae), p. 319-332.

Crivelli, A.J. 2006. In: *Aphanius iberus*. The IUCN Red List of Threatened Species 2006 [Internet]. Disponible a: http://dx.doi.org/10.2305/IUCN.UK.2006.RLTS.T1846A8299534.en

Darwin, C. (1859). On the Origin of Species by Means of Natural Selection Or the Preservation of Favoured Races in the Struggle for Life. John Murray, London.

Deacon, A. E., Ramnarine, I. W., & Magurran, A. E. (2011). How Reproductive Ecology Contributes to the Spread of a Globally Invasive Fish. PLoS ONE, 6(9), e24416.

De Buén, F., & De Buén, S. (1922). Adaptación en España de la Gambusia affinis. Archivos del Instituto Nacional de Higiene de Alfonso XIII, 13(1), 142-146.

De Buén, F. (1929). La invasion de nuestras aguas dulces por las Gambusias (*Gambusia holbrooki* Grd.). Revista de biología forestal y limnología, 1(1), 49-53.

Díaz, S., Settele, J., Brondízio, E. (2019). Summary for policymakers of the global assessment report on biodiversity and ecosystem services of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services. Advanced unedited version, 6 May 2019.

Díez-del-Molino, D., Araguas, R.-M., Vera, M., Vidal, O., Sanz, N., & García-Marín, J.-L. (2016). Temporal genetic dynamics among mosquitofish (*Gambusia holbrooki*) populations in invaded watersheds. Biological Invasions, 18(3), 841–855.

Díez-del-Molino, D., García-Berthou, E., Araguas, R. M., Alcaraz, C., Vidal, O., Sanz, N., & García-Marín, J. L. (2018). Effects of water pollution and river fragmentation on population genetic structure of invasive mosquitofish. Science of The Total Environment, 637-638, 1372–1382.

Didham, R. K., Tylianakis, J. M., Hutchison, M. A., Ewers, R. M., & Gemmell, N. J. (2005). Are invasive species the drivers of ecological change? Trends in Ecology & Evolution, 20(9), 470–474.

Dodson, S. I., Arnott, S. E., & Cottingham, K. L. (2000). The Relationship in Lake Communities between Primary Productivity and Species Richness. Ecology, 81(10), 2662-2679.

Edwards, T. M., Miller, H. D., & Guillette, L. J. (2006). Water Quality Influences Reproduction in Female Mosquitofish (*Gambusia holbrooki*) from Eight Florida Springs. Environmental Health Perspectives, 114(Suppl 1), 69–75.

Edwards, T. M., & Guillette, L. J. (2007). Reproductive characteristics of male mosquitofish (Gambusia holbrooki) from nitrate-contaminated springs in Florida. Aquatic Toxicology, 85(1), 40–47.

Elton, C. S. (1958). The Ecology of Invasions by Animals and Plants. Methuen.

El-Sabaawi, R. W., Frauendorf, T. C., Marques, P. S., Mackenzie, R. A., Manna, L. R., Mazzoni, R., … & Zandonà, E. (2016). Biodiversity and ecosystem risks arising from using guppies to control mosquitoes. Biology Letters, 12(10), 20160590.

European Commission. Council Directive 91/676/EEC of 12 December 1991 concerning the protection of waters against pollution caused by nitrates from agricultural sources. Official Journal of the European Communities 1991; 375, 1–8.

Fontúrbel, F. E. (2008). Contaminación ambiental y cultural en el lago Titikaka: estadoactual y perspectivas. Publicaciones integrales, Fundación Emege, La Paz.

Fridley, J. D., Stachowicz, J. J., Naeem, S., Sax, D. F., Seabloom, E. W., Smith, M. D., ... & Holle, B. V. (2007). The invasion paradox: reconciling pattern and process in species invasions. Ecology, 88(1), 3-17.

Fridley, J. D., & Sax, D. F. (2014). The imbalance of nature: revisiting a Darwinian framework for invasion biology. Global Ecology and Biogeography, 23(11), 1157–1166.

Fryxell, D. C., Arnett, H. A., Apgar, T. M., Kinnison, M. T., & Palkovacs, E. P. (2015). Sex ratio variation shapes the ecological effects of a globally introduced freshwater fish. Proceedings of the Royal Society B: Biological Sciences, 282(1817), 20151970.

García-Berthou, E. (1999). Food of introduced mosquitofish: ontogenetic diet shift and prey selection. Journal of Fish Biology, 55(1), 135–147.

García-Galán, M. J., Garrido, T., Fraile, J., Ginebreda, A., Díaz-Cruz, M. S., & Barceló, D. (2010). Simultaneous occurrence of nitrates and sulfonamide antibiotics in two ground water bodies of Catalonia (Spain). Journal of Hydrology, 383(1-2), 93–101.

Geiser, S. W. (1921). Notes on the Differential Death-Rate in *Gambusia*. Ecology, 2(3), 220–222.

Geyer, R. L., Smith, G. R., & Rettig, J. E. (2016). Effects of Roundup formulations, nutrient addition, and Western mosquitofish (*Gambusia affinis*) on aquatic communities. Environmental Science and Pollution Research, 23(12), 11729–11739.

Gobierno de España (2011). 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. Boletín Oficial del Estado, 23, Madrid.

Grabda, E., Einszporn-Orecka, T., Felińska, C., & Zbanyszek, R. (1974). Experimental methemoglobinemia in rainbow trout. Acta Ichthyologica et Piscatoria, 04(2), 43–71.

Grup de Defensa del Ter. (2019). In: Nitrate concentration in springs of Osona [Internet]. Disponible a: https://www.gdter.org/wp/wp-content/uploads/GDT-NITRATS-OSONA-01-2019.pdf.

Guillette, L. J. (2005). Is Nitrate an Ecologically Relevant Endocrine Disruptor in Vertebrates? Integrative and Comparative Biology, 45(1), 19–27.

Guttal, S. (2007). Development, Research and Change. IDS Bulletin, 38(2), 31–35.

Hamlin, H. J., Moore, B. C., Edwards, T. M., Larkin, I. L., Boggs, A., High, W. J., ... & Guillette Jr, L. J. (2008). Nitrate-induced elevations in circulating sex steroid concentrations in female Siberian sturgeon (*Acipenser baeri*) in commercial aquaculture. Aquaculture, 281(1-4), 118-125.

Handelsman, C. A., Broder, E. D., Dalton, C. M., Ruell, E. W., Myrick, C. A., Reznick, D. N., & Ghalambor, C. K. (2013). Predator-Induced Phenotypic Plasticity in Metabolism and Rate of Growth: Rapid Adaptation to a Novel Environment. Integrative and Comparative Biology, 53(6), 975–988.

Hansen, G. J. A., Vander Zanden, M. J., Blum, M. J., Clayton, M. K., Hain, E. F., Hauxwell, J., … & Sharma, S. (2013). Commonly Rare and Rarely Common: Comparing Population Abundance of Invasive and Native Aquatic Species. PLoS ONE, 8(10), e77415.

Hansen, M. J., Madenjian, C. P., Slade, J. W., Steeves, T. B., Almeida, P. R., & Quintella, B. R. (2016). Population ecology of the sea lamprey (*Petromyzon marinus*) as an invasive species in the Laurentian Great Lakes and an imperiled species in Europe. Reviews in Fish Biology and Fisheries, 26(3), 509–535.

Hatch, A. C., & Blaustein, A. R. (2003). Combined effects of UV-B radiation and nitrate fertilizer on larval amphibians. Ecological Applications, 13(4), 1083-1093.

Hart, B. T., Bailey, P., Edwards, R., Hortle, K., James, K., McMahon, A., … & Swadling, K. (1991). A review of the salt sensitivity of the Australian freshwater biota. Hydrobiologia, 210(1-2), 105–144.

Hickey, C. W., & Martin, M. L. (2009). A review of nitrate toxicity to freshwater aquatic species. Environment Canterbury.

Hildebrand, S.F. (1919) Fishes in relation to mosquito control in ponds. Rep. United States Comm. Fish. App.15, 1–15.

Hildebrand, S. F. (1927). Sex ratio in Gambusia. The Biological Bulletin, 53(5), 390–404.

Hinchliffe, C., Atwood, T., Ollivier, Q., & Hammill, E. (2017). Presence of invasive *Gambusia* alters ecological communities and the functions they perform in lentic ecosystems. Marine and Freshwater Research, 68(10), 1867.

Homski, D., Goren, M., & Gasith, A. (1994). Comparative evaluation of the larvivorous fish *Gambusia affinis* and *Aphanius dispar* as mosquito control agents. Hydrobiologia, 284(2), 137–146.

Honda, H., Watanabe, Y., Kikuchi, K., Iwata, N., Takeda, S., Uemoto, H., ... & Kiyono, M. (1993). High density rearing of Japanese flounder, *Paralichthys olivaceus* with a closed seawater recirculation system equipped with a denitrification unit. Aquaculture Science, 41(1), 19-26.

Hulme, P. E. (2006). Beyond control: wider implications for the management of biological invasions. Journal of Applied Ecology, 43(5), 835–847.

Jensen, F. B. (1995). Uptake and effects of nitrite and nitrate in animals. In: Walsh, P. J., Wright, P. A. (eds.). Nitrogen metabolism and excretion, 289-303.

Jensen, F. B. (1996). Uptake, elimination and effects of nitrite and nitrate in freshwater crayfish (*Astacus astacus*). Aquatic Toxicology, 34(2), 95–104.

Jeschke, J. M., & Genovesi, P. (2011). Do biodiversity and human impact influence the introduction or establishment of alien mammals? Oikos, 120(1), 57–64.

Johnstone, I. M. (1986). Plant invasion windows: a time-based classification of invasion potential. Biological Reviews, 61(4), 369–394.

Joosten, H. (2009). Human Impacts: Farming, Fire, Forestry and Fuel. In: Maltby, E., & Barker, T. The Wetlands Handbook, pp. 689–718.

Keane, R., & Crawley, M. J. (2002). Exotic plant invasions and the enemy release hypothesis. Trends in Ecology & Evolution, 17(4), 164–170.

Killen, S. S., Marras, S., & McKenzie, D. J. (2011). Fuel, fasting, fear: routine metabolic rate and food deprivation exert synergistic effects on risk-taking in individual juvenile European sea bass. Journal of Animal Ecology, 80(5), 1024–1033.

Kolar, C. S., & Lodge, D. M. (2001). Progress in invasion biology: predicting invaders. Trends in Ecology & Evolution, 16(4), 199–204.

Krumholz, L. A. (1948). Reproduction in the Western Mosquitofish, *Gambusia affinis affinis* (Baird & Girard), and Its Use in Mosquito Control. Ecological Monographs, 18(1), 1–43.

Lahti, K., Huuskonen, H., Laurila, A., & Piironen, J. (2002). Metabolic rate and aggressiveness between Brown Trout populations. Functional Ecology, 16(2), 167–174.

Landres, P. B., Morgan, P., & Swanson, F. J. (1999). Overview of the use of natural variability concepts in managing ecological systems. Ecological applications, 9(4), 1179-1188.

Larson, D. L., Phillips-Mao, L., Quiram, G., Sharpe, L., Stark, R., Sugita, S., & Weiler, A. (2011). A framework for sustainable invasive species management: Environmental, social, and economic objectives. Journal of Environmental Management, 92(1), 14–22.

Leprieur, F., Beauchard, O., Blanchet, S., Oberdorff, T., & Brosse, S. (2008). Fish invasions in the world's river systems: when natural processes are blurred by human activities. PLoS biology, 6(2), e28.

Levine, J. M., Adler, P. B., & Yelenik, S. G. (2004). A meta-analysis of biotic resistance to exotic plant invasions. Ecology Letters, 7(10), 975–989.

Lewis Jr, W. M., & Morris, D. P. (1986). Toxicity of nitrite to fish: a review. Transactions of the American fisheries society, 115(2), 183-195.

Lloyd, L. N., Arthington, A. H., & Milton, D. A. (1986). The mosquitofish – a valuable mosquito-control agent or a pest?. In: Kitching, R.L. (ed.), The Ecology of Exotic Animals and Plants Some Australian Case Histories. John Wiley & Sons, Brisbane, pp. 6–25.

Lowe, S., Browne, M., Boudjelas, S., & De Poorter, M. (2000) 100 of the World's Worst Invasive Alien Species A selection from the Global Invasive Species Database (Vol. 12). The Invasive Species Specialist Group, Auckland.

Lozano Morales, A. (1946). Técnicas de Lucha antipalúdica. Manuales de Medicina Práctica. Salvat, Barcelona.

Montijano Luna, V., & Lucena Santacreu, J. R. (2013). Un territorio d'aigua, aiguamolls i llacunes costaneres de Viladecans. VII Trobada de Centres d'Estudis i d'Estudiosos d'Eramprunyà. L'aigua, el territori i les persones, pp. 149-152.

MacDougall, A. S., & Turkington, R. (2005). Are invasive species the drivers or passengers of change in degraded ecosystems? Ecology, 86(1), 42–55.

Maceda-Veiga, A., López, R., & Green, A. J. (2017). Dramatic impact of alien carp *Cyprinus carpio* on globally threatened diving ducks and other waterbirds in Mediterranean shallow lakes. Biological Conservation, 212, 74–85.

Maluquer, J. (1916). Excursió al Prat de Llobregat. Butlletí de la Institució Catalana d'Història Natural, 13(4), 54.

Marchand, F., & Boisclair, D. (1998). Influence of fish density on the energy allocation pattern of juvenile brook trout (*Salvelinus fontinalis*). Canadian Journal of Fisheries and Aquatic Sciences, 55(4), 796–805.

Martin, B. T., Heintz, R., Danner, E. M., & Nisbet, R. M. (2017). Integrating lipid storage into general representations of fish energetics. Journal of Animal Ecology, 86(4), 812–825.

Mason, C. F., (1996). Biology of freshwater pollution. Longman.

McCoid, M. J., & Fritts, T. H. (1980). Observations of feral populations of *Xenopus laevis* (Pipidae) in southern California. Bulletin of the Southern California Academy of Sciences, 79, 82-86.

McNab, B. K. (2012). Extreme measures: the ecological energetic of birds and mammals. The University of Chicago Press, Chicago.

Meffe, G. K. (1986). Cannibalism, Food Availability, and Reproduction in Mosquitofish: A Critique. The American Naturalist, 127(6), 897–901.

Meffe, G. K. (1992). Plasticity of Life-History Characters in Eastern Mosquitofish (*Gambusia holbrooki*: Poeciliidae) in Response to Thermal Stress. Copeia, 1992(1), 94.

Meffe, G. K., & Snelson, F. F. (1989). An ecological overview of poeciliid fishes. In: Meffe, G. K., & Snelson, F. F. (eds.). Ecology and evolution of livebearing fishes (Poeciliidae), p. 13-31.

Millennium Ecosystem Assessment, 2005. Ecosystems and human well-being: wetlands and water Synthesis. World Resources Institute, Washington, DC.

Moreno, C. A., Morán, A. (1981). Sobre la introducción de *Gambusia affinis holbrooki* (Girard) y *Carassius carassius* (Linnaeus) en el río Maipo, Chile. Boletín de la Sociedad Biológica de Concepción, 52, 95-102.

Moss, B., Stephen, D., Alvarez, C., Becares, E., Bund, W. V. D., Collings, S. E., … Wilson, D. (2003). The determination of ecological status in shallow lakes - a tested system (ECOFRAME) for implementation of the European Water Framework Directive. Aquatic Conservation: Marine and Freshwater Ecosystems, 13(6), 507–549.

Moyle, P. B. (2014. Novel aquatic ecosystems: the new reality for streams in California and other Mediterranean climate regions. River Research and Applications, 30(10), 1335–1344.

Murphy, C. A., Grenouillet, G., & García-Berthou, E. (2015). Natural abiotic factors more than anthropogenic perturbation shape the invasion of Eastern Mosquitofish (*Gambusia holbrooki*). Freshwater Science, 34(3), 965–974.

Nájera, L. (1944). Sobre la identificación de la *Gambusia holbrooki*. Boletín de la Real Sociedad Española de Historia Natural, 42, 51-55.

Navarro-García, J. R. (2013). La familia de Buen y la introducción de la "Gambusia": consecuencias medioambientales de la lucha contra la malaria en España. Boletín de Malariología y Salud Ambiental, 53(1), 99-112.

Noga, E.J. (2010). Fish disease: diagnosis and treatment. John Wiley & Sons.

Nordlie, F. G. (2014). Influences of body mass, temperature, oxygen tension, and salinity on respiratory oxygen consumption of cyprinodontoid fishes of three families. Reviews in Fish Biology and Fisheries, 24(1), 269–315.

Nordlie, F., & Mirandi, A. (1996). Salinity relationships in a freshwater population of eastern mosquitofish. Journal of Fish Biology, 49(6), 1226–1232.

Norton, D. A. (2009). Species Invasions and the Limits to Restoration: Learning from the New Zealand Experience. Science, 325(5940), 569–571.

NVBDCP, 2019. In: Guidelines on the use of larvivorous fish for vector control. National Vector Borne Disease Control Programme, Ministry oh Health & Welfare, Governement of India [Internet]. Disponible a: www.nvbdcp.gov.in.

Oliver, P. (2017). In: Introducción en España de la gambusia en 1921 con el fin de combatir al mosquito transmisor del paludismo [Internet]. Disponible a: http://pereoliver.com

Pandey, R. B., Adams, G. L., & Warren, L. W. (2011). Survival and precopulatory guarding behavior of *Hyalella azteca* (Amphipoda) exposed to nitrate in the presence of atrazine. Environmental Toxicology and Chemistry, 30(5), 1170–1177.

Parker, H., & Rosell, F. (2014). Rapid rebound in colony number of an over-hunted population of Eurasian beaver *Castor fiber*. Wildlife Biology, 20(5), 267–269.

Pereira, A., Geraldes, P., Lima-Fernandes, E., Fernandes, I., Cássio, F., & Pascoal, C. (2016). Structural and functional measures of leaf-associated invertebrates and fungi as predictors of stream eutrophication. Ecological Indicators, 69, 648–656.

Pereira, A., Carvalho, A. P., Cruz, C., & Saraiva, A. (2017). Histopathological changes and zootechnical performance in juvenile zebrafish (*Danio rerio*) under chronic exposure to nitrate. Aquaculture, 473, 197–205.

Pittaluga. (1923). Enfermedades de los Países Cálidos y Parasitología Tropical. Calpe, Madrid.

Poulsen, R., Cedergreen, N., Hayes, T., & Hansen, M. (2018). Nitrate: An Environmental Endocrine Disruptor? A Review of Evidence and Research Needs. Environmental Science & Technology, 52(7), 3869–3887.

Preston, D. L., Hedman, H. D., & Johnson, P. T. J. (2018). Nutrient availability and invasive fish jointly drive community dynamics in an experimental aquatic system. Ecosphere, 9(3), e02153.

Prior, K. M., Adams, D. C., Klepzig, K. D., & Hulcr, J. (2017). When does invasive species removal lead to ecological recovery? Implications for management success. Biological Invasions, 20(2), 267–283.

Purcell, K. M., & Stockwell, C. A. (2014). An evaluation of the genetic structure and post-introduction dispersal of a non-native invasive fish to the North Island of New Zealand. Biological Invasions, 17(2), 625–636.

Pyke, G. H. (2005). A Review of the Biology of *Gambusia affinis* and *G. holbrooki*. Reviews in Fish Biology and Fisheries, 15(4), 339–365.

Rayner, T. S., & Creese, R. G. (2006). A review of rotenone use for the control of non-indigenous fish in Australian fresh waters, and an attempted eradication of the noxious fish, *Phalloceros caudimaculatus*. New Zealand Journal of Marine and Freshwater Research, 40(3), 477–486.

Ratmaya, W., Soudant, D., Salmon-Monviola, J., Cochennec-Laureau, N., Goubert, E., Andrieux-Loyer, F., … & Souchu, P. (2019). Reduced phosphorus loads from the Loire and Vilaine Rivers were accompanied by increasing eutrophication in Vilaine Bay (South Brittany, France). Biogeosciences Discussions, 1–29.

Reid, N. M., Proestou, D. A., Clark, B. W., Warren, W. C., Colbourne, J. K., Shaw, J. R., … Whitehead, A. (2016). The genomic landscape of rapid repeated evolutionary adaptation to toxic pollution in wild fish. Science, 354(6317), 1305–1308.

Reznick, D., & Ender, J. (1982). Erratum: The Impact of Predation on Life History Evolution in Trinidadian Guppies (*Poecilia reticulata*). Evolution, 36(3), 634.

Richardson, D. M., & Ricciardi, A. (2013). Misleading criticisms of invasion science: a field guide. Diversity and Distributions, 19(12), 1461–1467.

Ricklefs, R. E., & Wikelski, M. (2002). The physiology/life-history nexus. Trends in ecology & evolution, 17(10), 462-468.

Rincón, P. A., Correas, A. M., Morcillo, F., Risueno, P., & Lobon-Cervia, J. (2002). Interaction between the introduced eastern mosquitofish and two autochthonous Spanish toothcarps. Journal of Fish Biology, 61(6), 1560–1585.

Rolff, J. (2002). Bateman's principle and immunity. Proceedings of the Royal Society of London. Series B: Biological Sciences, 269(1493), 867-872.

Romo, S., Miracle, M. R., Villena, M.-J., Rueda, J., Ferriol, C., & Vicente, E. (2004). Mesocosm experiments on nutrient and fish effects on shallow lake food webs in a Mediterranean climate. Freshwater Biology, 49(12), 1593–1607.

Rosen, D. E., & Bailey, R. M. (1963). The poeciliid fishes (Cyprinodontiformes): their structure, zoogeography, and systematics. Bulletin of the American Museum of Natural History; v. 126, article 1.

Ruiz-Navarro, A., Torralva, M., & Oliva-Paterna, F. (2013a). Trophic overlap between cohabiting populations of invasive mosquitofish and an endangered toothcarp at changing salinity conditions. Aquatic Biology, 19(1), 1–11.

Ruiz-Navarro, A., Verdiell-Cubedo, D., Torralva, M., & Oliva-Paterna, F. J. (2013b). Removal control of the highly invasive fish *Gambusia holbrooki* and effects on its population biology: learning by doing. Wildlife Research, 40(1), 82.

Rupp, H. R. (1996). Adverse assessments of *Gambusia affinis*: an alternate view for mosquito control practitioners. Journal of the American Mosquito Control Association, 12(2), 155-166.

Seastedt, T. R., Hobbs, R. J., & Suding, K. N. (2008). Management of novel ecosystems: are novel approaches required? Frontiers in Ecology and the Environment, 6(10), 547–553.

Seebens, H., Blackburn, T. M., Dyer, E. E., Genovesi, P., Hulme, P. E., Jeschke, J. M., ... & Essl, F. (2017). No saturation in the accumulation of alien species worldwide. Nature communications, 8, 14435.

Servanty, S., Gaillard, J.-M., Ronchi, F., Focardi, S., Baubet, É., & Gimenez, O. (2011). Influence of harvesting pressure on demographic tactics: implications for wildlife management. Journal of Applied Ecology, 48(4), 835–843.

Simberloff, D. (2003). How Much Information on Population Biology Is Needed to Manage Introduced Species? Conservation Biology, 17(1), 83–92.

Smith, C. C., & Sargent, R. C. (2006). Female fitness declines with increasing female density but not male harassment in the western mosquitofish, *Gambusia affinis*. Animal Behaviour, 71(2), 401–407.

Snelson, F. F. (1989). Social and environmental controlof life history traits in poeciliid fishes. In: Meffe, G. K., & Snelson, F. F. (eds.). Ecology and evolution of livebearing fishes (Poeciliidae), p. 149-162.

Soga, M., & Gaston, K. J. (2018). Shifting baseline syndrome: causes, consequences, and implications. Frontiers in Ecology and the Environment, 16(4), 222–230.

Soucek, D. J., & Dickinson, A. (2011). Acute Toxicity of Nitrate and Nitrite to Sensitive Freshwater Insects, Mollusks, and a Crustacean. Archives of Environmental Contamination and Toxicology, 62(2), 233–242.

Stearns, S.C. (1992). The Evolution of Life Histories. Oxford University Press, Oxford, UK.

Steindachner, F. (1865). Ichthyologischer Bericht über eine nach Spanien und Portugal unternommene Reise. I. Zur Fischfauna des Albufera-Sees bei Valencia in Spanien. Sitzungsberichte der mathematisch-naturwissenschaftlichen Classe der kaiserlichen Akademie der Wissenschaften Wien, 52, 483-491.

Stephenson JF. 2014 The experimental evolution of parasite resistance in wild guppies: artificial selection, resource availability and predation pressure. Proceedings of the Royal Society B: Biological Sciences, 281, 20140541.

Stohlgren, T. J., Jarnevich, C., Chong, G. W., & Evangelista, P. H. (2006). Scale and plant invasions: a theory of biotic acceptance. Preslia, 78(4), 405-426.

Summerfelt, S. T., Adler, P. R., Glenn, D. M., & Kretschmann, R. N. (1999). Aquaculture sludge removal and stabilization within created wetlands. Aquacultural Engineering, 19(2), 81–92.

Tesh, A. E. C., Wilby, O. K., & Tesh, J. M. (1990). Effects of inorganic nitrates on growth and development of *Hydra*. Toxicology in Vitro, 4(4-5), 614–615.

Uliano, E., Cataldi, M., Carella, F., Migliaccio, O., Iaccarino, D., & Agnisola, C. (2010). Effects of acute changes in salinity and temperature on routine metabolism and nitrogen excretion in gambusia (*Gambusia affinis*) and zebrafish (*Danio rerio*). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 157(3), 283–290.

Vargas, M. J. (1993). Interacción entre *Aphanius iberus* y *Gambusia holbrooki* en el Delta del Ebro: sus ciclos biológicos y ecologías tróficas. Tesis Doctoral, Universitat de Barcelona.

Vargas, M. J., & de Sostoa, A. (1996). Life history of *Gambusia holbrooki* (Pisces, Poeciliidae) in the Ebro delta (NE Iberian peninsula). Hydrobiologia, 341(3), 215–224.

Vera, M., Díez-del-Molino, D., & García-Marín, J. L. (2016). Genomic survey provides insights into the evolutionary changes that occurred during European expansion of the invasive mosquitofish (*Gambusia holbrooki*). Molecular Ecology, 25(5), 1089-1105.

Wabnitz, C., Taylor, M., Green, E., & Razak, T. (2003). From Ocean to Aquarium. UNEP-WCMC, Cambridge, UK.

Walshe, D. P., Garner, P., Adeel, A. A., Pyke, G. H., & Burkot, T. R. (2017). Larvivorous fish for preventing malaria transmission. Cochrane Database of Systematic Reviews, 12, CD008090.

Winkler, P. (1975). Thermal Tolerance of *Gambusia affinis* (Teleostei: Poeciliidae) from a Warm Spring. I. Field Tolerance under Natural Stream Conditions. Physiological Zoology, 48(4), 367–377.

Zavaleta, E. S., Hobbs, R. J., & Mooney, H. A. (2001). Viewing invasive species removal in a whole-ecosystem context. Trends in Ecology & Evolution, 16(8), 454–459.

Zuk, M. (2009). The Sicker Sex. PLoS Pathogens, 5(1), e1000267.

ANNEXOS

Taula A1. Disrupció endocrina del nitrat en amfibis, peixos i macroinvertebrats aquàtics. Adapatat de Poulsen et al. (2018).

Alteracions en els nivells de les hormones esteroides circulants, la reproducció i el comportament

Increment de testosterona i altres esteroides en femelles d'esturió siberià *Acipenser paeri*, i de vitelogenina en mascles i femelles de salmó atlàntic *Salmo salar*.

Activitat reproductiva i pes dels embrions reduïts en femelles salvatges de *Gambusia holbrooki.*[*]

Acceleració de la maduració de les gònades en mascles de la granota lleopard *Lithobates pipiens.*

Desenvolupament embrionari anormal i reducció en el nombre de descendència de *Daphnia magna.*

Major activitat en el ritual d'aparellament del tritó palmat *Lissotriton helveticus*

El comportament en l'aparellament de l'amfípod *Hyalella azteca* es veié afectat negativament.

Alteracions en l'homeostasi de l'hormona tiroides

Inhibició de la captació de iodur per part de la tiroides en *Oncorhynchus mykiss*, *Perca fluviatilis* i *Cyprinus carassius*, però la seva funcionalitat no es veié afectada.

Creixement reduït en els ciprínids *Danio rerio* i *Gobiocypris rarus*.

Augment en el pes en capgrossos de l'anur *Xenopus laevis* i alteracions en el temps de metamorfosi dels anurs *Anaxyrus americanus* i *Hyla chrysoscelis*.

---

* Aquest és un estudi de camp correlatiu i per tant la coexistència d'altres compostos pot haver emmascarat les conclusions esmentades

Taula A2. Recopil·lació d'estudis sobre els trets d'història de vida afectats per alguna variable ambiental realitzats en gambúsia. S'inclou la procedència geogràfica dels peixos estudiats i si es troba dins el rang natural natiu (si no s'especifica vol dir que és introduït). ** Estudi fet amb *Gambusia affinis*.

Variable ambiental i localització	**Història de vida**	**Efecte**	**Referència**
Latitud (S – N) Costa mediterrània d'Espanya i el S de França	Prevalença de paràsits	(-)	Benejam et al. 2009
	Pes eviscerat	(+)	
	Esforç reproductor	(-)	
	Mida en maduresa	(-)	
	Mida de camada	(0)	
	Mida d'embrió	(0)	
Latitud (S – N) i **desembocadura – capçalera** Costa mediterrània d'Espanya i el S de França	Esforç reproductor	(-) amb latitud i capçalera	Carmona-Catot 2011
	Pes eviscerat	(+) amb latitud i capçalera	
	Mida del peix/edat	(+) capçalera, (0) amb latitud	
Salinitat Empordà, NE Península Ibèrica	Esforç reproductor	(+)	Alcaraz i García-Berthou 2007
	Mida de camada	(0)	
	Fecund. real	(+) fins a llindar	
	Mida d'embrió	(+)	
	Pes eviscerat	(-) en femelles	
	Maduració d'oòcits	S'avança	
Salinitat Texas, EUA. Rang natiu**	Esforç reproductor	(+)	Stearns i Sage 1980
	Mida de camada	(+)	
	Mida d'embrió	(-)	
	Pes	(-)	
Salinitat Florida, EUA. Rang natiu	Creixement	(-)	Zimmerer, 1983
Salinitat i **pH** Mississipi, EUA. Rang natiu**	Esforç reproductor	(+)	Brown-Peterson i Peterson, 1990
	Mida de camada	(+)	
	Mida d'embrió	(-)	
	Pes eviscerat	(0)	
	Maduració d'oòcits	S'avança	
Activitat agrícola Illa del Nord, Nova Zelanda**	Mida del peix	(0)	Lee et al., 2017
	Sex-ratio	(0)	

Taula A2 (continuació).

Variable ambiental i localització	Història de vida	Efecte	Referència
Disponibilitat d'aliment Austràlia Occidental, Austràlia	Creixement	(+)	Trendall 1983
	Esforç reproductor	(+)	
	Mida de camada	(+)	
	Mida d'embrió	(0)	
	Pes eviscerat	(+)	
Disponibilitat d'aliment Nord-est dels EUA. Rang natiu	Reserves lipídiques	(0)	Reznick i Braun, 1987
Disponibilitat d'aliment i temperatura California, EUA**	Creixement	(+) fins a 30ºC	Wurtsbaugh i Cech, 1983
	Mida i pes del peix	(+)	
Disponibilitat d'aliment i temperatura Califòrnia, EUA**	Esforç reproductor	(+)	Vondracek et al., 1988
	Mida de camada	(0) en Tª	
	Mida d'embrió	(+) en Tª	
	Pes del peix	(+)	
	Creixement	(+)	
Temperatura i estabilitat de Tª Nevada, EUA**	Mida en maduresa	(+)	Stockwell i Weeks 1999
	Contingut en lípids	(+)	
	Mida d'embrió	(+)	
Temperatura Carolina del Sud, EUA. Rang natiu	Esforç reproductor	(+)	Meffe 1991
	Mida de camada	(+)	
	Mida d'embrió	(-)	
	Mida del peix	(-)	
	Pes eviscerat	(-)	
Temperatura Carolina del Sud, EUA. Rang natiu	Esforç reproductor	(+)	Bennet i Goodyear 1978
	Fecund. real	(0)	
	Mida en maduresa	(0)	
	Mida del peix	(+) fins a llindar	
	Reserves lipídiques	(+) només en mascles	
Ambient estable – fluctuant Hawaii, EUA.**	Esforç reproductor	(+)	Stearns 1983
	Mida de camada	(+)	
	Mida d'embrió	(0)	
	Mida del peix	(+)	
	Pes eviscerat	(+)	

Taula A3. Resum de les basses i canals mostrejats del capítol 3, incloent-hi l'estimació visual semi-quantitativa d'abundància de *G. holbrooki* en superfície tant en el moment de la captura dels exemplars el maig de 2015 i posteriorment el maig de 2019.

Localitat	**Abundància 2015**	**Abundància 2019**	**Observacions**
G1	Alta	Baixa – Mitj.	
G2	Alta	**Sense peixos**	A l'estiu de 2015 ja s'havia assecat, bassa d'ús per a rec.
G3	Alta	**Sense peixos**	Bassa de rec reformada el 2016. Sense peixos des d'aleshores.
L1	Mitjana	**Sense peixos**	El 2016 ja gairebé sense aigua i no es veieren peixos. Zona d'inundació; a 150 m hi ha gambúsies el 2019.
L2	Molt alta	Molt alta	Sec l'estiu de 2016. Recolonitzada.
L3	Baixa	Mitjana	Només juvenils el 2019.
L4	Baixa	**Sense peixos**	
L5	Molt alta	Alta	
L6	Baixa	**Sense peixos**	Capa densa de *Lemna* sp. A la sèquia del costat sí que n'hi ha. Queden connectats quan hi ha molta aigua.
L7	Baixa	Alta	
L8	Mitjana	Alta	
L9	Alta	**Sense peixos**	
B1	Alta	Alta – M. alta	
B2	Mitjana	Baixa – Mitj.	
C1	Alta	Alta	
C2	Alta	**Sense peixos**	Es va assecar l'estiu de 2018 per a treure llots i retirar fauna exòtica.
V1	Mitjana	Baixa – Mitj.	Desapareguda a la zona on es va pescar, no a la cubeta central.
V2	Alta	Alta	

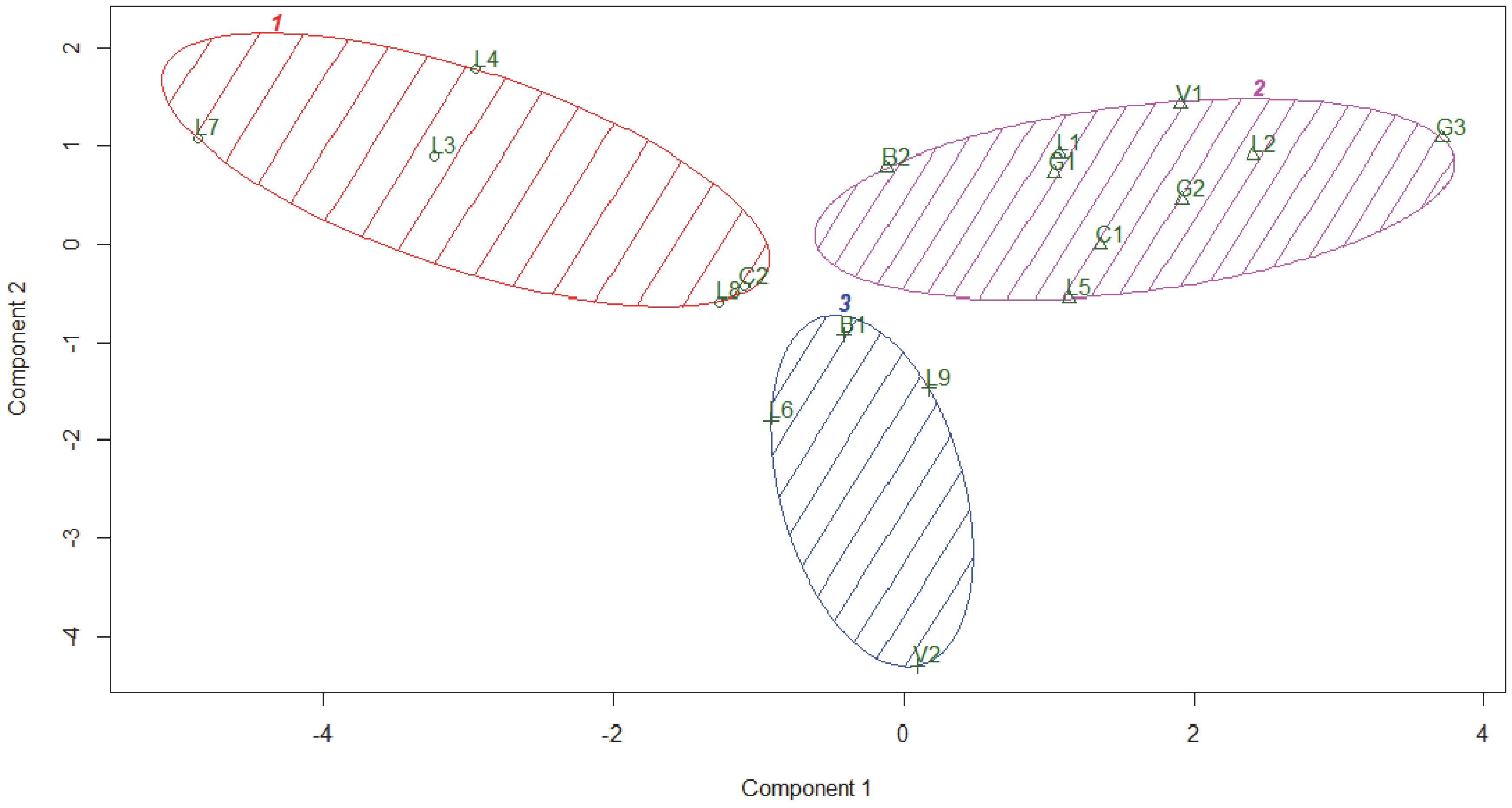


Figura A1. Distribució de les 18 localitats del capítol III segons l'anàlisi clúster de *k-mitjanes* dels trets d'història de vida de *Gambusia holbrooki* (veure Taula A4). Les dues components expliquen el 53,7 % de la variabilitat total.

Taula A4. Resum dels centroides de les tres agrupacions determinades en l'anàlisi clúster dels 12 trests d'història de vida de les gambúsies de les 18 basses i canals del capítol III. S'indica amb '+' o '-' el grup amb el valor més alt o més baix de cadascun dels trets.

Tret d'història de vida	**Clúster 1**		**Clúster 2**		**Clúster 3**	
Taxa de creixement ♂♂	0,35	+	0,05		-0,55	-
Taxa de creixement ♀♀	0,13		0,43	+	-1,13	-
Longituds retro-calculades ♂♂	0,57	+	-0,55	-	0,52	
Longituds retro-calculades ♀♀	-0,15		-0,61	-	1,55	+
Fecunditat potencial	1,10	+	-0,52	-	-0,21	
Fecunditat real	0,29	+	-0,28	-	0,27	
Nombre de descendents	1,15	+	-0,59	-	-0,11	
Mida de la descendència	-0,95	-	0,39	+	0,32	
Índex de massa escalat ♂♂	-0,65	-	0,47	+	-0,25	
Índex de massa escalat ♀♀	-0,93	-	0,71	+	-0,44	
Esforç reproductor ♂♂	1,24	+	-0,59	-	-0,22	
Esforç reproductor ♀♀	0,83	+	-0,39	-	-0,17	

VERSIÓ EN CASTELLÀ

INTRODUCCIÓN GENERAL

La globalización, definida como la integración del conjunto de economías locales en un único mercado global con una mínima regulación, ha multiplicado por diez el intercambio de bienes y servicios los últimos 50 años (Guttal, 2007; Díaz et al., 2019). Una de las consecuencias de este movimiento es la introducción de especies exóticas, entendida como el traslado por los humanos de una especie desde su región de origen hacia otra fuera de su rango nativo (Kolar y Lodge, 2001). Algunas introducciones pueden ser de forma accidental, como el mosquito tigre *Aedes albopictus* (Skuse, 1894), que llegó mediante transporte de neumáticos reciclados (Aranda et al., 2006), o deliberada, como la perca del Nilo *Lates niloticus* (Linnaeus, 1758) liberada para contrarrestar la pérdida de stocks de pesca en el lago Victoria (Lowe, 2000).

Una especie exótica se considera invasora cuando se establece fuera de su rango de distribución histórico y causa impactos sobre la biodiversidad nativa, fruto de la falta de un contexto de coevolución (Fig. 1; Kolar y Lodge, 2001). La mayoría de introducciones fracasan durante el establecimiento y la expansión en el ecosistema receptor, pero, en algunos casos, tienen éxito y completan las cuatro etapas, y es el análisis de los factores que lo determinan el objeto de estudio de la ciencia de las invasiones biológicas (Richardson y Ricciardi, 2013). Asimismo, dado que no es la especie la que deviene invasora, sino una población, se considera más adecuado emplear el término población invasora que no el de especie invasora. Por ejemplo, la lamprea de mar *Petromyzon marinus* (Linnaeus, 1758) es una especie con poblaciones nativas en regresión en la península ibérica e invasora en la región de los Grandes Lagos norteamericanos (Hansen et al., 2016). La tasa global de introducciones con éxito se ha incrementado desde 1950, llegando a valores que multiplican por 60 la tasa histórica preindustrial (Seebens et al., 2017). Entre estas introducciones, el pequeño porcentaje que se convierten en invasoras, junto con factores como la degradación de los hábitats y el cambio climático, representa una amenaza para la conservación de la biodiversidad (la tasa de extinción actual es hasta 100 veces superior a la tasa de los últimos 10 millones de años), pero también para la salud y la economía (Díaz et al., 2019).

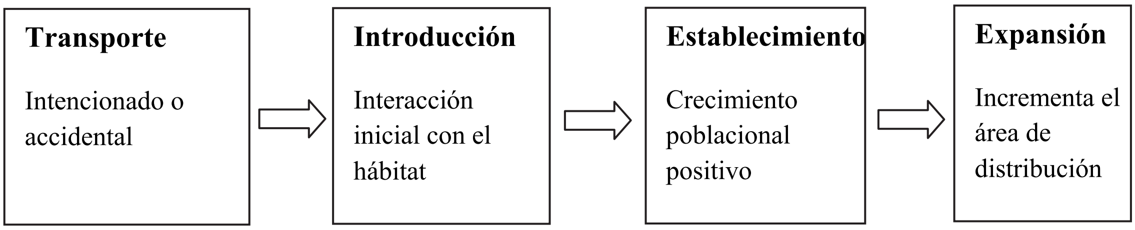


Figura 1. Etapas de invasión de una especie exótica en un nuevo hábitat receptor.

La ciencia de las invasiones biológicas es relativamente reciente y se habla de "ciencia" porque incluye factores sociológicos y económicos, además de los ecológicos, que determinan el éxito invasor de una especie exótica y sus impactos. Ahora bien, el concepto de invasión biológica es muy antiguo; Charles Darwin (1089 - 1882) y Alfred R. Wallace (1823 - 1913) ya hablaban de la naturalización de especies exóticas, aunque se atribuyen los fundamentos de la disciplina en el libro 'The Ecology of Invasiones by Animales and Plants' de Charles S. Elton (1900 - 1991):

> *Un sistema ecológico, como cualquier comunidad humana organizada, tiene sus propios centros de acción - como el suelo, el dosel arbóreo, el humedal y el arroyo, el tronco caído y el nido de pájaro - pero siempre en algún punto puedes encontrar conexiones entre todos ellos, lo que podría afectar el equilibrio entre poblaciones. El invasor se encuentra por tanto abriéndose paso como puede dentro de un sistema complejo, como un emigrante intenta encontrar un trabajo, una casa y establecer una familia en un nuevo país o ciudad. La manera más corta de describir esta situación [...] sería que el invasor se encuentra con una 'resistencia ecológica'.*
>
> *C.S. Elton, 1958*

Este concepto seminal de Elton fue la base de conceptos clave en biología de invasiones formulados posteriormente, que giran a su alrededor: i) hipótesis de la comunidad simple, que postula que es más fácil invadir comunidades pobres que ricas en especies (Elton, 1958; Johnstone, 1986). Esto va muy relacionado con la hipótesis de nichos[1]

---

[1] C.S. Elton lo define como el espacio que ocupa un individuo dentro del entorno biótico, su relación con el alimento disponible y los depredadores. Por lo tanto lo limita a un concepto trófico. El ecólogo G.E. Hutchinson (1903 – 1991) lo amplía a una serie de condiciones ambientales y de recursos que definen los requerimientos de un individuo para que la población persista.

vacíos, los cuales aumentan cuando se pierden especies o se generan nuevos por la actividad humana, y que aprovechan las especies introducidas para colonizar. ii) hipótesis de la carencia de enemigos, que asocia el éxito invasor a que una especie exótica se encuentra "libre" de los depredadores y patógenos que tenía en el hábitat de origen (Keane y Crawley, 2002). A partir de las ideas surgidas en la obra de Elton se han propuesto tres mecanismos diferentes para explicar los patrones de invasiones, que no se excluyen entre sí. En primer lugar, la actividad humana, que concierne a las cuatro etapas de la invasión. Una presión de propágulos[2] incrementada tanto en número como en frecuencia y el efecto de las perturbaciones de origen antrópico facilitarán el establecimiento de una especie no nativa (Leprieur et al., 2008; Chiron et al., 2009). En segundo lugar, la resistencia biótica, que concierne al establecimiento y la expansión únicamente. Las comunidades pobres en especies albergarán un mayor número de especies exóticas, ya que la especie no nativa se encontrará una competencia menor en comparación con una comunidad rica (Levine et al., 2004). Por último, la aceptación biótica, que concierne al establecimiento y la expansión únicamente, aparece en contraposición a las hipótesis de Elton. Se sugiere que el establecimiento de una especie no nativa será más probable en áreas ricas en especies nativas porque son zonas ricas en recursos y, por tanto, hay más energía y espacio físico disponibles (Stohlgren et al., 2006).

Ningún metaanálisis o evidencia experimental clara da un apoyo inequívoco para ninguna de las tres hipótesis. En el caso de los peces continentales, Leprieur et al. (2008) encontraron una estrecha relación entre la biogeografía de invasiones y la intensidad de la actividad humana exclusivamente. Otros estudios han encontrado el mismo patrón para otros grupos de animales y zonas geográficas (Chiron et al., 2009; Jeschke y Genovesi, 2011). Pero por otro lado, las otras dos hipótesis han tenido apoyo a escalas más pequeñas o en otros grupos taxonómicos (véase Levine et al., 2004; Fridley et al., 2007; Chen, 2012).

---

[2] Número de veces que se libera un número absoluto de individuos de una especie en una región no nativa.

Esta tesis se enmarca en el contexto de una invasión biológica en ecosistemas sometidos a fuerte degradación ambiental, en concreto las zonas húmedas[3] de los agroecosistemas del delta del Llobregat. Las zonas húmedas han estado tradicionalmente ligadas al desarrollo humano, pero son también zonas de gran valor ecológico amenazadas por la actividad agrícola (Joosten, 2009). En algunos casos, el cambio de usos de suelo como la desecación de tierras para uso agrícola y como medida de control de los mosquitos, vectores de enfermedades endémicas como el paludismo, ha transformado sustancialmente el ecosistema original. La modernización de la agricultura ha supuesto un incremento en el uso de fertilizantes, a menudo sintéticos (ej. sales de nitrógeno), que llegan a las masas de agua por escorrentía superficial o percolación en los acuíferos (Joosten, 2009). En consecuencia, los ecosistemas acuáticos experimentan eutrofización antrópica. La principal diferencia con la eutrofización natural radica en la intensidad de los efectos y los cambios que experimentan las masas de agua, incluyendo un aumento de la turbidez y una reducción de la concentración de oxígeno durante la noche que, en casos extremos, supone la muerte de toda la comunidad biótica. Así pues, si ha aparecido una degradación ambiental, se podría decir que la población invasora actúa como mero "pasajero con el billete comprado" en esta transición ambiental (Didham et al., 2005; MacDougall y Turkington, 2005). Pero la población invasora puede, según el contexto, ejercer una perturbación adicional en estos ecosistemas previamente degradados, alterando las interacciones entre las especies nativas y los servicios ecosistémicos que de éstas se derivan, por ejemplo el reciclaje de nutrientes o el control de plagas (Didham et al., 2005). La identificación de los cambios en la estructura de la comunidad y el funcionamiento del ecosistema por la presencia de múltiples factores de degradación y cómo estos indicadores se relacionan entre sí está muy poco estudiado en ecosistemas acuáticos.

La gestión de invasiones biológicas en ecosistemas “noveles”

Un ecosistema novel es un término de nuevo cuño, caracterizado por ser un ecosistema donde las condiciones ambientales y la composición de especies han sufrido un cambio tan marcado que no se parecen en nada a las características que había en un período histórico reciente (Seastedt et al., 2008; Moyle, 2014). Siempre ha existido una

---

[3] Zona húmeda o *wetland* en un sentido amplio. La definición del Convenio de Ramsar incluye lagos y ríos, acuíferos subterráneos, marismas, turberas, estuarios y masas de agua modificadas como embalses y balsas.

continuidad en la modificación de las condiciones de los ecosistemas, incluso su degradación, pero actualmente el gran cambio es que esta tasa de alteración se ha acelerado enormemente, impulsada a menudo por diversas perturbaciones actuando simultáneamente (Díaz et al., 2019; Fig. 2). Por ejemplo, con la construcción de una represa en un río se cambia de un hábitat lótico a otro léntico, a menudo acompañado de la liberación de especies exóticas relacionadas con la pesca (Moyle, 2014). Por lo tanto se hace difícil comparar las nuevas condiciones en cuanto a su biodiversidad y su funcionamiento con el estado de referencia, es decir, la variabilidad natural en un período y zona geográfica concretos que no se encuentran afectados por la actividad humana (Landres et al., 1999). Además, en zonas altamente desarrolladas es complicado encontrar zonas de referencia (Moss et al., 2003; Bonada et al., 2006). Con ello no se quiere dar la idea de que la restauración para acercarse al máximo a la situación de referencia sea imposible, o que se dé por bueno un incremento en la tolerancia a la degradación (Soga y Gaston, 2017).

La gestión de espacios naturales comprende desde políticas de *laissez-faire*, es decir, de mínima intervención independientemente del impacto, hasta las políticas muy intervencionistas. Estas últimas tradicionalmente se han centrado en el mantenimiento o restauración de los ecosistemas en el estado pre-impacto como podría ser la revegetación de una cantera abandonada o la erradicación de una especie exótica (Seastedt et al., 2008). En ecosistemas degradados, sin embargo, la semejanza con su estado natural histórico es tan bajo que las medidas de restauración deseadas tienen más posibilidades de fructificar si el objetivo fijado es realista y se tiene un conocimiento profundo de la ecología del medio y sus características socioeconómicas (Moyle, 2014). En estos hábitats enormemente alterados, el hecho de erradicar una especie invasora o mejorar la calidad del agua, pero manteniendo un régimen hidrológico alterado, puede suponer que el ecosistema siga sin recuperarse (Fig. 2). Concretamente, en determinadas situaciones como en el caso de vertebrados acuáticos de pequeño tamaño en coexistencia con múltiples fuentes de degradación, la probabilidad de éxito en la erradicación y la restauración del hábitat es baja (Prior et al., 2018).

Así pues, a pesar de que las invasiones biológicas están entre las causas de pérdida de biodiversidad del planeta (MEA, 2005; Díaz et al., 2019), la erradicación de especies exóticas de amplia distribución no es factible y sólo queda aplicar estrategias de control o mitigación de sus impactos a nivel local (Norton, 2009). Además, cuando tenemos una

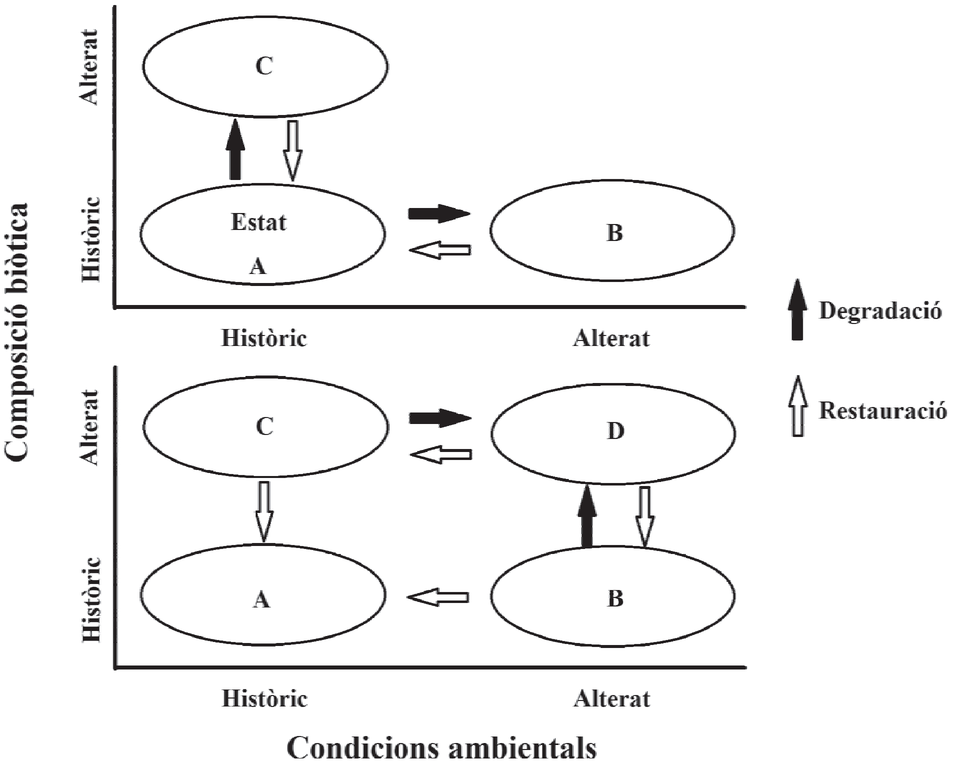


Figura 2. Alteraciones en las condiciones ambientales y la composición biótica. Un ecosistema, con su variabilidad natural (encajada dentro de la elipse A), que por la acción de un factor ambiental (A → B), o por la adición o pérdida de una especie (A → C) sufre una degradación. Una vez estamos en el nuevo estado (B o C), la adición de nuevas alteraciones bióticas o abióticas, o la misma reestructuración interna (pérdida de especies que no pueden tolerar las nuevas condiciones ambientales, o bien la degradación del hábitat por la pérdida de una especie clave en el funcionamiento del ecosistema), pueden llevar a un estado aún más degradado (D). Para volver al estado inicial (A), es probable que tengan que proveerse medidas de restauración que han de tener en cuenta tanto la composición de especies como las condiciones ambientales. El hecho de llevar a cabo una medida que no tenga en cuenta el conjunto del ecosistema, por ejemplo erradicar una especie invasora sin más, puede llevar a un estado (B) que no era el objetivo inicial de la restauración (A). Adaptado de Seastedt et al. (2008).

población exótica establecida desde hace muchos años, ésta puede estar fuertemente integrada en la comunidad nativa, puede haber establecido interacciones de competencia o depredación, y tener un papel funcional relevante incluso para las especies nativas (Zavaleta et al., 2001; Carroll, 2011). En estos casos la óptima gestión debería actuar no sólo en el control de la expansión de la población invasora, sino analizando el contexto ecológico para evitar que aparezcan "sorpresas ecológicas" derivadas de una cascada trófica (Caut et al., 2009). La eliminación de la especie exótica tendría un efecto negativo sobre depredadores nativos si estos dependen en gran medida de este recurso, o sobre otras especies nativas del mismo nivel trófico si los depredadores cambian de presa (Zavaleta et al., 2001). Esta aproximación ecosistémica no sólo puede prever efectos inesperados, sino que se puede utilizar como propia herramienta de control de la población invasora si se conocen las condiciones ambientales que reduzcan su éxito invasor. Ahora bien, no hay que olvidar que la mejor estrategia de manejo de las especies invasoras es la prevención, posiblemente a través de listados que prohíban la importación de especies con elevado riesgo de invasión y con programas de educación ambiental (Hulme et al., 2006).

La invasión de la gambusia *Gambusia holbrooki* (Girard, 1859)

Esta tesis utiliza la invasión por *G. holbrooki* como ejemplo de invasión difícil de gestionar una vez la especie se ha establecido y expandido por el territorio. Originaria de Norteamérica, *G. holbrooki* es un pez de agua dulce de la familia de los pecílidos, de pequeño tamaño, como máximo 35 mm en machos y 60-70 mm en hembras, de color plateado sobre todo en la zona ventral y una tonalidad gris - verdosa el resto del cuerpo (Vargas y de Sostoa, 1996; Pyke, 2005). Tiene una sola aleta dorsal, y una aleta caudal redondeada. El dimorfismo sexual, aparte del tamaño, es muy evidente (Fig. 3). Los machos tienen gonopodio, que es la aleta anal modificada en forma de órgano copulador (Meffe y Snelson, 1989). Es una especie ovovivípara, o sea, los huevos fecundados permanecen en el interior de la hembra hasta eclosionar. El tamaño de camada típica de una hembra es de 5 a 100 juveniles, la cual depende en gran parte del tamaño de la hembra y la disponibilidad de alimento (Meffe, 1986; Vargas y de Sostoa, 1996). Se trata de una especie muy prolífica, con camadas cada tres o cuatro semanas y juveniles que maduran sexualmente a la edad de entre tres y diez semanas (Meffe, 1992). La longevidad es corta, típicamente menos de 3 años en hembras y menos de 2 años en machos (Vargas y de Sostoa, 1996). Las poblaciones tienen una proporción de sexos, aunque con excepciones temporales, sesgada hacia las hembras con una media de un macho por cada cuatro hembras (Hildebrand, 1927; Vargas, 1993).

Es una especie ecológicamente generalista, pero con una preferencia por aguas someras y cálidas (31-35 ºC), de corriente nula o muy baja, con una buena presencia de vegetación sumergida (Pyke, 2005) y moderada salinidad (< 20 ‰, Chervinski, 1983; Alcaraz y García-Berthou, 2007). Además, se han convertido en muy abundantes en masas de agua cercanas a núcleos urbanos, por ejemplo en estanques urbanos, albercas y canales de zonas agrícolas, los cuales suelen tener problemas de contaminación (Lloyd et al., 1986; Arthington y Lloyd, 1989).

Su dieta incluye artrópodos terrestres que reposan o caen en la superficie acuática, crustáceos, oligoquetos, nemátodos, moluscos, larvas y pupas de invertebrados acuáticos, algas y detritos, diatomeas y pequeños peces, incluyendo juveniles de la misma especie (Vargas, 1993; García-Berthou, 1999). Experimentalmente se ha demostrado que se pueden alimentar también de huevos y larvas de anfibios o causar serias lesiones a estos o de otras especies nativas de pequeño tamaño (Pyke, 2005; Preston et al., 2018), aunque sus impactos sobre el funcionamiento de los ecosistemas todavía son bastante desconocidos (véase Hinchliffe et al., 2017).

Figura 3. Hembra y macho adultos de *Gambusia holbrooki*. Fotografías cortesía de Chris Appleby, Fishbase.org.

El rango de distribución natural de *G. holbrooki* es la costa este de Estados Unidos, desde la costa de Nueva Jersey hasta Florida (Rosen y Bailey, 1963). Sin embargo, tanto la especie de estudio *G. holbrooki* como su especie hermana, *Gambusia affinis* (Baird & Girard, 1853), han sido ampliamente distribuidas por todos los continentes excepto la Antártida. La invasión de *G. holbrooki* tiene su origen en el control biológico de mosquitos en zonas de presencia endémica del paludismo (Hildebrand, 1919; De Buén y De Buén, 1922). Sin embargo, casi no hay datos cuantitativos sobre la eficacia de *G. holbrooki* en el control de mosquitos en condiciones ambientales variables, por tanto su introducción mundial podría haber sido innecesaria, tal como pronosticaron ictiólogos de la época (De Buén, 1929). A pesar de ser extensa la literatura sobre cómo afectan los cambios ambientales en la biología de *G. holbrooki* (Tabla A2 en el anexo), es bastante desconocido cómo le afecta la eutrofización, bastante común en las masas de agua donde prolifera una de las especies de mosquito de más amplia distribución: el mosquito común *Culex pipiens* (Linnaeus, 1758).

Tabla 1. Principales fuentes de nitrógeno de origen antrópico, que puede llegar a los ecosistemas principalmente por dos vías. Adaptado de Camargo y Alonso (2006).

Fuentes puntuales
Aguas residuales procedentes de la actividad ganadera.
Emisiones de la industria de acuicultura.
Efluentes de aguas residuales, procedente de las estaciones depuradoras de aguas residuales (EDAR) y de zonas industriales.
Desborde de alcantarillas en episodios de lluvia torrencial.
Fuentes difusas
Cultivo de plantas fijadoras de nitrógeno.
Uso de estiércol y fertilizantes de nitrógeno inorgánicos (ej. salitre) en agricultura, y la posterior escorrentía.
Escorrentía procedente de zonas urbanas, zonas de vertido de residuos, lixiviados sépticos y de zonas de actividad minera.
Nitrógeno almacenado en aguas subterráneas que alimentan las aguas superficiales (ríos, lagos, estuarios).
Emisiones hacia la atmosfera por volatilización de los fertilizantes o la quema de combustibles y la posterior deposición atmosférica.
Movilización de nitrógeno almacenado en ecosistemas que funcionan como sumideros de nitrógeno, como la tala y quema de bosques y la desecación de zonas húmedas.

El nitrógeno: de nutriente a contaminante

La producción primaria depende de los compuestos de nitrógeno, entre otros nutrientes, que están en el medio natural como sales inorgánicas. Las formas principales de nitrógeno inorgánico son el amonio, tanto ionizado (NH_4^+) como no ionizado (NH_3), el nitrito (NO_2^-) y el nitrato (NO_3^-), todos ellos presentes en muy baja concentración en los ecosistemas acuáticos a menos que haya fertilización antrópica, supere su capacidad de asimilación natural y cause eutrofización (Tabla 1). En condiciones naturales la concentración de los diferentes compuestos de nitrógeno viene determinada por el ciclo del nitrógeno (Fig. 4). Los microbios heterótrofos (ej. bacterias, hongos) degradan la materia orgánica como heces y restos vegetales y generan NH_3 que se oxida a NO_2^- y NO_3^- por acción de las bacterias nitrificantes. Los dos primeros compuestos son muy tóxicos para la fauna acuática en bajas concentraciones[4] (ej. 1 mg/l) y, además, dado que su oxidación es rápida, incluso a bajas concentraciones de oxígeno, su presencia indica un vertido reciente de aguas con elevado contenido de materia orgánica (Jensen, 1995). Por el contrario, el nitrato es más estable en el medio y se acumula, a menos que sea asimilado por plantas y algas, o haya desnitrificación, es decir, una reducción a nitrógeno molecular (N_2) en condiciones anóxicas (Jensen, 1995; Camargo et al., 2005). De hecho, su concentración máxima permitida es de 50 mg/l, dos órdenes de magnitud mayor que el recomendado para el nitrito (Directiva 91/676 / CE; Noga, 2010). Por lo tanto, los organismos acuáticos han desarrollado más tolerancia a su presencia, que puede llegar a ser excepcionalmente superior a 300 mg/l en el medio natural o de 2000 mg/l en aguas de piscifactoría (Honda et al., 1993; ACA, 2014; Grup de Defensa del Ter, 2019).

Las administraciones públicas han desarrollado diferentes mecanismos para controlar los valores de nitratos en aguas superficiales, subterráneas y de consumo humano para proteger la fauna acuática pero sobre todo para garantizar la salud pública. Viendo que la problemática se puso de manifiesto especialmente durante los años 70 y 80, la Unión Europea implementó la Directiva 91/676/CE, de 12 de diciembre, relativa a la protección de las aguas contra la contaminación producida por nitratos procedentes de fuentes agrarias. Además de establecer un valor máximo para el agua de consumo humano, la Directiva Nitratos también obliga a los Estados miembros a elaborar un código de buenas prácticas agrarias, de aplicación voluntaria, y se introdujo el concepto de zona vulnerable a los nitratos, delimitación territorial donde sería necesario establecer un programa de actuación de obligado cumplimiento para prevenir la contaminación por nitratos. La Directiva 91/676/CE no fue transpuesta a la legislación española hasta 1996, con el Real Decreto 261/1996, donde se trasladaba a las comunidades autónomas la elaboración de los planes de actuación. A pesar de los esfuerzos para controlar los valores de nitrato, la realidad es que en zonas de alta actividad agrícola la normativa se encuentra repetidamente vulnerada (Figs. 5 y 6).

---

[4] En este trabajo todas las concentraciones se dan en mg/l total, por ejemplo mg NH_4^+/l, en contraste con la bibliografía norteamericana donde se acostumbra a dar en mg N-NH_4^+/l.

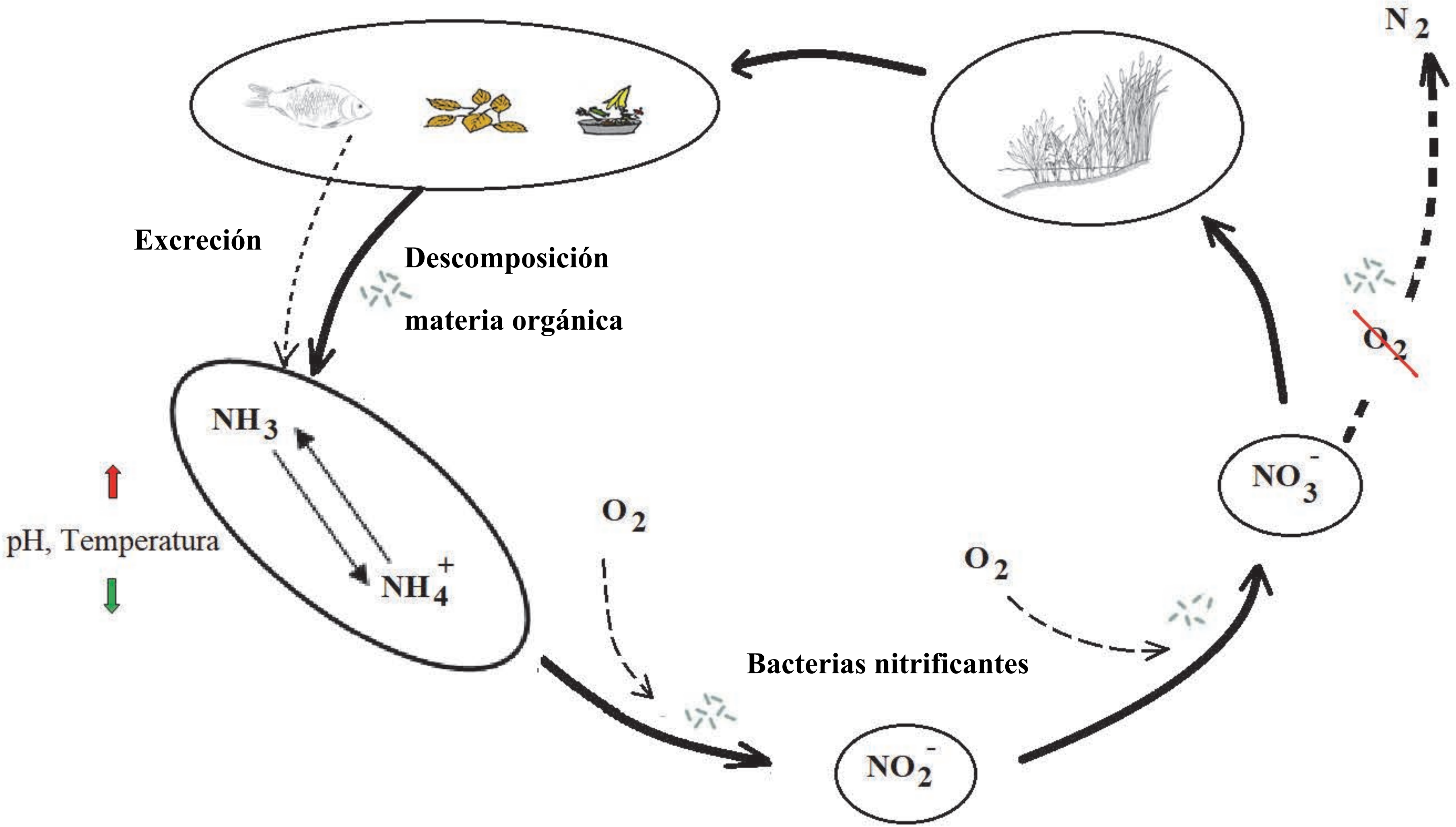


Figura 4. Fuentes principales de entrada y salida de nitrógeno en un ecosistema acuático.

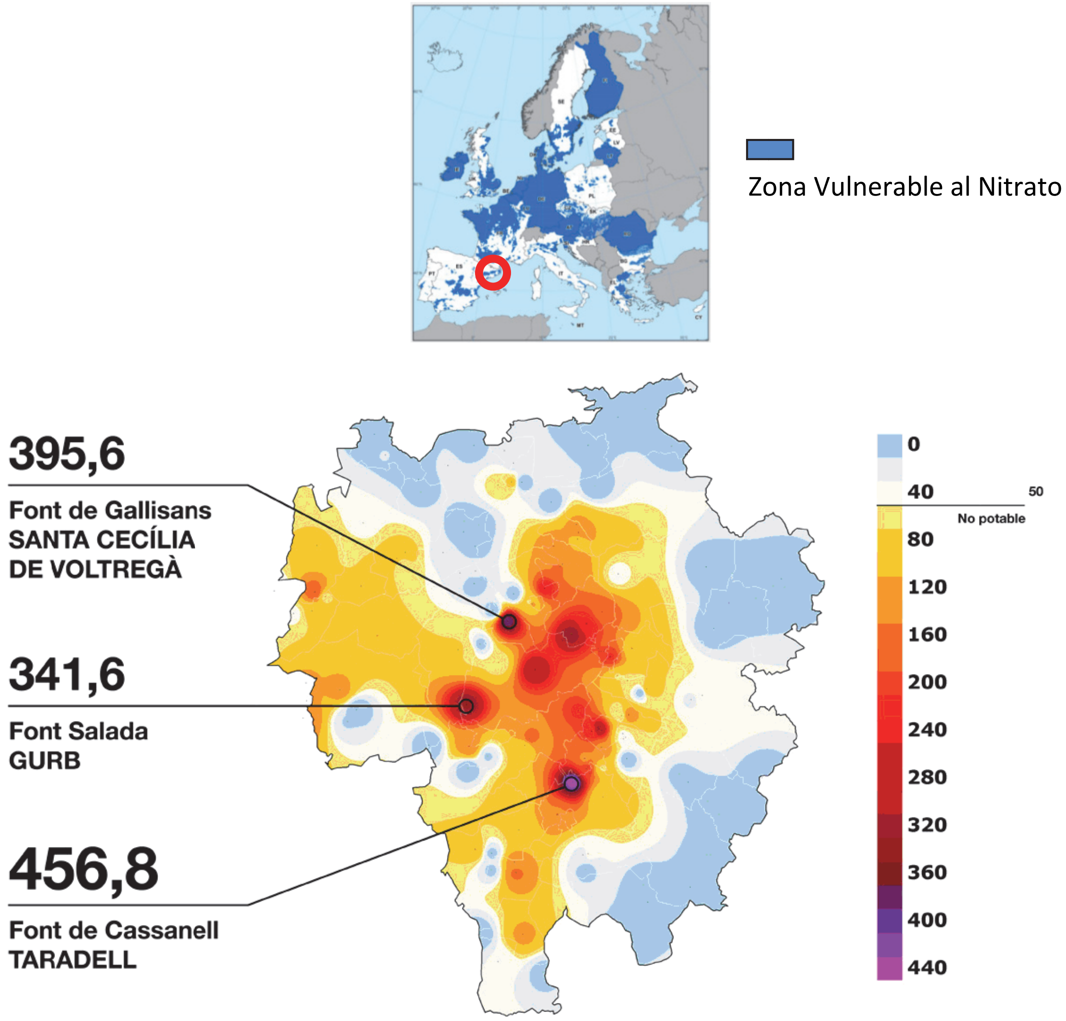
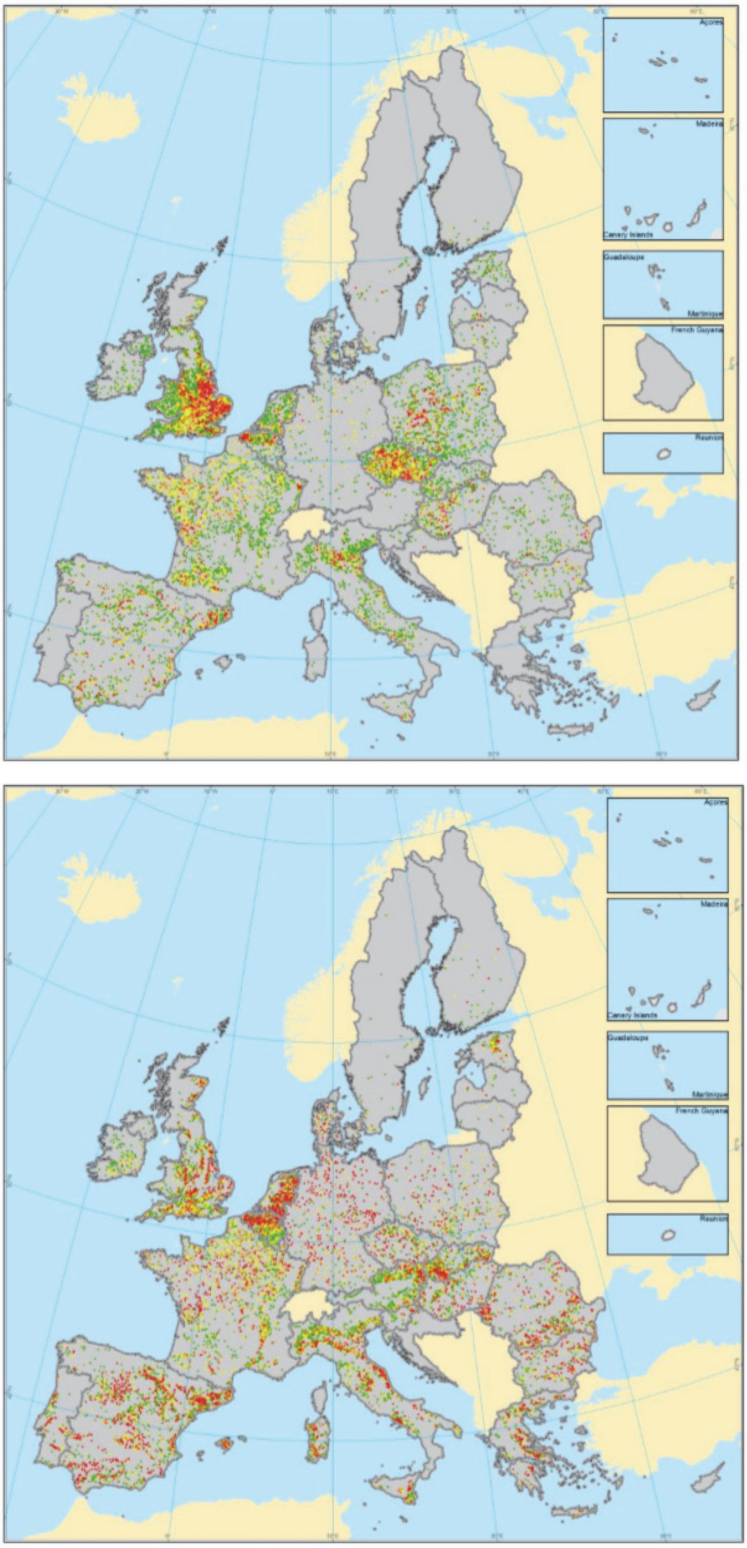


Figura 5. Mapa de las concentraciones de nitratos medidas en el 2019 en las fuentes de la comarca de Osona y Lluçanès, una zona declarada como vulnerable a los nitratos, debido a la alta actividad ganadera y la gestión ineficiente de los purines. Fuente: Grup de Defensa del Ter, 2019.

A pesar de su interés en estudios de eutrofización, la toxicidad del nitrato está poco estudiada en relación a la del amoníaco y el nitrito, y aún menos en condiciones de exposición crónica. Una buena parte de los estudios de toxicidad del nitrato han buscado la concentración letal (LC_{50}) en ensayos de 48 a 120 horas (Camargo et al., 2005; Hickey y Martin, 2009), aunque se han realizado algunos estudios de toxicidad crónica con parámetros no letales (ej. Hamlin et al., 2008; Adelman et al., 2009; Pereira et al., 2017). Se cree que el principal mecanismo de toxicidad no es debido al nitrato en sí, ya que tiene una baja permeabilidad de entrada por vía branquial, sino cuando éste se reduce a nitrito de forma endógena, principalmente en el tracto digestivo de los



◀**Figura 6**. a) Concentraciones máximas de nitrato (mg/l) durante el periodo 2012 – 2015 en aguas superficiales y subterráneas. De todos los ríos monitorizados en los 28 estados miembros, el 12 % y el 7 % se clasificaron como eutróficos e hipereutróficos, respectivamente. Los países con >50 % de ríos con esta categorización son Austria, Luxemburgo, España, Lituania, República Checa, Bélgica, Croacia, Malta e Inglaterra. Entre los lagos monitorizados, el 18 % se catalogaron como eutróficos y el 8 % como hipereutróficos. Los países con peores registros son Bulgaria, Croacia y Polonia. En estuarios, sólo 10 de los estados miembros tienen programas de seguimiento, con 6 estados del total de 10 que reportaron el 100% de sus localidades como eutróficas o hipereutróficas.

b) Concentraciones máximas de nitrato (mg/l) durante el periodo 2012 – 2015 en aguas subterráneas.

Concentración máxima

● **10 – 25 mg/l** ● **25 – 40 mg/l**

● **40 – 50 mg/l** ● **>= 50 mg/l**

Fuente: Comisión Europea (2018). Informe para el periodo 2012 – 2015 de la Comisión al Consejo y el Parlamento Europeos sobre la implementación de la Directiva 91/676/EEC sobre la protección de aguas contra la contaminación por nitratos causada por la agricultura.

vertebrados (Jensen, 1995). El nitrito, tanto endógeno como el que captan activamente los animales acuáticos a través de las células del cloruro de sus branquias, entra en el torrente sanguíneo y oxida los iones de Fe(II) a Fe(III) de la hemoglobina (Hb), la proteína responsable de transportar el oxígeno desde los órganos respiratorios hasta los tejidos en vertebrados (Lewis y Morris, 1986). La proteína resultante, la metahemoglobina (MetHb), que da a la sangre un característico color marrón, tiene muy poca afinidad con el oxígeno, provocando dificultades respiratorias al animal (Noga, 2010).

Si bien la toxicidad del nitrato en exposición crónica es bastante desconocida, hay evidencias de que renacuajos de sapo común *Bufo bufo* (Linnaeus, 1758), ciertos gammáridos y juveniles de trucha arco iris *Oncorhynchus mykiss* (Walbaum, 1792) estarían entre los taxones más afectados, incluso a concentraciones por debajo del umbral máximo establecido (Grabda et al., 1974; Baker y Waights, 1993; Camargo et al., 2005). Sin embargo, estos estudios también ponen de manifiesto que la toxicidad del nitrato depende de la salinidad, la dureza del agua, el tamaño corporal y la preexposición previa al nitrato, que aumentan la tolerancia (Johansson et al., 2001; Camargo et al., 2005; Baker et al., 2017). Por el contrario, se han descrito efectos sinérgicos con herbicidas (Pandey et al., 2011; Geyer et al., 2016) y la radiación ultravioleta (Hatch y Blaustein, 2003). Por lo tanto, todavía es pronto para calificar el nitrato de relativamente inocuo. Además de la mencionada metahemoglobinemia, el resto de alteraciones conocidas descritas en vertebrados acuáticos están principalmente relacionadas con afectaciones en la reproducción y el desarrollo por una disrupción endocrina (Tabla A1 en el anexo). Los mecanismos de acción del nitrato serían los siguientes: a) afectaciones en la osmorregulación por interferencia en la concentración intracelular de cloruro y yoduro, al ser el nitrato un ión monovalente que afectaría el transporte a través de la membrana celular, b) alteraciones neuroendocrinas por conversión de NO_3^- a óxido nítrico (NO), que es un neurotransmisor (Guillette y Edwards, 2005; Poulsen et al., 2018).

En gran parte de los estudios hechos con nitrato, de toxicidad aguda o crónica, la variable respuesta ha sido o bien mortalidad y crecimiento (véase Hickey y Martin, 2009; Adelman et al., 2009), o alteraciones a nivel bioquímico como el balance iónico y hormonal (Jensen, 1996; Hamlin et al., 2008). Entre las dos aproximaciones aparece un compromiso entre la relevancia ecológica y la rapidez de reacción al tóxico. El uso combinado de diferentes biomarcadores permite hacer un análisis más preciso del efecto del tóxico, ya que algunas alteraciones no suponen un peligro para la viabilidad del individuo, pero podrían estar impidiendo un correcto funcionamiento fisiológico. Sin embargo, estamos lejos de entender la correspondencia entre biomarcadores de naturaleza diversa, ni las conclusiones extraídas sobre la salud del animal (Colin et al., 2016). La incertidumbre se hace más patente si sólo se estudian los estadios inmaduros (ej. Pereira et al., 2017) o alguno de los dos sexos (Hamlin et al., 2008), ya que la ventana de toxicidad puede variar entre estos (Adelman et al., 2009). La integración de varios biomarcadores de respuesta temporal variante en el conjunto de edades y sexos de la población podría ayudar a dilucidar esta incertidumbre sobre el alcance del nitrato, por ejemplo en aspectos tan poco estudiados y de gran relevancia ecológica como alteraciones en la captura los nutrientes y en el almacenamiento de reservas energéticas (Guillette y Edwards, 2005; Poulsen et al., 2018).

Sólo aquellos rasgos biológicos, como los parámetros fisiológicos o la fecundidad, que varían dentro de una misma especie pueden responder rápidamente a la selección impuesta por el ambiente (Stearns, 1992). Condicionantes como la depredación o la calidad del hábitat influyen en el desarrollo de estos rasgos, que al final determinarán su eficacia biológica o *fitness* (Ricklefs y Wikelski, 2002). Así, la variabilidad interpoblacional resultado de la variabilidad ambiental se ha utilizado para estudiar qué condiciones favorecen en una dirección u otra determinados rasgos como el crecimiento individual, el tamaño y número de la descendencia entre otros (Côté et al., 1999; Benejam et al., 2009; Bassar et al., 2012). El estudio de los rasgos de historia de vida está ampliamente utilizado para entender el éxito invasor de una especie (ver Tabla S2 en el anexo). Ahora bien, del mismo modo que se puede aumentar la salinidad para limitar la expansión de *G. holbrooki*, el control del grado de eutrofización de las aguas, las características físicas del hábitat o el favorecer la presencia de depredadores naturales podría ayudar en el control de esta especie invasora o mitigar los efectos ecológicos derivados de su introducción (Larson et al., 2011).

OBJETIVOS

Objetivo general

El objetivo general de esta tesis es el estudio de la influencia que tiene la eutrofización en general y el exceso de nitratos en particular sobre la biología y ecología del pez invasor *G. holbrooki*. Es una tesis multidisciplinar que, a través de estudios observacionales de campo y experimentales en laboratorio y mesocosmos, explora la hipótesis de si la eutrofización estaría entre las causas que promueven el éxito invasor de *G. holbrooki*. En concreto, se dirá que *G. holbrooki* no ve afectado su éxito invasor debido al nitrato si tiene la capacidad fisiológica de tolerarlo y si el impacto de la invasión por *G. holbrooki* sobre especies y ecosistemas son comparables en presencia y ausencia del mismo.

Objetivos específicos

1. Evaluar el impacto de la introducción de *G. holbrooki* sobre la estructura de las comunidades acuáticas y los procesos ecosistémicos en condiciones de eutrofización por nitrato. Esperamos que la adición de nitrato incremente la producción primaria y que *G. holbrooki* incremente el efecto de la eutrofización al alimentarse de zooplancton. Sin embargo, su carácter omnívoro podría hacer que al depredar sobre el plancton, bentos y los detritos neutralice el impacto neto sobre las comunidades.

2. Evaluar el impacto de la exposición crónica del nitrato sobre la biología de *G. holbrooki* utilizando biomarcadores. La concentración de nutrientes es la variable sobre la que actúan los gestores para evitar la eutrofización, si bien en el experimento 1 la toxicidad directa por nitrato queda enmascarada por los efectos ecosistémicos. Es por ello que el objetivo 2 busca determinar los mecanismos fisiológicos de la toxicidad por nitrato, así como confirmar la hipótesis de que las poblaciones invasoras de *G. holbrooki* tienen una gran tolerancia a cambios ambientales como la fertilización por nitrato.

3. Estudiar los rasgos de historia de vida de *G. holbrooki* para explorar medidas de gestión efectivas en el control de su expansión. Conocidos los efectos directos e indirectos de la contaminación por nutrientes sobre *G. holbrooki*, este capítulo plantea cuál es la importancia relativa de estos en el conjunto de factores ambientales que pueden determinar el éxito invasor de la especie, como la complejidad de la hábitat físico o la abundancia de depredadores como las aves. Los gestores han de desgranar la importancia relativa de estos factores a la hora de establecer una aproximación ecosistémica para la gestión de las poblaciones invasoras de *G. holbrooki*.

4. Cuantificar la prevalencia de macroparàsits en las poblaciones invasoras de G. holbrooki. Junto con las variables de calidad del agua y de depredación por vertebrados, otro de los factores no analizados en el capítulo 3 que podría determinar el éxito invasor de la especie es la falta de parásitos según la hipótesis de la falta de enemigos naturales. Un primer apoyo para esta hipótesis sería que la prevalencia de parásitos y de lesiones en los individuos de *G. holbrooki* analizados fuera baja.

5. Comparar la efectividad en el control biológico de mosquitos en diferentes condiciones de turbidez entre *G. holbrooki* y un pez nativo amenazado. Sea por eliminación directa o a través de una aproximación ecosistémica, *G. holbrooki* es una especie a erradicar de acuerdo con la legislación vigente. Este capítulo plantea si especies nativas como el fartet *Aphanius iberus* (Valenciennes 1846) podrían sustituir *G. holbrooki* en el servicio ecosistémico del control de mosquitos en condiciones de eutrofización. Por otra parte, también se analiza si la eutrofización daría ventaja competitiva a la especie nativa en relación a la invasora.

DISCUSIÓN GENERAL

Esta tesis doctoral pretende profundizar sobre el conocimiento de los efectos directos e indirectos de la eutrofización por nitrato en *G. holbrooki*, una de las especies con más poblaciones invasoras en todo el mundo (Meffe y Snelson, 1989; Lowe, 2000). Los resultados más relevantes de sus cinco capítulos se exponen de forma integrada a continuación como recordatorio para el lector antes de la discusión final.

Gambusia holbrooki es una especie con un rango de tolerancia amplio a condiciones ambientales diversas (Homski et al., 1994; Pyke, 2005), y los capítulos I, II, y III confirman que la contaminación por nitrato, en el rango de concentraciones testado aquí (<10-425 mg / l), no le supone ningún problema grave. Sin embargo, la reducción en condición física y biomasa de *G. holbrooki* del capítulo I, probablemente debida a la pérdida de abundancia de presas en los tanques con nitrato podría tener otras causas añadidas, como un efecto tóxico directo del nitrato sobre el pez (capítulo II), cambios en los rasgos de historia de vida de *G. holbrooki* a consecuencia de la eutrofización (capítulo III) o cambios en la turbidez del agua que dificultarían la captura de presas (capítulo V). En los experimentos de toxicidad directa las afectaciones sólo aparecieron en machos y juveniles, y de carácter leve (capítulo II), pues si se encuentra generalizado en otras condiciones ambientales, podría explicar la proporción de sexos sesgada hacia las hembras en poblaciones invasoras y nativas (Hildebrand, 1927; Vargas, 1993). En cambio, sí se encontró una variabilidad interpoblacional en los rasgos de historia de vida, pero no debido a la disponibilidad de nutrientes sino regulada sobre todo por la densidad de *G. holbrooki* en cada localidad (capítulo III), patrón visto también en otros pecílidos (Bassar et al., 2012). Esto, junto a la ratio de sexos dominada por las hembras y la baja prevalencia de parásitos externos (capítulo IV), hace difícil el control de una especie tan prolífica, más allá de la eliminación directa de poblaciones enteras (ej. Ruiz-Navarro et al., 2013b). En caso de que algunas de estas actuaciones fueran viables localmente, habría que asegurarse de que con la desaparición de *G. holbrooki*, la propia comunidad nativa mantendría el servicio ecosistémico de control de mosquitos. Las condiciones turbias de eutrofización dificultaron la captura de mosquitos a *G. holbrooki* y *A. iberus*, y éste, si bien mostró una inferioridad competitiva, alcanzó junto con la comunidad de invertebrados por sí sola una reducción progresiva de la abundancia de mosquitos.

Integrando la representatividad ecológica y niveles de organización biológica crecientes

Es importante destacar el énfasis que se ha intentado establecer en este trabajo para incluir tanto estudios de campo observacionales, cuyos resultados son lo más cercano posible a la realidad que se quiere estudiar, pero a la vez complementado con estudios experimentales en mesocosmos y en laboratorio. Por otra parte, también se ha intentado integrar diferentes niveles de organización biológica, una aproximación recomendable siguiendo la premisa de que las perturbaciones afectan desde el individuo hasta el ecosistema, con un tiempo de respuesta creciente en efectos crónicos (Colin et al., 2016). La especialización creciente en la ciencia tiende a aislar el foco de estudio, es decir, la histopatología y la fisiología sitúan al individuo como unidad de estudio, mientras la ecología tiende a obviar los cambios individuales y se fija más en los flujos de materia y energía. Con la aproximación aislada de un histopatólogo se diría que las altas concentraciones de nitrato han tenido un efecto irrelevante, contradiciendo las conclusiones de un ecólogo, que diría que el nitrato ha hecho descender la condición física, la biomasa total de *G. holbrooki* y su efectividad en la depredación de mosquitos, derivados de un efecto indirecto.

Los experimentos de laboratorio permiten una máxima replicabilidad y aislamiento de efectos concretos que permiten una explicación mecanicista del efecto testado. Podemos inferir que las relaciones vistas y validadas estadísticamente se deben a una relación de causa-efecto, es decir, tienen una validez interna, pero desconocemos el grado de validez externa, que es el grado de confianza en los resultados obtenidos para poder generalizarse a otros animales, poblaciones o áreas geográficas (Campbell y Stanley, 1963). Concretamente, las inferencias de la depredación sobre mosquitos en condiciones de laboratorio podrían cambiar de forma en condiciones naturales donde las larvas de mosquito son un componente más de la comunidad de invertebrados. Los resultados de laboratorio y mesocosmos, sin embargo, fueron coincidentes. Posibles factores que amenazarían la validez externa en los experimentos de laboratorio descritos aquí serían por ejemplo la falta de interacciones macho - hembra porque estaban separados por sexos, que representarían un coste energético extra, o la imposibilidad para los individuos experimentales de escaparse de condiciones ambientales desfavorables, reacción que en determinadas circunstancias sí pueden hacer los animales en condiciones naturales. Es por ello que la realización de ensayos experimentales y estudios observacionales con escalas temporales y espaciales diferentes permite reducir ligeramente la incertidumbre sobre la validez externa a la vez que obtener un máximo conocimiento mecanicista.

La contaminación por nutrientes: un problema persistente 200 años después

Pese a haber un creciente interés en los últimos años en estudiar los efectos producidos por nuevos contaminantes emergentes como los microplásticos y los productos farmacéuticos o de cosmética, el viejo problema de la contaminación por nutrientes es uno de los principales problemas de degradación de los ecosistemas acuáticos en la actualidad (MEA, 2005). Los primeros problemas de contaminación orgánica graves aparecieron con la Revolución Industrial en Gran Bretaña, cuando el crecimiento poblacional, la creciente urbanización y la emisión directa de los efluentes de desecho sobrepasaron la capacidad natural de depuración de los ríos. El caso más célebre es la histórica contaminación del Támesis alrededor de Londres, el cual perdió la población de peces durante buena parte de los siglos XIX y XX, y hasta que no se mejoró el tratamiento de aguas residuales con la inclusión del tratamiento secundario no lo pudieron recolonizar (Mason, 1996). La entrada excesiva de nutrientes sigue siendo un problema actual en áreas donde no hay un tratamiento de aguas residuales, por ejemplo ciertas bahías del lago Titicaca (Fontúrbel, 2008) o en áreas de alta actividad agrícola como Osona y la Bretaña (Grup de Defensa del Ter, 2019; Ratmaya et al., 2019). En concreto, esta tesis ha examinado la toxicidad directa de uno de los nutrientes menos estudiados, el nitrato, y además pretende ser uno de los estudios más completos sobre las consecuencias de la exposición crónica del nitrato sobre un pez: la gambusia *G. holbrooki*.

La interacción de la gambusia con los nitratos

Los primeros dos capítulos de la tesis han tratado sobre el efecto directo de los nitratos sobre la fisiología de *G. holbrooki* y la interacción de ésta con el ecosistema receptor. La batería de biomarcadores a diferentes niveles de complejidad biológica sugieren una rápida aclimatación de *G. holbrooki* a una subida de la concentración de nitratos, pero ésta es más efectiva en hembras. Esta variabilidad entre sexos y estadios de vida tienen una relevancia ecológica nada despreciable. Muchas poblaciones silvestres de pecílidos tienen una distribución de sexos sesgada hacia las hembras (Snelson, 1989), incluyendo *G. holbrooki* (Hildebrand, 1927; Vargas, 1993). Teniendo en cuenta que al nacer, la ratio de sexos en pecílidos raramente se desvía de 1:1 (Snelson, 1989), este sesgo sería atribuible a una predisposición a sufrir una mayor mortalidad. Hay un puñado de estudios en gambusia donde los machos sucumbieron más rápido a diferentes estreses ambientales, incluyendo temperaturas altas, superpoblación, inanición e hipoxia (Krumholz, 1948; Winkler, 1975; Cech et al., 1985). Sin embargo, la falta de mortalidad en el experimento del capítulo II, junto con lo que se expondrá a continuación sugieren que la toxicidad del nitrato tendría unas consecuencias casi nulas a nivel poblacional. El experimento de laboratorio no estudió los efectos sobre la reproducción porque es uno de los aspectos de la toxicidad del nitrato mejor documentados (Edwards et al, 2006; Edwards y Guillette, 2007). No obstante, en caso de aparecer alteraciones en la población invasora estudiada, se cree que tendrían poco efecto ya que un solo macho dominante puede fertilizar varias hembras (Constantz, 1989). Además, las hembras almacenan esperma viable durante meses, por lo que una mortalidad repentina de la mayoría de machos por una exposición aguda a amonio o nitrato podría ser contrarrestada con el nacimiento de nuevos machos en la siguiente cohorte. Por último, una alimentación alterada en machos consecuencia del nitrato tendría un efecto menor a nivel del ecosistema. Experimentalmente, Fryxell et al. (2015) demostraron que las poblaciones de *G. holbrooki* con mayor proporción de hembras producían fuertes cascadas tróficas en comparación con poblaciones con mayor proporción de machos, causando en el primer caso reducciones sustanciales en la abundancia de zooplancton y el aumento del fitoplancton, la productividad, el pH y la temperatura. Esta mayor susceptibilidad en machos se encuentra extendida en muchos animales, incluso en los propios humanos, donde la esperanza de vida es mayor en mujeres. Las hembras ganarían aptitud incrementando su longevidad, que da la posibilidad de tener más camadas, mientras los machos ganarían aptitud incrementando las posibilidades de reproducirse (Bateman, 1948; Rolff, 2002). Para cumplirse esto las hembras deberían invertir más recursos en inmunidad (Rolff, 2002; Zuk, 2009) que proporciona, entre otros, resistencia a la polución ambiental.

El experimento de laboratorio, además de ayudar a comprender mejor que las poblaciones de *G. holbrooki* pueden estar sesgadas hacia las hembras, también permitió estudiar los mecanismos fisiológicos de la tolerancia al nitrato de *G. holbrooki*. En concreto, se menciona brevemente en la discusión del capítulo II, que los juveniles redujeron la acumulación de reservas energéticas, sin implicaciones en su crecimiento, lo que apunta a una prioridad en asignar la energía asimilada en crecer rápidamente (Fig. 1). La etapa de juvenil es la que tiene más riesgo de depredación, y dado que las redes tróficas están fuertemente estructuradas por tamaño (fitoplancton < zooplancton < zooplanctívoros < depredadores), el hecho de priorizar el crecimiento y alcanzar una determinada talla puede ser una medida de defensa para escapar de depredadores (Martin et al., 2017). Esta plasticidad para asignar la energía asimilada a uno u otro compartimento según las condiciones ambientales supone una ventaja en la colonización de un ambiente nuevo (Handelsman et al., 2013).

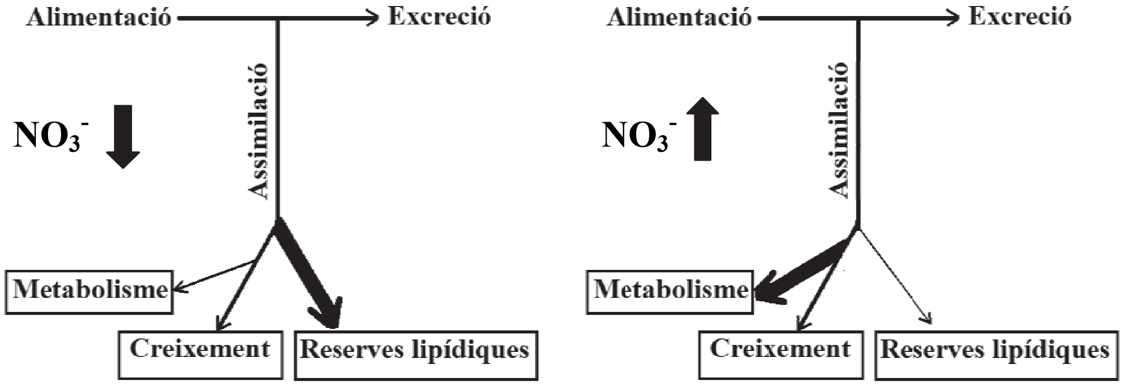


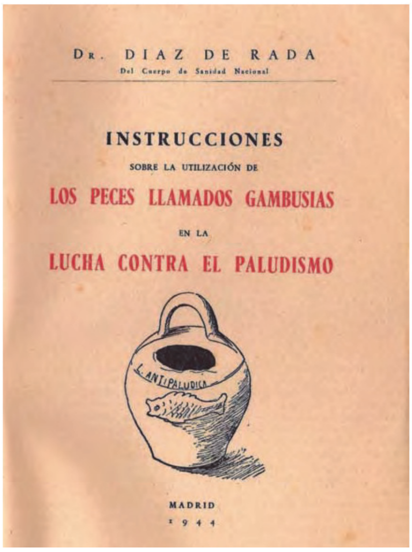
Figura 1. Esquema representativo de la hipótesis sobre la distribución de energía asimilada entre los costes de metabolismo, la energía destinada al crecimiento y la destinada a la reserva de lípidos. A la izquierda se muestra la situación de un hipotético individuo en un entorno bajo en nitratos, mientras la situación de la derecha representa el mismo individuo en un entorno contaminado.

Los resultados de los capítulos I y III contradicen, en parte, las conclusiones de un trabajo sobre los efectos del nitrato en el rango nativo de la especie (Edwards et al, 2006; Edwards y Guillette, 2007). Estos autores encontraron una correlación entre las concentraciones crecientes de nitrato (1-22 mg/l) y un aumento en el número de hembras no reproductoras, un descenso en el peso medio embrionario, así como un descenso en el número de espermatozoides y un aumento en el índice gonadosomático masculino. En el capítulo I el número de juveniles aumentó de forma marginal en los tanques con nitrato, mientras en el capítulo III los nutrientes totales, incluyendo el nitrato, hizo subir el peso medio embrionario. Los valores máximos de nitrato en las lagunas de Florida eran 2 y 19 veces inferiores a los de las localidades del presente trabajo y del experimento en mesocosmos, respectivamente, con lo cual es posible que haya una variabilidad interpoblacional en cuanto a la tolerancia a los nitratos, acentuada además por la condición de población nativa o introducida tal como se explicará a continuación.

Las translocaciones de peces como "filtros" de selección artificial

Los primeros envíos de peces para control biológico, sobre todo *G. holbrooki*, *G. affinis* y *Poecilia reticulata* (Peters, 1859) se hicieron a principios de siglo XX, a menudo en viajes transoceánicos que duraban semanas, como el del primer envío de *G. holbrooki* hacia Europa desde los Estados Unidos en 1921. Parece que el envío iba dirigido a Italia, pero ante la alta mortalidad sufrida se decidió detener el viaje y aclimatarlas previamente en España (Nájera, 1944; Navarro-García, 2013). Del lote inicial de 200 ejemplares solo llegaron 40, es decir, sufrieron una mortalidad del 80%, y antes de la liberación al medio natural se aclimataron en los acuarios del Instituto Español de Oceanografía en Madrid (Oliver, 2017). El transporte se hizo mediante bidones de hojalata, con oxigenación insuficiente y sin filtración, con cambios de agua parcial para evitar el deterioro excesivo del agua. Sin embargo, y teniendo en cuenta que los peces seguramente estaban confinados en grandes densidades, es probable que se acumularan grandes cantidades de compuestos nitrogenados y la concentración de oxígeno fuera baja, lo que explica la gran mortalidad, también expresada por otros autores en transportes más cortos (36-41% en un transporte de 48 h, Geiser, 1921). Actualmente las condiciones de transporte en el comercio de peces ornamentales han mejorado pero se siguen dando mortalidades que seleccionarían ciertos rasgos que proferirían una mayor resistencia (Wabnitz et al., 2003; Deacon et al., 2011).

Es probable que las últimas 12 gambusias supervivientes liberadas en Talayuela (Cáceres) fueran las más resistentes, o con más capacidad de aclimatación en condiciones de anoxia o eutrofización. Este efecto fundador, que efectivamente redujo la variabilidad genética de las poblaciones introducidas (Vera et al., 2016; Díez-del-Molino, 2018), podría no tener efectos deletéreos sobre su capacidad de invasión y de adaptación a nuevas condiciones ambientales si retiene una cierta variabilidad genética. La muerte de peces durante el transporte habría actuado como una especie de selección artificial inicial de individuos más resistentes a la toxicidad de compuestos nitrogenados, reforzada por las posteriores translocaciones entre localidades, realizadas en recipientes pequeños (Calero et al., 2014; Fig. 2) donde la acumulación de amoniaco también sería altamente probable. Una exposición repetida a condiciones tóxicas puede inducir una aclimatación y procesos microevolutivos que ayudarían a sobrevivir en estas condiciones poco favorables (Reid et al., 2016).



DR. DIAZ DE RADA
Del Cuerpo de Sanidad Nacional

INSTRUCCIONES
SOBRE LA UTILIZACIÓN DE
LOS PECES LLAMADOS GAMBUSIAS
EN LA
LUCHA CONTRA EL PALUDISMO

MADRID
1944

Figura 2. Folleto informativo distribuido por la Dirección General de Sanidad del Ministerio de Gobernación para difundir el uso de *G. holbrooki* como agentes de control biológico.

Esta selección artificial ocurrida durante el transporte podría haber afectado también la resistencia a infecciones por parásitos. Tal como explica Stephenson (2014), en un experimento con *P. reticulata* de Trinidad se introdujeron cuatro nuevas poblaciones desde una zona con fuerte depredación y parasitismo hacia otra con baja depredación y libre de parásitos. Los resultados no fueron los esperados, es decir, no se observó una resistencia a los ectoparásitos monógeneos menor en las poblaciones introducidas, sino que fueron más resistentes, hecho atribuido a la selección artificial durante el transporte y aclimatación de los individuos translocados. No se sabe si la baja prevalencia de parásitos externos observado en el capítulo IV se debe a la falta de parásitos específicos en áreas fuera de su rango nativo o por una mayor resistencia de las poblaciones invasoras, pero el hecho es que parece que les proporciona una ventaja en su éxito invasor.

Posibles interacciones con otros compuestos iónicos

Otro aspecto que podría haber afectado a los resultados sobre la toxicidad del nitrato en los capítulos I y II es que la fuente de nitrato fue la sal nitrato de sodio ($NaNO_3$). Aunque uno de los fertilizantes más utilizados en agroecosistemas actualmente es el nitrato de amonio (NH_4NO_3), este hubiera podido enmascarar los resultados obtenidos experimentalmente, ya que estaríamos vertiendo amonio a la vez que nitrato. Tradicionalmente, la mezcla de nitrato de potasio (KNO_3) y nitrato de sodio era el principal fertilizante nitrogenado mineral utilizado, el salitre o nitrato de Chile (Fig. 3).

No obstante, para facilitar los cálculos estequiométricos en todos los experimentos aquí descritos se ha utilizado nitrato de sodio exclusivamente. La presencia de sodio, sin embargo, tiene el efecto colateral de aumentar la salinidad del medio, lo que no es neutro a nivel fisiológico ni ecológico y merece ser discutido.

Figura 3. Característico anuncio del nitrato de Chile en España.

Ciertamente, en algunos estudios de toxicología donde se evalúa el efecto de algún ión, además de los controles con agua de red sin cloro, también incluyeron un control evaluando el efecto del ión con carga opuesta que forma el enlace iónico, el sodio en este caso. El estudio de Pereira et al. (2017) con pez cebra *Danio rerio* (Hamilton, 1822) incluyó este control de sodio, añadiendo NaCl para obtener una conductividad similar a su tratamiento más alto con nitrato, lo que dio una salinidad equivalente a ~ 2 ‰. En estas réplicas la evaluación histopatológica de branquia, piel, riñón e intestino no fue diferente que el de agua control. El único órgano con una afectación moderada fue el hígado, pero no afectó ni la supervivencia ni el crecimiento tal como pasó en el capítulo II. *Gambusia holbrooki* es un pez con una mayor tolerancia a la salinidad que *D. rerio* (Uliano et al., 2010), de hecho, individuos de *G. holbrooki* expuestos a salinidades de hasta el 20 ‰ tuvieron variaciones mínimas en la tasa de metabolismo en reposo (Akin y Neill, 2003). Además, el estudio de aclimatación a salinidades crecientes de Nordlie y Mirandi (1996) tuvo una supervivencia del 95% a 15 ‰ de salinidad. Con estos estudios previos podemos descartar un efecto significativo del sodio sobre *G. holbrooki* en las concentraciones utilizadas en el presente trabajo: al final del experimento en mesocosmos, los depósitos tenían una salinidad estimada a partir de la medida de conductividad de 0,80 ‰ para los controles y 1,18 ‰ para los de nitrato. En el laboratorio la salinidad de los acuarios a 250 mg/l de nitrato se estimó inferior a 0,85 ‰.

Respecto al experimento en mesocosmos, la subida de salinidad por influencia del sodio sí pudo haber influido moderadamente en la composición de invertebrados. Muchos géneros de oligoquetos, los pólipos de *Hydra* y ciertos grupos de crustáceos como cladóceros, copépodos y ostrácodos de agua dulce son muy sensibles a salinidades superiores a 1 ‰ (Tesh et al., 1990; Hart et al., 1991). Es posible pues que el sodio haya tenido un papel determinante en la pérdida de biomasa de invertebrados en el capítulo I, aunque los nitratos son también particularmente tóxicos para *Hydra* spp. (Tesh et al., 1990). Otra de las diferencias entre las condiciones experimentales del capítulo I y II es que en el de laboratorio había instalado en cada acuario un sistema de filtración biológica que reducía la concentración de amonio y nitritos al mínimo. En los mesocosmos del capítulo I se quiso evaluar el efecto del nitrato en condiciones más naturales donde el fitoplancton, los macrófitos y la desnitrificación fueran los responsables del mantenimiento de la calidad del agua. Los análisis en cromatografía líquida indicaron una concentración alta de nitritos (7,4 mg/l NO_2^- en las réplicas de nitrato, < 0,1 mg/l en los controles, de media), con lo cual hubo una reducción de nitrato a nitrito que no fue compensada por la nitrificación. Análogamente, en sedimentos de estuario se ha medido una mayor tasa de reducción de NO_3^- a NH_4^+ que tasa de desnitrificación a N_2, y un incremento notable de este patrón después de enriquecer el medio con nitrato (An y Gardner, 2002). Estas concentraciones de nitrito pueden ser efectivamente tóxicas, aunque las concentraciones de cloruro superiores a 300 mg/l presentes en los tanques actúan como agente protector (Lewis y Morris, 1986). Además, la aclimatación a la metahemoglobinemia a largo plazo hace que las diferencias entre las toxicidades crónicas y agudas sean mínimas (Lewis y Morris, 1986). La toxicidad de nitrito en invertebrados puede ser altamente variable, desde grupos más sensibles como los efemerópteros y gammáridos hasta los más tolerantes, oligoquetos y moluscos (Soucek y Dickinson, 2012). La presencia de nitritos evidentemente aporta ruido en la evaluación del efecto neto de los nitratos sobre la comunidad, y no se descarta que disminuyese la condición física y crecimiento de los peces expuestos, pero se ganó en verosimilitud de los resultados. De hecho en el estudio de campo del capítulo III, en las muestras donde el nitrato salió alto, gran parte de veces este fue acompañado de valores de nitrito por encima del promedio (hasta cuatro veces > 2 mg/l NO_2^-). En cualquier caso, estos procesos de oxidación y reducción de compuestos de nitrógeno podrían ocurrir de manera natural si se produjera un vertido de aguas con una elevada concentración de nitrógeno, tal y como sucede después de un derrame de agua de piscifactoría donde practiquen la recirculación (ej. 1036 mg/l N_{total}, Summerfelt et al., 1999), después de una recarga de agua superficial utilizando agua de pozo contaminada por nitrato (ej. 400 mg/l NO_3^-, García-Galán, 2010) o, también, la escorrentía de zonas con intensificación agrícola (ej. > 50 mg/l NO_3^-, Comisión Europea, 2018). De hecho, estas entradas de nutrientes llevan de rebote otros iones; así pues se cree que el diseño experimental utilizado tiene un realismo ecológico razonable.

El efecto del nitrato sobre los determinantes bióticos de la invasión de *G. holbrooki*

Esta tesis demuestra que *G. holbrooki* tiene una elevada tolerancia al nitrato y a las condiciones de eutrofización, si bien parece que la *fitness* de los individuos expuestos a eutrofización por exceso de nitrato sería menor (Capítulo I - menos biomasa y condición física). Además, se constató que el nitrato produjo un empobrecimiento de las comunidades de productores secundarios. Sin embargo, la pregunta que deja abierta la presente tesis es como la eutrofización puede afectar la resistencia biótica a la invasión de *G. holbrooki* ejercida por la presencia de especies nativas. En los experimentos de mesocosmos no se consideró la exposición de *A. iberus*, quizás la especie más estudiada donde se demuestre el desplazamiento debido a *G. holbrooki*, por dos motivos principales. Uno, el ético, *A. iberus* es una especie catalogada en peligro de extinción según la IUCN y en el Catálogo Nacional de Especies Amenazadas (Real Decreto 139/2011; Crivelli, 2006). En segundo lugar, la disponibilidad de réplicas, ya que se prefirió que hubiera al menos cinco réplicas por condición experimental que no tres, aumentando así la potencia estadística. Sin embargo, los experimentos de depredación de mosquitos en el capítulo V, junto con los resultados de los capítulos I y II, permiten hacer ciertas inferencias al respecto. No se disponen de comparaciones directas de toxicidad a nitrato en especies desplazadas por *G. holbrooki*, ni la tolerancia a la metahemoglobina, pero en el estudio de Homski et al. (1994) compararon la alimentación y mortalidad en condiciones de hipoxia del ciprinodóntido *Aphanius dispar* (Rüppell, 1829) y el pecílido *G. affinis*. Las dos especies toleraron concentraciones tan bajas como 1 mg/l, pero incluso *A. dispar* fue más resistente a < 1 mg/l. Cuando los expusieron dos semanas en las aguas eutróficas del río Alexander (Israel), la mortalidad de *A. dispar* fue ligeramente menor. En nuestro estudio *G. holbrooki* ha mostrado gran capacidad de aclimatación a la entrada excesiva de nitrógeno, pero a partir de los resultados del presente trabajo y los de Homski et al. (1994) tenemos indicios para pensar que el hecho de tener aguas eutrofizadas con condiciones de hipoxia y con una turbidez mayor no deberían suponer una ventaja directa para *G. holbrooki*. Posiblemente sea más importante la desalinización de las lagunas del delta del Llobregat debido a los regímenes hídricos dependientes del regadío, por ejemplo la Ricarda y la Murtra, que perjudican claramente *A. iberus*, más eurihalino. Sin embargo, en experimentos en mesocosmos de competencia trófica entre *A. iberus*, el samarugo *Valencia hispanica* (Valenciennes, 1846) y *G. holbrooki* las dos especies nativas presentaron un porcentaje mayor de digestivos vacíos, y *G. holbrooki* además tuvo un consumo absoluto de presas y una voracidad mayores (Caiola y de Sostoa, 2005). Ruiz-Navarro et al. (2013a) encontraron resultados similares en poblaciones naturales de la zona del Mar Menor (Murcia). El porcentaje de presas capturadas en acuario fue también mayor en *G. holbrooki* respecto a *Aphanius fasciatus* (Valenciennes, 1821), *A. iberus* y *V. hispanica*, así como la agresividad interespecífica fue siempre mayor en la especie no nativa, incluso en aguas de salinidad moderada (Rincón et al., 2002; Alcaraz et al., 2008). De hecho, hay observaciones sobre la mortalidad sufrida por la especie nativa cuando se pone en un mismo acuario con *G. holbrooki* (De Buén, 1929; Moreno y Morán, 1981). Hay por tanto una clara ventaja competitiva de la especie norteamericana bastante generalizada en un rango amplio de condiciones ambientales.

Además de los peces, no se puede olvidar otros vertebrados, como los anfibios, los reptiles o las aves que podrían ejercer resistencia biótica. En relación a las últimas, en el capítulo III se observó una reducción en el tamaño de los embriones y de los machos a abundancias crecientes de depredadores potenciales, correlación demostrada experimentalmente en *P. reticulata* de Trinidad, un pecílido muy utilizado como modelo evolutivo (Reznick y Endler, 1982; Stearns, 1992). En relación a los reptiles, si bien hay *Natrix maura* (Linnaeus, 1758) por la zona, no se dispuso de suficientes datos cuantitativos para incluir en los modelos. Y en relación a los anfibios, pueden ejercer competencia trófica o en el caso de los anuros depredación directa sobre juveniles de *G. holbrooki* (McCoid y Fritts, 1980). Mas todos estos organismos sufrirían las consecuencias de la toxicidad directa del nitrato como lo demuestran los trabajos de Camargo et al. (2005) y Hickey y Martin (2009). Además, algunos de estos depredadores potenciales depredan visualmente (Barlow, 1998), por tanto, la turbidez del agua dificultaría la captura de presas. Sin embargo, el conocer como favorecer el control *top-down* en las poblaciones invasoras de *G. holbrooki* en los ambientes degradados, donde normalmente habita, sería de gran interés en la lucha biológica.

Por último, conviene destacar en este apartado de resistencia biótica la competencia intraespecífica, que como se vio en el capítulo III es uno de los factores más importantes que afectan el ciclo reproductor de *G. holbrooki*. Está bastante demostrado el efecto negativo de la densidad sobre el crecimiento, el número de descendientes e incluso los costes energéticos (Marchand y Boisclair, 1998; Smith y Sargent, 2006); aún así, si *G.*

holbrooki mantiene una capacidad competitiva mayor que las especies del ecosistema receptor su potencial invasor se vería poco afectado.

Otros posibles mecanismos favorables a la invasión de *G. holbrooki*

Nordlie (2014) apunta la posibilidad de que las poblaciones de distribución reducida, por ejemplo en islas pequeñas o estanques y marismas aisladas, tengan unas tasas metabólicas inferiores que las de islas grandes o continentales, fenómeno visto también en aves y quirópteros (McNab 2012). Queda como incógnita si *G. holbrooki*, de amplia distribución, tiene una tasa metabólica superior a las especies endémicas ibéricas con un área de distribución más reducida y diseminada. Pero con los datos de captura de mosquitos del capítulo V y el resto de trabajos bibliográficos podemos intuir que así es. Como norma general, los individuos con una tasa metabólica superior tendrán un requerimiento mayor de alimentos y una agresividad incrementada (Lahti et al., 2002; Killen et al., 2011). Como se ha visto en el capítulo I, el exceso de nutrientes y productividad primaria ha comportado una pérdida de biomasa y riqueza de invertebrados, hecho también descrito en ríos y lagos someros cuando se supera un cierto umbral (Dodson et al., 2000; Romo et al., 2004; Pereira et al., 2016). ¿Una menor disponibilidad de presas facilitaría la invasión de una especie con unos requerimientos metabólicos superiores y una superioridad competitiva evidente? El autor sugiere que especies con una superioridad competitiva, en condiciones de escasez de alimentos obtendrán mayoritariamente los pocos recursos que quedan, excluyendo las menos competitivas.

Las especies que tengan también una mayor capacidad de convertir los recursos en reproducción alcanzarán una densidad mayor en el ecosistema, atesorando la mayoría de recursos energéticos y espaciales en detrimento de los competidores (Fig. 4). Es difícil, sin embargo, de medir el potencial reproductor, ya que requiere medir la productividad de la población. Una estimación grosera sería el índice gonadosomático: en un estudio hecho en el delta del Ebro, se comparó el índice gonadosomático de individuos de *A. iberus* y *G. holbrooki* capturados en la misma laguna y por tanto con una disponibilidad de recursos similar (Vargas, 1993). Tanto los machos como hembras de *G. holbrooki* presentaron índices gonadosomáticos superiores a *A. iberus*, de casi el doble en el mes de máxima actividad reproductora. Sin embargo, el hecho de ser ovovivípara le da a *G. holbrooki* una protección a las crías que podría explicar en parte su éxito reproductor. En el trabajo de Homski et al. (1994) 50 ejemplares de *G. affinis* y *A. dispar* cada uno se escaparon de la jaula experimental por una subida del nivel del agua y al cabo de seis meses la población de *G. affinis* se había reproducido satisfactoriamente hasta alcanzar grandes densidades, mientras *A. dispar* fracasó en reproducirse. No son las mismas especies que las de la península ibérica, pero sirven para inferir los potenciales mecanismos de superioridad competitiva. El hecho es que tal y como se muestra en la tabla S2 del capítulo V, las densidades de *Gambusia* spp. buscadas en la bibliografía fueron ligeramente mayores que las de *Aphanius* spp, apoyando el patrón de abundancias de especies nativas - introducidas a nivel global (Hansen et al., 2013). En este metaanálisis la diferencia entre las medias de abundancia fue de 2,7 veces superior para las poblaciones introducidas, una diferencia muy similar a la expuesta en la tabla S2.

Aunque la biología de invasiones ha girado en gran parte en torno a la propuesta de ecosistemas desequilibrados de Elton, la teoría de selección natural de Darwin ya había establecido un marco teórico para explicar ciertos patrones de invasiones biológicas en las que la superioridad competitiva se hacía evidente:

> *La selección natural actúa por competencia, adapta los habitantes de cada región sólo en relación con el grado de perfeccionamiento de sus asociados. [...] No nos deberíamos sorprender que los habitantes de cualquier región, aunque supusiéramos que están especialmente creados y adaptados para este hábitat, se encontraran batidos y suplantados por otros organismos producidos naturalmente en otro territorio.*
>
> *C. Darwin, 1859*

Zonas con una alta diversidad genética serían por tanto más propensas a ser fuentes de organismos invasores, mientras zonas con menor diversidad filogenética serían más propensas a ser invadidas, lo que se conoce como hipótesis del desequilibrio evolutivo (Fridley y Sax, 2014). Este patrón se ha comprobado por ejemplo en la asimetría entre el número de especies intercambiadas entre el mar Rojo y el mar Mediterráneo, siendo este último el mayor receptor.

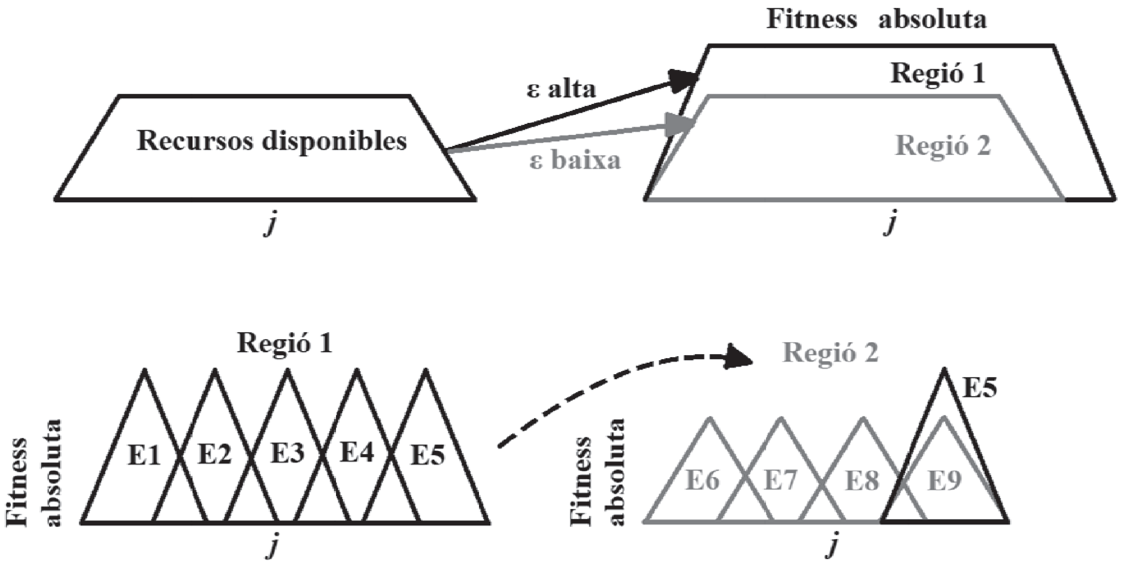


Figura 4. El potencial invasor varía entre regiones, como resultado de aptitudes o *fitness* absolutas diferentes. La variación genética (tamaño de población, área) y la intensidad competitiva (número de competidores) podrían provocar desequilibrios evolutivos, manifestados en su potencial reproductor (ε) variable entre regiones. Así, las especies (E) de la región 1 tienen un ε mayor que las de la región 2, es decir, en un gradiente ambiental (*j*), la capacidad de convertir los recursos en descendencia es mayor, y aumenta la *fitness* absoluta. Cuando por acción humana se introduce una especie de la región 1 (E5) a la región 2, esta es competitivamente superior y puede suplantar especies de la región 2 que utilicen un abanico de recursos parecido. Adaptado de Fridley y Sax (2014).

La gestión de las poblaciones de *G. holbrooki* invasoras en el área de estudio

El autor cree que esta tesis ha contribuido al conocimiento mecanicista de los efectos de la contaminación por nitratos y la invasión por *G. holbrooki*, pero también pretende contribuir a la conservación del área de estudio. A menudo estos mensajes de gestión regional quedan difuminados en los artículos internacionales y la tesis es un buen foro para hacerles mención. La gestión de las invasiones requiere un conocimiento de las características biológicas con el objetivo de predecir el potencial invasor (Simberloff, 2003). El hecho de que la propia densidad de *G. holbrooki* sea el factor más determinante en regular su estrategia reproductiva hace muy difícil la reducción efectiva de las poblaciones, ya que pueden recuperar en poco tiempo sus abundancias previas si la extirpación no es completa, fenómeno conocido como "efecto rebote" y que se ha descrito también en especies sujetas a caza como los jabalíes y castores (Servanty et al., 2011; Parker y Rosell, 2014). Esta influencia tan alta de la densidad intraespecífica sobre los rasgos de historia de vida se ha descrito también en *P. reticulata* de Trinidad. Se comprobó que modificando experimentalmente las densidades de *P. reticulata* en cada una de las pozas naturales y en mesocosmos los peces respondían incrementando o disminuyendo la supervivencia, el crecimiento, el número de embriones y la actividad reproductora para adecuar la tasa de crecimiento poblacional a las nuevas condiciones impuestas (Bassar et al., 2012).

Por tanto, las acciones más efectivas serían las actuaciones repetidas para erradicar la población (Ruiz-Navarro et al., 2013b). Ahora bien, en un hábitat degradado y en presencia de varias especies invasoras como la tortuga de Florida *Trachemys scripta elegans* (Wied, 1839) y el cangrejo americano *Procambarus clarkii* (Girard, 1852) en el delta del Llobregat, la simple erradicación de *G. holbrooki*, sin afrontar de forma integrada el resto de problemas, no llevará a una recuperación ecológica del ecosistema (Prior et al., 2018). En determinadas situaciones la recuperación ecológica es muy rápida, como en el caso de la eliminación de la carpa europea *Cyprinus carpio* (Linnaeus, 1758) de las lagunas de Medina y Zóñar en Andalucía. En pocos meses se restauró la cobertura de macrófitos y la comunidad de aves acuáticas original (Maceda-Veiga et al., 2017). Hay que tener en cuenta, sin embargo, las diferencias entre intentar eliminar la población en un espacio pequeño y confinado, donde un simple secado del estanque podría ser suficiente, o grande y con múltiples metapoblaciones. Incluso, en albercas aisladas se podría valorar la posibilidad de no intervenir en la población si no supone una fuente de nuevos propágulos, es decir, si no es de acceso público. En el caso de la zona muestreada del delta del Llobregat, hay un número grande de poblaciones estables y otras temporales que colonizan nuevos hábitats y desaparecen siguiendo el ciclo estacional hidrológico, en el caso por ejemplo de la localidad L1 en la Ricarda, o poblaciones situadas en canales que se encuentran sujetos a los calendarios de riego. Para comprobar el estado de las 18 localidades del capítulo III se volvieron a visitar en mayo de 2019 ex profeso, cuatro años después de hacer el muestreo. Dadas las limitaciones de formato exigidas en una tesis por publicaciones, se ha decidido incluir estos resultados en este apartado por ser considerados de interés para mejorar la gestión de esta especie exótica. El hecho de que en ocho de las dieciocho localidades los peces hayan desaparecido definitiva o de forma intermitente en el período 2015 - 2019 (Tabla A3), ya sea por acción humana o por dinámicas hidrológicas, indica que la viabilidad de algunas poblaciones es altamente estocástica. En estos casos sería recomendable hacer un inventario previo de las poblaciones estables y las que tienen una mayor capacidad de dispersión, para evitar acciones de control innecesarias en localidades donde posiblemente desaparecerían a corto plazo. Por ejemplo, si se agrupan en clústeres las diferentes localidades del capítulo III según rasgos de historia de vida se pueden diferenciar aquellas en que los peces tuvieran un mayor esfuerzo en reproducción y en la producción de propágulos (clúster 1), de las localidades donde los peces invirtieron más recursos en el mantenimiento somático y el crecimiento en hembras (clúster 2). En cambio, el clúster 3 se caracterizó por un crecimiento menor en la época de muestreo, seguramente porque ya habían alcanzado la asíntota de crecimiento (Fig. A1 y Tabla A4). Este agrupamiento según tipologías, sugerido como simple ejemplo que habría que perfeccionar y desarrollar a mayor escala, permitiría al gestor localizar los estanques o canales que contengan individuos con un fenotipo que favorezca altas fecundidades, lo que alertaría sobre una posible mayor tasa de dispersión en caso que se hiciera alguna translocación con individuos originarios de estas balsas. Esto permitiría focalizar las medidas de control en localidades concretas, al tiempo que optimizar la gestión de recursos en la lucha biológica de las invasiones.

Aunque en esta tesis no se han podido encontrar factores ambientales que permitan controlar el crecimiento poblacional de *G. holbrooki*, la gestión de las invasiones mediante esta aproximación ecosistémica puede tener resultados satisfactorios en casos concretos, sobre todo en distribuciones a gran escala en que la erradicación es imposible, muy cara y potencialmente contraproducente (Zavaleta et al., 2001; Carroll, 2011). Por ejemplo, en espacios abiertos o si la vegetación es abundante el uso de rotenona ha supuesto un fracaso en la eliminación de pecílidos (Rayner y Creese, 2006). En cambio, si se tienen en cuenta otras acciones, como la restauración de las especies nativas para incrementar la competencia con la invasora y favorecer la coexistencia, o restaurar un balance de nutrientes desequilibrado que estuviera favoreciendo una determinada especie en detrimento de las nativas, la recuperación puede ser gradual y en algunos casos exitosa (Hulme, 2006; Carroll, 2011).

Aphanius iberus, el fartet atlántico *Aphanius baeticus* (Doadrio, Carmona & Fernández-Delgado, 2002) y otros peces de la misma posición trófica como *V. hispanica*, y el espinoso, *Gasterosteus aculeatus* (Linnaeus, 1758), han sufrido una regresión paulatina en sus distribuciones naturales. *Aphanius iberus* fue descrito como muy abundante en áreas como el delta del Llobregat (Maluquer, 1916) y la Albufera de Valencia (Steindachner, 1865), zonas donde actualmente *G. holbrooki* se encuentra ampliamente distribuida. Realmente, discernir a posteriori si *G. holbrooki* fue un oportunista que aprovechó la degradación de los hábitats originales o fue un agente activo de cambio en las comunidades bióticas es difícil de decir, incluso con los resultados de esta tesis, ya que las dos perturbaciones se dieron generalmente al mismo tiempo. La gran transformación de la zona húmeda del Llobregat se produjo durante el primer tercio del siglo XX, con el aumento de la población (entre 1920 y 1936 se pasó de cerca de sesenta mil habitantes a más de noventa y cinco mil, con unas instalaciones de alcantarillado y depuración inexistentes), el cambio a un régimen agrícola de regadío, la desecación de zonas húmedas y la construcción del aeropuerto en 1916 (Montijano y Lucena, 2013). Paralelamente, en 1921 *G. holbrooki* se introdujo en Europa, concretamente en la provincia de Cáceres y se fue distribuyendo sobre todo en las provincias con mayor incidencia del paludismo, y en 1942 ya tenemos constancia de una distribución similar a la actual (Lozano, 1946; Fig. 5). La creación de viveros en varias provincias facilitó la dispersión gratuita promovida por diferentes instituciones administrativas de Sanidad, por las confederaciones hidrográficas y posteriormente por el Instituto Nacional de Colonización franquista (Navarro-García, 2013). Esta evidencia clara de la estrecha relación entre la distribución endémica histórica del paludismo y la de *G. holbrooki* actual es quizás la prueba fehaciente de que por encima de los factores abióticos como la temperatura, la salinidad, la contaminación por nitratos o la degradación del hábitat, la dispersión por acción humana es el principal factor facilitador de la invasión de *G. holbrooki*. El flujo genético entre diferentes poblaciones no conectadas, descrito en diferentes poblaciones ampurdanesas y neozelandesas es una evidencia de que las translocaciones por acción humana son frecuentes y contribuyen en gran medida a su expansión (Purcell y Stockwell, 2014; Díez-del-Molino, 2016). Siguiendo estas políticas de distribución gratuita de *G. holbrooki* por parte de los Institutos Provinciales de Higiene y los Dispensarios antipalúdicos en el siglo pasado (Calero et al., 2014), el autor propone la creación de un vivero de *A. iberus*, como el que gestionaba el Consorcio para la Protección de los Espacios Naturales del Delta del Llobregat, y que ahora continúa por medio de la Fundación Barcelona Zoo, para promocionar su reintroducción en estanques de ciudadanos o entidades interesadas en participar de la conservación de una especie amenazada. La remesa de *A. iberus* debería hacerse siempre con un control oficial para saber el destino de las reintroducciones y evitar la introgresión en otras poblaciones que harían perder la diversidad de genotipos locales.

Por último, y sin restarle importancia, hay que recordar que la prevención de nuevas introducciones o translocaciones y una buena campaña informativa ciudadana sobre la liberación de animales domésticos son elementos esenciales. En un momento de máximo volumen de pasajeros y mercancías y con la progresiva subida de temperaturas es posible que ciertas enfermedades transmitidas por mosquitos amplíen su rango de distribución, aparezcan brotes epidémicos como los de los virus Zika y Chikungunya en América del Sur y el Caribe, o reaparezcan casos endémicos de enfermedades que ya habían sido erradicadas localmente (Costello et al., 2009; Azevedo-Santos et al., 2016). Esta situación puede conllevar nuevas introducciones de peces como arma de control biológico en nuevos países, a menudo impulsada por las mismas administraciones. Actualmente se están realizando nuevas introducciones de *P. reticulata* para combatir la expansión del dengue en Brasil y Pakistán (El-Sabaawi et al., 2016), o *G. affinis* en la India (NVBDCP, 2019). Incluso en una búsqueda rápida en una página de compraventa por Internet en España aparecen particulares que venden y envían individuos de *G. holbrooki* en línea. Sin embargo, la mejora de las condiciones sanitarias, de la salubridad de las aguas circundantes, evitar la acumulación de agua en pequeños volúmenes de agua estancados y la mejora de los tratamientos son medidas más efectivas para evitar la proliferación de mosquitos (Azevedo-Santos et al., 2016). Aunque no se sabe todavía a ciencia cierta si la presencia de peces larvífagos contribuyen en gran medida a la reducción de la incidencia de estas enfermedades (Walshe et al., 2017), si se quiere utilizar peces como agentes de control biológico debería ser siempre mediante las especies nativas de cada región (Courtenay y Meffe, 1989; Rupp, 1996).

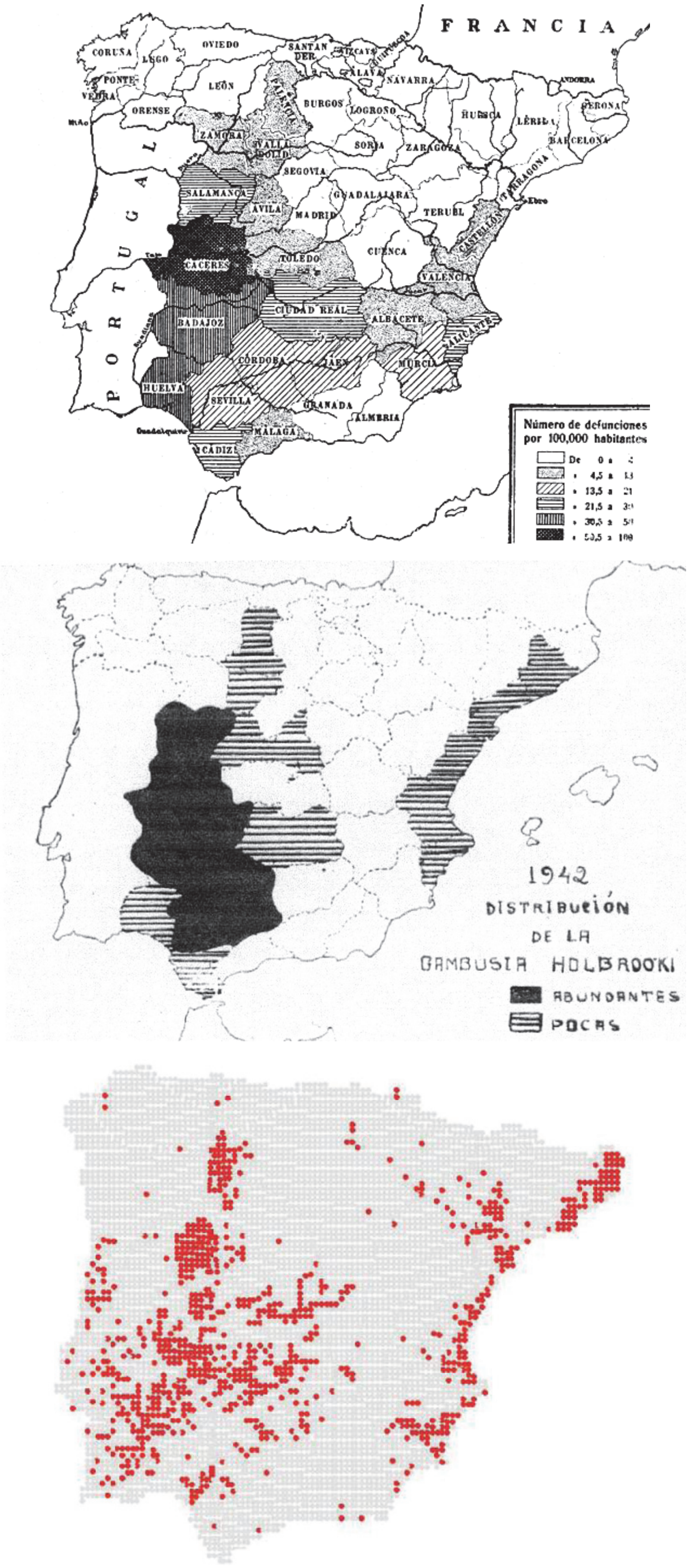


Figura 5. a) Defunciones causadas por las fiebres palúdicas en la España peninsular durante el año 1921. b) Distribución de *Gambusia holbrooki* en España el año 1942. c) Mapa de registros de distribución actual de *G. holbrooki* a fecha de 2015. Fuentes: Pittaluga, 1923; Lozano, 1946; Murphy et al., 2015.

CONCLUSIONES

1. El experimento en mesocosmos mostró que los efectos de la introducción de *G. holbrooki* sobre la comunidad biótica y los procesos ecológicos fueron comparables en presencia o ausencia de la fertilización por nitratos, produciendo cambios en la estequiometría del material en descomposición y la producción primaria, pero de una magnitud menor en comparación a los cambios provocados por los nitratos y la sucesión temporal.

2. El nitrato redujo la biomasa pero no la abundancia de *G. holbrooki*, probablemente debido a la menor disponibilidad de presas, un efecto tóxico directo del nitrato o la plasticidad en la reproducción que haga variar el número y tamaño de la descendencia.

3. El experimento de laboratorio confirmó que la exposición crónica a nitrato afecta a la capacidad de *G. holbrooki* de capturar presas y de almacenar energía, sin cambios en la condición física. Estos efectos se observaron en juveniles y machos, pero no en hembras.

4. Esta tolerancia podría explicar que la proporción de sexos de las poblaciones de *G. holbrooki* estén, en general, sesgadas a favor de las hembras, aunque esta tesis no estudió los efectos directos de la contaminación sobre la reproducción de *G. holbrooki*.

5. El estudio de los condicionantes ambientales que determinan el éxito invasor *G. holbrooki* ofrecieron pocas esperanzas a una estrategia de gestión basada en cambios en la calidad del agua o del hábitat físico en estanques. No obstante, favorecer la presencia de depredadores nativos podría ayudar al control cuando la erradicación directa no fuera posible.

6. La propia densidad de *G. holbrooki* puede limitar su crecimiento poblacional, pero se vislumbra una gran plasticidad en los rasgos de historia de vida. Las infecciones parasitarias parecen jugar un papel poco importante en las poblaciones introducidas, aunque la presencia de muchos individuos típicamente se asocie a una mayor posibilidad de contagios.

7. La introducción de *G. holbrooki* no parece justificada por el control de mosquitos dado que una especie de tamaño similar y amenazada, el fartet (*Aphanius iberus*), mostró una efectividad similar, pero más lenta, en condiciones de eutrofización o de aguas limpias. De hecho, una comunidad de invertebrados nativos por sí sola puede ejercer este control biológico.

8. La turbidez derivada de la eutrofización no dio superioridad competitiva a *A. iberus*, quien, en todas las condiciones experimentales, fue superado en velocidad de captura de presas por *G. holbrooki*, considerada la principal especie responsable de su regresión.

9. Esta tesis confirma, proveyendo de evidencia experimental en eutrofización, que *G. holbrooki* es una especie con una amplia tolerancia a las condiciones ambientales.

10. Conocida la dificultad de gestionar las poblaciones invasoras de *G. holbrooki*, es prioritario desarrollar campañas de educación ambiental para evitar más introducciones y que pongan en valor servicios ecosistémicos como el control de mosquitos que suministran las especies nativas.