



Universitat de Lleida

Latitudinal and altitudinal variation of life history traits in natterjack toads (*Bufo calamita*): genetic adaptation vs. phenotypic plasticity

Neus Oromí Farrús

ADVERTIMENT. La consulta d'aquesta tesi queda condicionada a l'acceptació de les següents condicions d'ús: La difusió d'aquesta tesi per mitjà del servei TDX (www.tesisenxarxa.net) ha estat autoritzada pels titulars dels drets de propietat intel·lectual únicament per a usos privats emmarcats en activitats d'investigació i docència. No s'autoritza la seva reproducció amb finalitats de lucre ni la seva difusió i posada a disposició des d'un lloc aliè al servei TDX. No s'autoritza la presentació del seu contingut en una finestra o marc aliè a TDX (framing). Aquesta reserva de drets afecta tant al resum de presentació de la tesi com als seus continguts. En la utilització o cita de parts de la tesi és obligat indicar el nom de la persona autora.

ADVERTENCIA. La consulta de esta tesis queda condicionada a la aceptación de las siguientes condiciones de uso: La difusión de esta tesis por medio del servicio TDR (www.tesisenred.net) ha sido autorizada por los titulares de los derechos de propiedad intelectual únicamente para usos privados enmarcados en actividades de investigación y docencia. No se autoriza su reproducción con finalidades de lucro ni su difusión y puesta a disposición desde un sitio ajeno al servicio TDR. No se autoriza la presentación de su contenido en una ventana o marco ajeno a TDR (framing). Esta reserva de derechos afecta tanto al resumen de presentación de la tesis como a sus contenidos. En la utilización o cita de partes de la tesis es obligado indicar el nombre de la persona autora.

WARNING. On having consulted this thesis you're accepting the following use conditions: Spreading this thesis by the TDX (www.tesisenxarxa.net) service has been authorized by the titular of the intellectual property rights only for private uses placed in investigation and teaching activities. Reproduction with lucrative aims is not authorized neither its spreading and availability from a site foreign to the TDX service. Introducing its content in a window or frame foreign to the TDX service is not authorized (framing). This rights affect to the presentation summary of the thesis as well as to its contents. In the using or citation of parts of the thesis it's obliged to indicate the name of the author.

**Latitudinal and altitudinal variation of
life history traits in natterjack toads
(*Bufo calamita*):
genetic adaptation vs. phenotypic plasticity**

DISSERTATION

To obtain the degree of Doctor at the University of Lleida

MEMÒRIA DE TESI

Per optar al grau de Doctor per la Universitat de Lleida

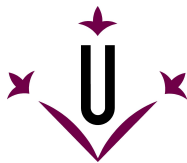
By

Per

NEUS OROMÍ FARRÚS

October 2011

Octubre 2011



Universitat de Lleida
Escola Tècnica Superior
d'Enginyeria Agrària



Cover designer: Ares Juclà Reche
Cover photograph: Albert Gispert

DIRECTORS:

Dr. Delfí Sanuy Castells.

Departament de Producció Animal. Universitat de Lleida.

Prof. Dr. Ulrich Sinsch.

Universität Koblenz-Landau.

Dr. Joan Fibla Palazón.

Departament de Ciències Mèdiques Bàsiques. Universitat de Lleida.

A la meva família,

*A man who knows how little he knows is well,
a man who knows how much he knows is sick.*

Lao Tzu as interpreted by Writter Bynner

Agraïments

Ja fa uns anys que em vaig endinsar en el món de la investigació perquè creia que podria trobar respostes a algunes preguntes. Però, la veritat, ha estat tot al contrari. Se m'han obert nous horitzons i els petits dubtes que tenia, la majoria no només hi segueixen sent, sinó que s'han fet més complexes. M'imagino que això és "fer ciència".

Voldria agrair a tots aquells qui han fet possible la realització de la meva tesis el seu suport incondicional:

Primerament, als meus tutors Delfí Sanuy, Joan Fibla i Ulrich Sinsch per el seu suport en tot el desenvolupament de la tesis.

Als meus pares, Pepe i Dolors, i germans, Mireia i Josep Ramon, i a l'Albert, per ser els millors. També a tota la meva família.

Als meus companys de camp i laboratori: a l'Emile per haver estat sempre al meu costat, corregint-me el meu catalanitzat anglès i acompanyar-me en tots els mostrejos; i a la Marina, per la seva paciència, per aguantar el meu desordre al laboratori i per ser més que una companya, una bona amiga. També als estudiants Pau, Joan, Gispi i Miquel.

Als "botànics", Xavi i Alex, per acompanyar-me al camp i aconsellar-me tant professionalment com personalment. I a les "tresines" Carolina i Gemma, i als respectius "tresinos" Manel i Aaron per donar-me tan bones vetllades i tan apreciats consells. I al "tresinet" Pau, per fer-me entendre que les coses senzilles són les més valuoses.

A la meva gran amiga Laura per estar sempre al meu costat, i a la Paula, per deixar-me ser encara una nena. A la Maria, a la Mònica, a l'Elena, a la Núria, al Joan, a l'Ares, al Jaume, a l'Anival... i a tots aquells que compartim tan bones estones.

A la Carolina, la Tresa i l'Anna del Departament de Producció Animal i a la Imma i l'Anna de la Facultat de Medicina pels seus consells. A la Lidon, l'Emi i el Sergi del Departament d'Enginyeria Agroforestal.

A tot el Departament de Producció Animal i a la Universitat de Lleida per la beca FPI-UdL i per haver finançat la meva estada a Alemanya. Al Departament de Ciències Mèdiques Bàsiques i a la Universitat de Koblenz-Landau.

Finalment, al Ministerio de Ciencia e Innovación pel projecte "Variación genética adaptativa y plasticidad fenotípica en tolerancia térmica-Impacto del calentamiento global en las poblaciones de anfibios sobre gradientes de altitud" CGL2009-12767-C02-01, que ha finançat gran part de la tesis.

ABSTRACT

The aim of the present study is to analyse the latitudinal and altitudinal variations of life history traits (thermal behaviour, migratory range, age structure and size variation) in natterjack toads (*Bufo calamita*) and its relationship with genetic variability. This species is an excellent model organism because of its wide distribution that ranges from 36° to 58° of latitude and from sea level up to 2540 m. Life history traits of *B. calamita* seem to depend on the contraction of the annual activity period along latitudinal and altitudinal gradient. The substrate type determines the shelter used by toads for hibernation that affects the size variation and the migratory ranges. Females respond more sensitively than males to environmental changes. Whereas the pattern of size variation suggests a covariance of females' size and potential reproductive lifespan (PRLS) to optimize the lifetime fecundity rather in a latitudinal cline, lifetime fecundity decreases at the upper altitudinal ranges because PRLS is similar to the lowland population but females are smaller. This reduction in lifetime fecundity can explain the inability of natterjack toads to colonize elevations exceeding 2500 m and probably to expand geographical range further north. The high gene flow between populations suggest genetic cohesion of metapopulations systems as predicted the migratory capacity of dispersing natterjacks estimated in this study. It remains currently open, whether or not observed variability of the life history traits studied is mainly due to the phenotypic plasticity of a "general purpose genotype".

RESUM

El present estudi pretén analitzar la variació latitudinal i altitudinal dels caràcters del cicle vital (comportament tèrmic, rang de migració, estructura d'edats i variació en mida) del gripau corredor (*Bufo calamita*) i la seva relació amb la variabilitat genètica. Aquesta espècie és un organisme model excel·lent per al seu estudi ja que presenta una àmplia distribució geogràfica, trobant-se des dels 36° als 58° en latitud i des del nivell del mar fins als 2540 m en altitud. La variació dels caràcters del cicle vital del *B. calamita* sembla dependre de la reducció del període d'activitat anual al llarg del gradient altitudinal i latitudinal. El tipus de sòl determina el refugi que utilitzen els gripaus per a la hibernació que afecta a la variació en la seva mida corporal i al seu rang de migració. Les femelles són més sensibles que els mascles als canvis ambientals. El patró de variació de la mida corporal de les femelles suggereix una covariància entre la mida i el període potencial reproductiu (PRLS) per tal d'optimitzar la fecunditat total (fecunditat al llarg de la vida de l'individu) en la clina latitudinal. En el rang altitudinal, la fecunditat total decreix en les poblacions més elevades ja que el seu PRLS és similar al de les poblacions de zones baixes, però la mida de les femelles és inferior. Aquesta reducció en la fecunditat total pot explicar la inhabilitat del gripau corredor per colonitzar elevacions que excedeixin els 2500 m i probablement, la impossibilitat d'ampliar el seu rang geogràfic més al nord de la seva distribució actual. L'elevat flux genètic entre les poblacions suggereix cohesió genètica dels sistemes metapoblacionals tal i com prediu l'elevada capacitat migratòria dels individus dispersors. Els anàlisis genètics efectuats no permeten afirmar amb certesa si la variació observada en els caràcters del cicle vital de l'espècie és majoritàriament conseqüència de la plasticitat fenotípica d'un genotip general ("general purpose genotype").

RESUMEN

El presente estudio pretende analizar la variación latitudinal y altitudinal de los caracteres del ciclo vital (comportamiento térmico, rango de migración, estructura de edades y variación en tamaño) del sapo corredor (*Bufo calamita*) y su relación con la variabilidad genética. Esta especie es un modelo excelente para su estudio ya que presenta una amplia distribución geográfica encontrándose desde los 36° a los 58° de latitud y desde el nivel del mar hasta los 2540 m en altitud. La variación de los caracteres del ciclo vital de *B. calamita* parecen depender de la reducción del período de actividad anual a lo largo del gradiente altitudinal y latitudinal. El tipo de suelo determina el refugio que utilizan los sapos para la hibernación y afecta a la variación de su tamaño corporal y su rango de migración. Las hembras son más sensibles que los machos a los cambios ambientales. El patrón de variación en el tamaño corporal de las hembras en la clina latitudinal, sugiere una covarianza entre el tamaño y el período potencial reproductivo (PRLS) con el fin de optimizar la fecundidad total (fecundidad a lo largo de la vida del individuo). En el rango altitudinal, la fecundidad total decrece en las poblaciones más elevadas ya que su PRLS es similar al de las poblaciones de zonas bajas, pero el tamaño corporal es inferior. Esta reducción en la fecundidad total, puede explicar la incapacidad del sapo corredor para colonizar hábitats que excedan los 2500 m y probablemente, la imposibilidad de ampliar su rango geográfico más al norte de su distribución actual. El elevado flujo genético entre las poblaciones sugiere cohesión genética de los sistemas metapoblacionales como revela la elevada capacidad migratoria de los individuos dispersores. Los análisis genéticos efectuados no permiten afirmar con certeza si la variación en los caracteres de vida de la especie es mayoritariamente consecuencia de la plasticidad fenotípica de un genotipo general (“general purpose genotype”).

The Thesis includes the following papers:

- OROMI, N.; SINSCH, U.; SANUY, D. (2010) Thermal ecology of natterjack toads (*Bufo calamita*) in a semiarid landscape. *Journal of Thermal Biology*, 25:34-40.
- SINSCH, U.; OROMI, N.; MIAUD, C.; DENTON J.; SANUY, D. Connectivity of local amphibian populations: modelling the migratory range of radio-tracked natterjack toads. *Animal Conservation*. Submitted manuscript.
- SINSCH, U.; MARANGONI, F.; OROMI, N.; SANUY, D.; TEJEDO, M. (2010) Proximate mechanisms determining size variability in natterjack toads. *Journal of Zoology*, 281: 272-281.
- OROMI, N.; SINSCH U.; SANUY, D. Altitudinal variation of demographic life-history traits does not mimic latitudinal variation in natterjack toads (*Bufo calamita*). *Zoology*. Accepted manuscript.
- OROMI, N.; RICHTER-BOIX, A.; SANUY, D; FIBLA, J. Genetic variability of geographical populations of the natterjack toad (*Bufo calamita*). Manuscript in preparation.

Papers accepted for publication are reproduced with the permission of all co-authors.

Papers related to the thesis and previously published by the author of this thesis:

Leskovar, C., Oromi, N., Sanuy, D. & Sinsch, U. (2006). Demographic life history traits of reproductive natterjack toads (*Bufo calamita*) vary between northern and southern latitudes. *Amphibia-Reptilia* 27, 365-375.

Sinsch, U., Oromi, N. & Sanuy, D. (2007). Growth marks in natterjack toad (*Bufo calamita*) bones: histological correlates of hibernation and aestivation periods. *Herpetological Journal* 17, 129-137.

INDEX OF CONTENTS:

INTRODUCTION Introducció (Català)	3
MAIN OBJECTIVES	13
STUDY AREAS	15
CHAPTER 1: Thermal ecology of natterjack toads (<i>Bufo calamita</i>) in a semiarid landscape	29
CHAPTER 2: Connectivity of local amphibian populations: modelling the migratory range of radio-tracked natterjack toads.	37
CHAPTER 3: Proximate mechanisms determining size variability in natterjack toads	63
CHAPTER 4: Altitudinal variation of demographic life-history traits does not mimic latitudinal variation in natterjack toads (<i>Bufo calamita</i>)	75
CHAPTER 5: Genetic variability of geographical populations of the natterjack toad (<i>Bufo calamita</i>)	99
GENERAL DISCUSSION	123
CONCLUSIONS Conclusions (Català)	131

INTRODUCTION

The aim of the present study is to analyse the latitudinal and altitudinal variations of life history traits (thermal behaviour, migratory range, age structure and size variation) in the natterjack toads (*Bufo calamita*) and its relationship with genetic variability. This species is an excellent model organism because its wide distribution that ranges from 36° to 58° of latitude and from sea level up to 2540 m (Sinsch, 2008). Moreover, like most other amphibian species, *B. calamita* has a complex biological cycle that can differ locally, depending on the environmental conditions. Intra- and interspecific variations of life history traits among populations allow to understand how they evolve along time and how they can adapt to environmental conditions, which can be limiting factors for the species survival.

Amphibians, as ectotherm animals, lack a significant physiologic mechanism of temperature control and water balance. For this reason, they depend on environmental temperatures for thermoregulation (Brattstrom, 1963; Hutchinson & Dupré, 1992). The studies of body temperature variations of ectotherm animals are crucial to understand their ecology because ambient temperature affects their development, physiology and behaviour (Huey, 1982; Smits, 1984). The importance of terrestrial habitat selection in amphibian dispersal or in some species, during the hibernation, has been studied by different authors (e.g. Bartelt, 2000; Muths, 2003; Pilliod et al., 2002; Semlitsch, 1998). In species with a wide geographical distribution, we expect that latitudinal variation of the local climate causes modifications in the annual activity period promoting different thermoregulatory strategies.

The information about the influence of environmental temperature on the behaviour of natterjack toad is limited to two moist and temperate sites located

in Germany (Sinsch, 1998; Leskovar & Sinsch, 2001). In addition, there isn't much information about the thermoregulatory behaviour of *B. calamita* in water stress conditions. In this work, the thermal ecology of natterjack toads in a semiarid landscape is described and compared with previously published studies in order to determine the differences in behaviour and thermal range preference of the species in different environmental conditions (Chapter 1).

The migratory range of an amphibian species determines its ability to disperse, i.e. to colonise or to re-colonise breeding sites, and to maintain genetic cohesion among neighbouring local populations within a metapopulation framework (Sinsch, 1990; Smith & Green, 2005). The study of migratory range variability among individuals of different populations allows to understand the metapopulation dynamics and to define purposes of conservation management of local amphibian populations. In the present study, data of natterjack toads movements from eight localities along a latitudinal range was combined to analyse the differences in their migratory ranges which can be influenced by local habitat features such latitude, altitude, temperature or precipitation (Chapter 2).

The amphibian biology, like that of other organisms, is characterized by patterns of growth development, storage and reproduction (Begon et al., 1996). The theory of life history traits explains the subjacent relations of life cycle of organism living in the same environment. The demography is crucial to understand the biology of an organism. This theory comprises various demographic traits, such as age at maturity, longevity, potential reproductive lifespan and age-size relationship. These demographic traits interact depending on the local environmental factors and are often connected by trade-offs (Stearns, 1992, 2000).

Variation in life history traits among anuran populations is explained by differences in habitat quality and climate (Hastings, 1997) that vary along

altitudinal and latitudinal gradients (review in Morrison & Hero, 2003). The latitudinal effects in *B. calamita* have been recently studied (Leskovar et al., 2006). Age at maturity and longevity show a tendency to increase with latitude, whereas potential reproductive lifespan (PRLS) of females significantly increases from south to north (Leskovar et al., 2006). However, the geographical pattern of size variation is important to analyse the fitness among populations in the latitudinal gradient, but it has not yet been evaluated. In addition, altitudinal variation neither has been explored in *B. calamita*. Although life history theory predicts that latitudinal and altitudinal effects cause similar patterns of variation, inconsistent results are published to date (e.g. the Morrison & Hero, 2003). In the present study, the causes of the body size variation in the latitude (Chapter 3) and the life history traits variation in the altitude (Chapter 4) are evaluated in order to determine the variation of these traits in the total geographical distribution of *B. calamita* and the proximate factors (temperature, rainfall, food supply, predation, competition, etc.) that can cause this variability.

Structure and dynamic of metapopulations are important features of the amphibian's biology. Behavioural studies of populations living in a specific area are essential to know the strategies adopted by these populations, especially in habitats with limiting conditions (Sinsch, 1992). Moreover, metapopulations can be genetically different as a consequence of their evolution, which depends on the interconnections between the different metapopulations and their extinction rate (e.g. Rowe et al., 2000). Genetic diversity is the substrate of the evolution that emerges from genetic processes, such as mutation and selection, and stochastic processes, such as genetic drift and genetic flow. Each life trait can be defined as a result of environmental and genetic components. The genetic component refers to the DNA that codes a particular trait. The environmental component reflects the influences of ambient characteristics on the phenotype and favours evolutionary changes. To understand the process that generates a phenotypic variation in a particular trait, it is necessary to evaluate its genetic

component and the interaction with environment and other traits (Stearns, 1989).

Studies of phenotypic plasticity and species genetic and environmental differentiation are essential to understand the evolution and the adaptive nature of changes that occur in a population (Berven, 1982ab). Thus, the final study of the present work is the evaluation of the genetic variation and differentiation among population of *B. calamita* in order to determine if altitudinal and latitudinal effects in life history traits found in the previous chapters are a consequence of genetic adaptation, phenotypic plasticity or a combined effect of both (Chapter 5).

This thesis includes various published, accepted or submitted manuscripts, which have been integrated in the corresponding chapters. Each chapter has been structured in the following sections: introduction, aims, material and methods, results and discussion.

REFERENCES

- Bartelt, P.E. (2000) A biophysical analysis of habitat selection in Western toads (*Bufo boreas*) in southeastern Idaho, Ph.D. Dissertation, Idaho State University, Pocatello, Idaho.
- Begon, M., Harper, J.L., Townsend, C.R. (1996) Ecology: individuals, populations and communities. Blackwell Science Ltd, Oxford.
- Berven, K.A. (1982a) The genetic basis of altitudinal variation in the wood frog *Rana sylvatica*. I. An experimental analysis of life history traits. *Evolution*, 36: 962-983.
- Berven, K.A. (1982b) The genetic basis of altitudinal variation in the frog *Rana sylvatica* II. An experimental analysis of larval development. *Oecologia*, 52:360-369.

- Brattstrom, B.H. (1963) A preliminary review of thermal requirements of amphibians. *Ecology*, 44: 238-255.
- Hastings, A. (1997) Evolution of mixed maturation strategies in semelparous life histories: the crucial role of dimensionality of feedback environment. Philosophical Transaction of the Royal Society. *Biological Science*, 353: 1647-1655.
- Huey, R.B. (1982) Temperature, physiology, and the ecology of reptiles. In: Gans, C., Pough, F.H. (Eds.), *Biology of the Reptilia*, vol. 12. Physiology C Academic Press, London.
- Hutchison, V.H., Dupré, R.K. (1992) Thermoregulation. In: Feder, M. E., Burggren, W. W. (Eds), *Environmental physiology of the amphibians*. The University of Chicago Press: 206-249.
- Leskovar, C., Oromí, N., Sanuy, D., Sinsch, U. (2006) Demographic life history traits of reproductive natterjack toads (*Bufo calamita*) vary between northern and southern latitudes. *Amphibia-Reptilia*, 27: 365-375.
- Leskovar, C., Sinsch, U. (2001) Hibernation behaviour of radiotracked natterjack toads *Bufo calamita* and green toads *Bufo viridis*. *Biota*, (Suppl.) 2: 33-34.
- Morrison, C., Hero, J.M. (2003) Geographic variation in life-history characteristics of amphibians: a review. *Journal of Animal Ecology*, 72: 270-279.
- Muths, E. (2003) Home range and movements of boreal toads in undisturbed habitat. *Copeia*, 161-165.
- Pilliod, D.S., Peterson, C.R., Ritson, P.I. (2002) Seasonal migration of Columbia spotted frogs (*Rana luteiventris*) among complementary resources in a high mountain basin. *Canadian Journal of Zoology*, 80: 1849-1862.
- Rowe, G., Beebee, T. J. C., Burke T. (2000) A microsatellite analysis of natterjack toad, *Bufo calamita*, metapopulations. *Oikos*, 88: 641-651
- Semlitsch, R.D. (1998) Biological delineation of terrestrial buffer zones for pond-breeding salamanders. *Conservation Biology*, 12: 1113-1119.

- Sinsch, U. (1990) Migration and orientation in anuran amphibians. *Ethology Ecology & Evolution*, 2: 65-79.
- Sinsch, U. (1992) Structure and dynamic of a natterjack toad metapopulation (*Bufo calamita*). *Oecologia*, 90: 489-499.
- Sinsch, U. (1998) Biologie und Ökologie der Kreuzkröte. Bochum, Laurenti Verlag.
- Sinsch, U. (2008) *Bufo calamita* Laurenti, 1768 - Kreuzkröte. *Handbuch der Amphibien und Reptilien Europas* (ed. By K. Grossenbacher), pp. 339-413. Aula Verlag. 5/1, Wiesbaden.
- Smits, A.W. (1984) Activity patterns and thermal biology of the toad *Bufo boreas halophilus*. *Copeia*, 689-696.
- Smith, M.A., Green, D.M. (2005) Dispersal and the metapopulation paradigm in amphibian ecology and conservation: are all amphibian populations metapopulations? *Ecography*, 28:110-128.
- Stearns, S.C. (1989) The evolutionary significance of phenotypic plasticity. *Bioscience*, 39: 436-445.
- Stearns, S.C. (1992) The evolution of life histories. Oxford University Press, London, pp 123-211.
- Stearns, S.C. (2000) Life history evolution: successes, limitations, and prospects. *Naturwissenschaften*, 87: 476-486.

INTRODUCCIÓ (Català)

El present estudi pretén analitzar la variació latitudinal i altitudinal dels caràcters del cicle vital (comportament tèrmic, rang de migració, estructura d'edats i variació en mida) del gripau corredor (*Bufo calamita*) i la seva relació amb la variabilitat genètica. Aquesta espècie és un organisme model excel·lent per al seu estudi ja que presenta una àmplia distribució geogràfica, trobant-se des dels 36° als 58° en latitud i des del nivell del mar fins als 2540 m en altitud. D'altra banda, el gripau corredor, com a espècie amfíbia, té un cicle biològic complex que pot presentar diferències en la seva plasticitat fenotípica o genotípica tot depenent de les condicions ambientals. L'estudi de les variacions intra o interespecífiques dels caràcters del cicle vital d'una espècie permet entendre com les poblacions sobreviuen al llarg del temps i s'adapten a les diferents condicions ambientals, especialment en aquells hàbitats on les condicions poden ser limitants per la seva supervivència.

Els amfibis, en ser animals ectotèrmics, depenen directament del medi extern per a la regulació de la seva temperatura corporal i de la seva humitat ja que no tenen mecanismes fisiològics efectius per al seu control (Brattstrom, 1963; Hutchinson & Dupré, 1992). L'estudi de les variacions en la temperatura corporal dels animals ectotèrmics és crucial per entendre la seva ecologia ja que la temperatura afecta al seu desenvolupament, fisiologia i comportament (Huey, 1982; Smits, 1984). La importància de l'elecció d'hàbitats terrestres per part dels amfibis per a la dispersió, i en algunes espècies, per a la hibernació, ha estat constatada per diferents autors (per ex. Bartelt, 2000; Muths, 2003; Pilliod et al., 2002; Semlitsch, 1998). En espècies amb un rang geogràfic de distribució ampli cal esperar que la variació latitudinal del clima causi modificacions en l'activitat anual de l'espècie tot promovent diferents estratègies de termoregulació.

Els coneixements sobre la influència de la temperatura ambiental en el comportament del gripau corredor es limiten als estudis realitzats en dos zones temperades i humides d'Alemanya (Sinsch, 1998; Leskovar & Sinsch, 2001). Es desconeix però, quin és el comportament termoregulador del *B. calamita* en condicions d'estrès hídric. En aquest treball es descriu l'ecologia tèrmica del gripau corredor en un ambient semiàrid i es compara amb la literatura prèviament publicada, per tal de determinar les diferències en comportament i en preferència tèrmica de l'espècie en diferents condicions ambientals (Capítol 1).

El rang de migració d'una espècie amfibica determina la seva habilitat de dispersió per tal de colonitzar o re-colonitzar punts de reproducció i mantenir la cohesió genètica entre les poblacions locals veïnes dins un marc de metapoblació (Sinsch, 1990; Smith & Green, 2005). L'estudi de la variabilitat en el rang o àrea de migració entre individus de diferents poblacions permet comprendre la dinàmica de la metapoblació i realitzar una bona gestió per a la conservació de les poblacions locals d'amfibis. Per aquest motiu, es varen obtenir les dades dels moviments de vuit poblacions de gripau corredor per tal d'analitzar les diferències en el seus rangs de migració; possiblement influenciats per les característiques locals de l'hàbitat com p. ex. la latitud, l'altitud, la temperatura i la precipitació (Chapter 2).

La biologia dels amfibis, igual que la dels altres organismes, es regeix per patrons de creixement, desenvolupament, capacitat de reserves i reproducció (Bengon et al., 1996). La teoria de la estratègia vital o "life history" permet comprendre les relacions subjacents entre organismes, els seus cicles vitals i el medi en el que viuen. La demografia és clau per a la teoria de la biologia dels organismes i inclou un conjunt de caràcters que es barregen en resposta a factors ambientals (Stearns, 1992). L'edat a l'arribada a la maduresa sexual, la longevitat, el potencial reproductiu i la relació entre l'edat i la mida dels

individus d'una població són caràcters demogràfics que poden estar sovint connectats per “trade-offs” (Stearns, 1992, 2000). Les variacions en els caràcters del cycle vital entre poblacions de la mateixa espècie són freqüentment explicades per diferències en la qualitat de l'hàbitat o del clima (Hastings, 1997). En els amfibis, aquests caràcters responen també a variacions interpoblacionals en latitud i altitud (Morrison & Hero, 2003)

Recentment, s'han estudiat els efectes de la latitud en els caràcters del cycle vital de *B. calamita* (Leskovar et al., 2006). L'edat a la maduresa sexual i la longevitat presenten una tendència a incrementar-se amb la latitud, mentre que el potencial reproductiu (Potential Reproductive Lifespan: PRLS) de les femelles s'incrementa significativament de sud a nord (Leskovar et al., 2006). El patró geogràfic de variació de la mida corporal dels individus presenta gran importància per tal d'avaluar la “fitness” entre poblacions al llarg de la distribució de l'espècie. Malgrat això, la variació d'aquest caràcter no ha estat encara estudiada. A més, es desconeix la variació dels caràcters del cycle vital de l'espècie en el rang altitudinal tot i que es preveu que ambdues variacions, altitudinal i latitudinal, segueixin patrons similars (e.g. Morrison & Hero, 2003). En aquest estudi, es pretén determinar la variació de la mida corporal dels gripaus en funció de la latitud (Capítol 3) i de l'altitud, i els caràcters demogràfics del cycle vital del gripau corredor al llarg d'un gradient altitudinal (Capítol 4). Es pretén avaluar també, els factors locals (temperatura, precipitacions, alimentació, predació, competència, etc.) que poden causar aquesta variabilitat.

Un altre aspecte important en la biologia dels amfibis és l'estudi de les estructures metapoblacionals i la seva dinàmica. L'estudi comportamental de les poblacions en un territori és essencial per a conèixer les seves estratègies, especialment sota condicions ambientals limitants (Sinsch, 1992). A més, les metapoblacions poden ser diferents genèticament com a conseqüència de la seva evolució, la qual depèn de la interconnexió entre les diferents metapoblacions i

la seva taxa d'extinció (e.g. Rowe et al., 2000). La diversitat genètica és el substrat per a l'evolució que emergeix de processos genètics, com la mutació i la selecció, i de processos estocàstics, com la deriva genètica o el flux genètic. Qualsevol caràcter vital es pot definir com a resultat d'una component ambiental i d'una component genètica, que són factors intrínsecs de l'organisme. La component genètica representa l'ADN que codifica un determinat caràcter. Mentre que la component ambiental fa referència a la influència del medi en el desenvolupament del fenotip a partir d'un genotip determinat, afavorint els canvis evolutius. Per aquest motiu, és necessari conèixer els processos que generen variació fenotípica en un determinat caràcter i com interactuen amb altres caràcters i amb el medi (Stearns, 1989).

Per tant, la distinció entre la variació genètica i la variació induïda per l'ambient és fonamental per tal de poder interpretar l'evolució i la naturalesa adaptativa dels canvis que es poden produir dins una població (Berven, 1982ab). Per aquest motiu, l'últim capítol del present treball versa sobre l'avaluació de la variació genètica i la diferenciació entre poblacions de *B. calamita* per tal de determinar si els efectes latitudinals i altitudinals trobats en els caràcters del cicle vital de l'espècie estan basats en adaptació genètica o en plasticitat fenotípica, o en una combinació d'ambdós factors (Capítol 5).

La tesis està formada per un recull d'articles publicats, acceptats o en revisió que s'inclouen en els diferents capítols. Cada capítol s'estructura en els següents apartats: introducció, objectius, materials i mètodes, resultats i discussió.

MAIN OBJECTIVES

The natterjack toads (*Bufo calamita*) inhabits in a wide geographical range of distribution in which exists a gradual change in their activity cycle. Previous studies show differences in the life history traits of *B. calamita* in a latitudinal gradient. Environmental heterogeneity may increase phenotypic plasticity of traits and/or favour local adaptation by selection under extreme conditions. The principal aim of this study is the analysis of the different mechanisms that can explain the altitudinal and latitudinal variation of life history traits of *B. calamita*.

The specific aims of the present work are:

1. To analyse the thermal ecology of natterjack toads in a semiarid landscape:
 - a. To describe the thermoregulatory behaviour and the range of body temperature experienced by adults in the semiarid zone of Mas de Melons.
 - b. To analyse the temperature- and moisture- related shelter used during the dry summer period and during hibernation.
 - c. To discuss the latitudinal variation strategies employed to cope with environmental temperatures and hydration stress.
2. To analyse the connectivity of local *B. calamita* populations with the modelling of the migratory range of radio-tracked toads:
 - a. To describe the migratory range of different localities along a latitudinal range.
 - b. To model the relationship between movement distance and proportion of adult population.
 - c. To quantify the influence of local habitat features on movement distances.

3. To evaluate the size variation of natterjack toads in a latitudinal gradient:
 - a. To qualify the influence of climate on the age-adjusted body size variation.
 - b. To evaluate the hypothesis that the near-clinal body size variation is a by-product to co-variation between juvenile growth rate, longevity and lifetime fecundity.
4. To study the altitudinal variation of life history traits of natterjack toads:
 - a. To determine several life history traits such as age at maturity or longevity in different localities in an altitudinal range.
 - b. To analyse the proximate mechanisms that cause variation in life history traits in the altitudinal and latitudinal gradient.
5. To analyse genetic variability of geographical populations of natterjack toads:
 - a. To characterise the genetic diversity of several populations in the altitudinal gradient of the Sierra de Gredos.
 - b. To analyse the altitudinal and latitudinal genetic variation of the species using genetic diversity parameters from our data and those published in the literature.

STUDY AREAS

In this study, different zones of natterjack toad distribution are considered in order to analyse the ecological strategies of the species in different environments. We focused my experiments in the zones that can present limitant factors for the species development or survival (Mas de Melons and Sierra de Gredos). The principal characterizes of the zones used in the present study are summarized in Table 1 (Fig.1). Climate variables used were minimum and maximum annual air temperature, average annual air temperature, and precipitation [mm/year]. Temperature (15-year averages) and precipitation data (20-year averages) for the nine study areas were derived from the “Atlas climático digital de la Península Ibérica” (Ninyerola et al., 2005), except for precipitation data (30-year average) for Balaguer and Mas de Melons (meterological station: Lleida – Observatorio 2, 192 m a.s.l., period 1971-2000; Instituto Nacional de Meteorología, pers. comm.).

The most important studied zones, Mas de Melons and Sierra de Gredos, have been accurately described in two different sections because they are essential for understand the general discussion.

Table 1: Location and climate features.

Locality	Coordinates	Altitude [m a.s.l.]	Mean temperature (Min-Max) [°C]	Precipitation [mm]
Palamós	41°51'49"N 03°07'59"W	10	15 (+11 - 20)	844
Balaguer	41°46'30"N 00°46'50"W	220	14 (+8 - 21)	369
Mas de Melons	41°30'50"N 00°43'30"W	240	14(+8 - 20)	369
Navaluenga	40°24'57"N 04°40'47"W	752	13 (+7 - 20)	735
El Pinós	38°22'44"N 00°58'40"W	760	15 (+9 - 20)	313
Dehesa del Barraco	40°27'53"N 4°38'12"W	920	13 (+7 - 19)	676
La Cedrera	40°30'33"N 4°39'16"W	1472	10 (+3 - 16)	652
Cavadores	40°16'19"N 5°15'12"W	2100	7 (0 - 14)	1172
Navasomera	40°15'07"N 5°15'49"W	2270	6 (-1 - 13)	923

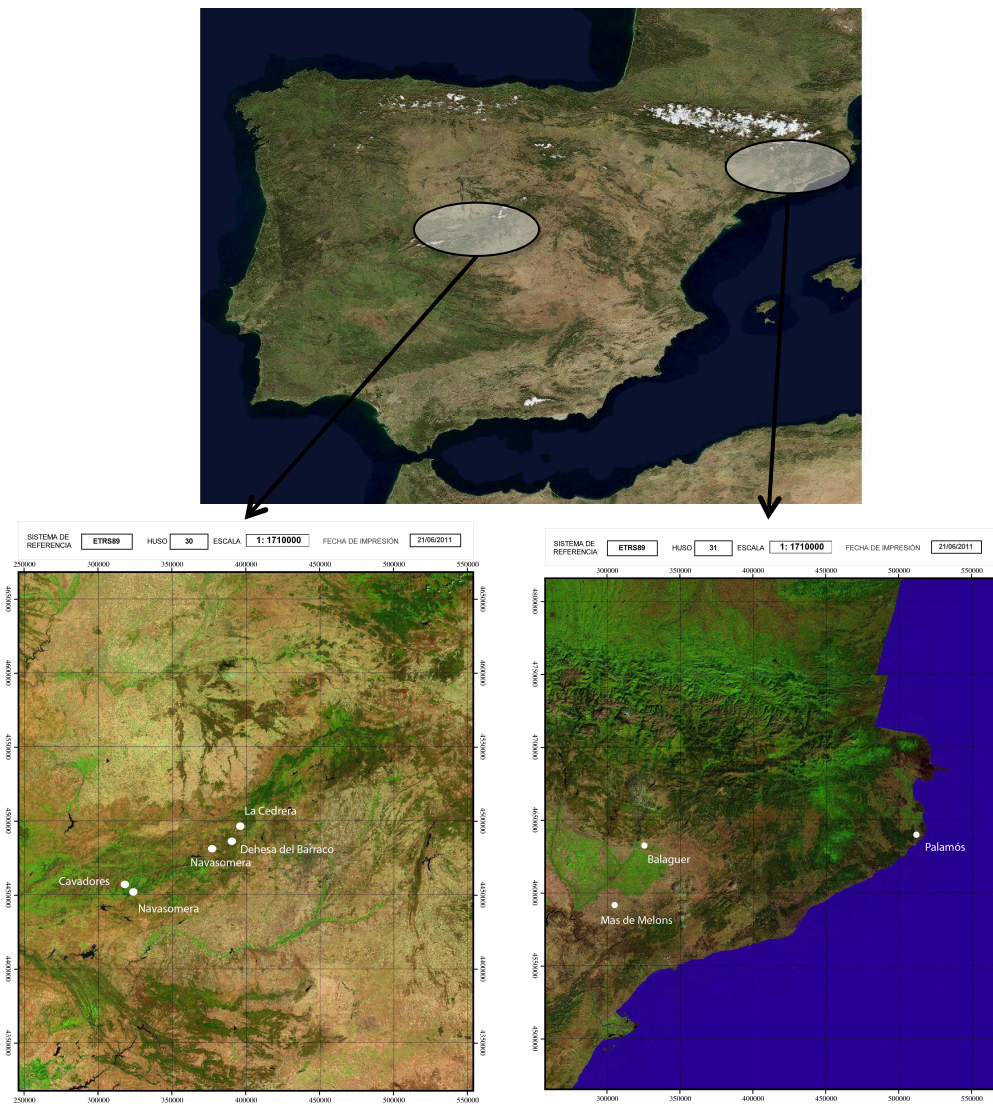


Figure 1. Map of the situation of the studied zones.

SEMIARID ZONE OF MAS DE MELONS

The semiarid zone of Mas de Melons (41.51°N, 0.74°E; 240 m asl) is situated in the plain region of Lleida (Ebro river valley: northwest of Iberian Peninsula) at a distance of about 15 Km southeast of the city of Lleida. This area is delimited by the Pre-Pyrenees in the north and by the Catalan Coastal Range ("Serralada Litoral") in the south. The study zone is located in the eastern part of the Ebro depression, concretely in the southern area of Lleida.

Mas de Melons is a semiarid zone (Bolòs, 1973) considered as a protected area by the Habitat Directive of EU Nature legislation (92/43/CEE). The aim of this Directive is to promote the maintenance of biodiversity, taking account of economic, social, cultural and regional requirements. The protected area occupies a total of 2733.84 ha. A part of this area, 1432.55 ha, is also included in Bird Directive (2009/147/EC) that identify species and sub-species with particularly threatened and in need of special conservation measures. Another protected figure of the zone is the PEIN (special landscape of natural interest) considered by the Generalitat de Catalunya since 1985 (1985/12; actual modification 2006/12). This protected figure wants to promote the biodiversity conservation with a sustainable use of natural resources in agriculture.

The depression of the Ebro valley was formed by the flexure of the continental lithosphere induced by vertical loading of the Pyrenees orogenic wedge. As the most foreland basis, the Ebro depression has a marked asymmetrical geometry. Throughout the tecto-sedimentary evolution of the basin, the main evaporitic formations have developed in the most actively subsiding depocenters. This resulted in a general onlap of successively younger stratigraphical units onto the foreland. In the initial sedimentary stage (Paleocene-Eocene), the depression was open to the sea. In Upper Eocene existed an endorheic stage that lost this character at the end of Miocene. The transition from endorheic to exorheic conditions was a crucial event in the

development of gypsum karst that characterizes actually the geomorphology of the zone. The most antic gypsum of the depression has been found in Mas de Melons because this is the highest area of the valley depression where gypsum comes to the surface. The soils are clayey and dry.

This zone is the area of maximum aridity of the Ebro depression and is characterized by a hot and dry summer. The climate is Mediterranean and moderately Continental with average summer temperatures of 24.7°C (July) and winter temperatures of 5.3°C (January). The average number of days with temperatures below 0°C is 37 per year and the total annual average precipitation 369 mm (meteorological station of Lleida, Obs.2. period 1971-2000).

The study was carried out in a rainfed agricultural area. The current vegetation belongs to the mediterranean flora characterized by two climatic dominia: the continental *Rhamno lycioidis-Quercetum cocciferae* subass. *cocciferetosum* in the interior plains, and the mediterranean *Quercetum rotundifoliae* in the southern sector (Conesa et al., 1994; Conesa, 2006). These communities are fragmented because of the agricultural activity and the landscape is a patchwork of cereal, olive and almonds fields. Nowadays, *Rosmarinus officinalis*, *Linus suffruticosum*, *Cytisus fontanessi* and *Cistus clussi* scrubs occupy most part of the non-agricultural surface. In low open areas appears *Sideritetum cavanillesii* and in the oriental slopes *Delphinio gracilis-Lygeetum sparti*. Nitrophilous and halo-nitrophilous vegetation with *Suaedetum brevifoliae* communities can appear in low areas. In cereal fields *Roemerio hybridae-Hypecoetum penduli* community is highly represented.

The amphibians present in the study area of Mas de Melons are: the common green frog (*Pelophylax perezi*), the natterjack toad (*Bufo calamita*), the common toad (*Bufo bufo*) and the western spadefoot toad (*Pelobates cultripes*).



A



B

Figure 2. Amphibians observed at Mas de Melons. A: *Pelobates cultripipes* and B: *Bufo calamita*.

Toads reproduce in many ephemeral water bodies, which are distributed within the winter cereal fields, and in permanent eutrophic cattle ponds (Fig. 3). Behavioural ecology of natterjacks in this area was previously described (Miaud et al., 2000; Miaud & Sanuy, 2005). Reproductive period begins on March or April depending on the climatic conditions and responding to a heavy rainfall. Aboveground activity continues until June and ceases in absence of precipitations. Some years, toads can reproduce in autumn season if a significant rainfall occurs in this period.



A



B

Figure 3. A: Permanent eutrophic pond, B: Ephemeral water body in a field track.

SIERRA DE GREDOS

The study zone of Sierra de Gredos is situated in the Iberian Peninsula. It occupies an extension of 140 km², bordering at north by the Adaja river and to the south by the Tietar River. The Alberche river cuts this mountain range in two alignments with a WSW/ NSW orientation. The topography of the mountain is asymmetric between the north and south due to the tectonic constitution with a fault orientated towards the south (Parrillas & Palacios, 1995). Sierra de Gredos's highest summit is the Pico Almanzor (2592 m asl).

The Sierra de Gredos was given Regional Park Status (Boletín Oficial de Castilla y León, 124/29 June 1996) in order to conserve the ecosystem biodiversity. The prior objective of this Regional Park is to maintain the agropecuarian practices in the natural ecosystems. The major part of the regional park zone (87160 ha) is constituted by glaciers like the Laguna de Gredos (1980 m asl) or Navasomera (2363 m asl). This study is limited to the North face.

The Sierra de Gredos granite formation dates from the Hercynian Orogenesis in the Palaeozoic period. The present orography was formed in the Alpine orogenesis (40-20 millions of years ago), when the main mountain range of the earth was formed: the Himalayas, the Rocky Mountains, the Alps and the Pyrenees. A few differences exist between the north and the south face especially in the abrupt landscape of the highest peaks, more pronounced in the south. The present relief of the Sierra of Gredos is due to the erosion in the Quaternary Ice Age (10000 years ago). The result of this erosion was the creation of glacier with lagoons and canyons surrounded by high peaks. As a consequence of the high mountain climatic conditions, the Sierra de Gredos preserves an important flora and fauna from the glacial period. Actually, the

substrate is made of plutonic rocks, mostly granites and metamorphic rocks in lower elevations. The soils are deep and siliceous.

The Sierra de Gredos has a Mediterranean climate which corresponds to the fitoclimatic subtypes: oroarticoid thermoxeric, altitude > 1900m; oroborealoid subnemoral, between altitudes of 1400 and 1900 m; and nemoromediterranean genuine, between 700 and 1400 m of altitude (Allué, 1990). The average mean temperatures range from 6 to 12 °C with a mean annual temperatures between 0 and 2 °C during the coldest months (December, January and February) and 20 to -22 °C during the hottest months (July and August) (Ninyerola et al., 2005). Storms are frequent in spring and autumn and its can be heavy. Precipitations vary depending on the altitude between 1000 mm to >2000 mm (Palacios et al., 2003, unpublished data of Spain's Instituto Nacional de Meteorología).

Dense forests of *Quercus pyrenaica* once covered this area, but were destroyed by over-grazing and fires. The scrub *Cytisus multiflori* and the grasses such as *Festuca elegans* have replaced the trees. The actual vegetal community is composed by grassland with patches of *Juniperus communis ssp. alpina* and *Cyperus oromediterraneus* with a *Pinus sylvestris* tree line. The mire vegetation consists of a mosaic of oligotrophic communities (*Caricetum carpetanae*) dominated by *Carex carpetana*, *C. demissa*, *C. echinata*, *Parnassia palustris*, *Sphagnum*, *Drosera rotundifolia* and *Viola juressi* (Sánchez-Mata, 1989; Escudero & Sánchez-Mata, 1996). The vegetation of Gredos includes a mosaic of open pastures, scrublands, wooded pastures and pine stands (Rodríguez-Rojo & Sánchez-Mata, 2004).

The amphibian species that inhabit in the Sierra de Gredos are: in the high zones, the common toad (*Bufo bufo gredosicola*), the natterjack toad (*Bufo calamita*), the Iberian endemic brown frog (*Pelophylax iberic*), the common green frog (*Pelophylax perezi*) and the salamander (*Salamandra salamandra almazoris*); and in low altitude zones *Hyla arborea*, *Hyla meridionalis*, *Pelophylax*

perezi, *Pleurodeles waltl*, *Triturus marmoratus*, *Alytes cisternasii*, *Alytes obstetricans*, *Discoglossus galganoi*, *Bufo calamita* and *Bufo bufo*. In streams of medium and high mountains also inhabits *Triturus boscai*.

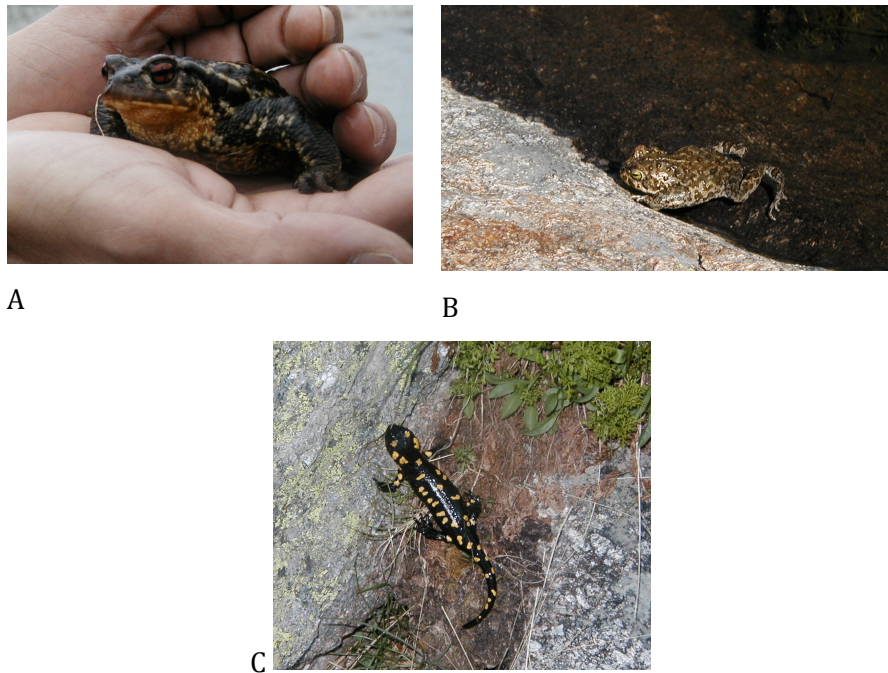


Figure 4. Amphibians observed at high altitudes. A: *Bufo bufo gredosicola*, B: *Bufo calamita* and C: *Salamandra salamandra almanzoris*.

B. calamita is present everywhere in the Iberian Peninsula, from sea level to the high mountain areas. In Gredos, natterjack toad is found from the base of the mountain (700-900 m) to the top, 2400 m (Lizana, et al., 1988, 1991). The breeding period begins depending on annual climatic conditions. It usually takes place in the middle of April and continues until June or July depending on the altitude. In high altitudes, the zone is covered by snow for a period of 6 to 7 months depending on the year, and the thaw determines the beginning of the breeding period. In low altitude, the reproductive period depends on the temperature and precipitation conditions. The reproductive sites of *B. calamita*

in the altitudinal gradients of Sierra de Gredos are humid meadows and ponds or glacial lagoons in the top of the mountains (see Fig.5)



A



B



C



D

Figure 5. Reproductive sites in the altitudinal gradient of Sierra de Gredos. A: Glaciars of Navasomera, 2300 m, B: La Cedrera, 1470 m, C: Dehesa del Barraco, 920 m and D: Navaluenga, 720 m).

REFERENCES

Allué, J.L. (1990) Atlas fitoclimático de España. Ministerio de Agricultura, Pesca y Alimentación (I.N.I.A.). Madrid. 221 pp.

- Bolòs, O. (1973) Algunas consideraciones sobre las especies esteparias en la Península Ibérica. *Anales Instituto Botanico, Cavanilles*, 10: 445-453.
- Conesa, J. A. (2006) La vegetació: La fauna vertebrada de les terres de Lleida. Casals & Sanuy Eds. Universitat de Lleida. Lleida.
- Conesa, J. A., Moyoral, A., Pedrol, J. & Recasens, J. (1994) El paisatge vegetal dels espais d'interès natural de Lleida: àrea meridional. I.E.I., Lleida.
- Escudero, A., Sánchez-Mata, D. (1996) Las fitocenosis de interés pascícola y su diversidad en el Parque Regional de la Sierra de Gredos (Ávila, España). *Studia Botanica*, 15: 47-67.
- Lizana, M., Ciudad M.J., Pérez-Mellado, V. (1988). Distribución altitudinal de la herpetofauna en el Macizo central de la Sierra de Gredos. *Revista Española de Herpetología*, 3: 55-67.
- Lizana, M., Ciudad, Gil, M.J., Guerrero, F., Pérez-Mellado, V., Martín-Sánchez, R. (1991) Nuevos datos sobre la distribución de la herpetofauna en el Macizo central de la Sierra de Gredos. *Revista Española de Herpetología*, 6: 61-80.
- Miaud, C., Sanuy, D. (2005) Terrestrial habitat preferences of the natterjack toad during and after the breeding season in a landscape of intensive agricultural activity. *Amphibia-Reptilia*, 26: 359-366.
- Miaud, C., Sanuy, D., Avriillier J.N. (2000) Terrestrial movements of natterjack toad *Bufo calamita* (Amphibia, Anura) in a semi-arid, agricultural landscape. *Amphibia-Reptilia*, 21: 357-369.
- Ninyerola, M., Pons, X., Roure, J.M (2005) Atlas Climático Digital de la Península Ibérica. Metodología y aplicaciones en bioclimatología y geobotánica. Universidad Autónoma de Barcelona, Bellaterra.
- Palacios, D., Garca, R., Rubio, V., Vigil R. (2003) Debris flows in a weathered granitic massif: Sierra de Gredos, Spain. *Catena*, 51: 115-140.
- Parrilla, G., Palacios, D. (1995) Colada de depósitos en Gredos y su significado climático: el caso de la Albarea (1989). In: Aleixandre, T., Pérez, A. (Eds.), Reconstrucción de

paleoambientes y cambios climáticos durante el cuaternario. C.S.I.C., Madrid, pp. 205–214.

Rodríguez-Rojo, M.P., Sánchez-Mata, D. (2004) Mediterranean hay meadow communities: diversity and dynamics in mountain areas throughout the Iberian Central Range (Spain). *Biodiversity and Conservation*, 13: 2361–2380.

Sánchez-Mata, D. (1989) Flora y vegetación del Macizo Oriental de la Sierra de Gredos (Ávila). Diputación Provincial de Ávila, Institución Gran Duque de Alba, Ávila

Thermal ecology of natterjack toads (*Bufo calamita*) in a semiarid landscape

Published in: *Journal of Thermal Biology*, 25 (2010) 34-40.

Authors: OROMÍ, N.; SANUY, D.; SINSCH, U.

The semiarid zones of the Lleida plain are characterized by hot and dry summers. In previous skeletocronological studies carried out in populations of Mas de Melons area (Sinsch et al., 2007), the bone section of adult toads showed the formation of multiple lines of arrested growth. These results suggest the interruption of hibernation during the hot periods of winter. This phenomenon seems to indicate activity of toads during winter. The Chapter 1 explains the thermal ecology of *B. calamita* in a semiarid zone. A radio-tracking experience was carried out in different years using a temperature-sensitive radio transmitter, implanted in the abdominal cavity of adult toads, which measured their body temperature. The results were compared with others found by Sinsch et al. (1998) and Leskovar et al. (2001) in a moisture area from the Rhine river valley. The features of the occupied shelter explain the thermal ecology of the species in a semiarid zone.

Author contribution: First authorship reflects that I was the main contributor to the paper. I have personally developed the concept, radiotracked the toads and analysed the data. I have written the paper in close collaboration with Prof. Drs Ulrich Sinsch and Delfi Sanuy.



Contents lists available at ScienceDirect

Journal of Thermal Biology

journal homepage: www.elsevier.com/locate/jtherbioThermal ecology of natterjack toads (*Bufo calamita*) in a semiarid landscapeNeus Oromí^a, Delfi Sanuy^a, Ulrich Sinsch^{b,*}^a Escola Tècnica Superior Enginyeria Agrària, Departament Producció Animal (Fauna Silvestre), University of Lleida, Spain^b Institute of Integrated Sciences, Department of Biology, University of Koblenz-Landau, Universitätsstr. 1, D-56070 Koblenz, Germany

ARTICLE INFO

Article history:

Received 30 July 2009
Accepted 20 October 2009

Keywords:

Behavioural thermoregulation
Water balance
Shelter site use
Radio-tracking

ABSTRACT

- (1). To monitor the seasonal variation of body temperature (t_b) in free-ranging adult *Bufo calamita*, 15 toads were radio-tracked at Mas de Melons (Catalonia, Spain) using temperature-sensitive transmitters implanted to the abdominal cavity.
- (2). t_b varied between +0.3 °C during winter and 32.2 °C during summer demonstrating behavioural avoidance of ambient temperature extremes by choosing moist and temperature-buffered microhabitats.
- (3). Frost avoidance included frequent changes of shelter sites by aboveground dispersal, distinguishing this population from conspecifics evading frost by burrowing.
- (4). The control of water balance superposed behavioural efforts to optimize t_b identifying natterjacks as thermal conformers.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

In temperate zone amphibians, the annual activity pattern of adults comprises a summer period characterized by significant growth and aboveground activities such as breeding and foraging, and a winter period usually spent underwater, underground or beneath leaf litter, without growth and little to none aboveground movements (Jørgensen, 1992; Pinder et al., 1992). The proximate causes of this pattern are the variations of ambient temperature and of water availability, as amphibians lack a significant physiological control of temperature and water balance (Brattstrom, 1963; Hutchison and Dupré, 1992). Still, many amphibians have a limited behavioural control of body temperature by interplaying water economy (e.g. controlled mucus discharges) and thermoregulation to allow them to be more active for longer duration (Lillywhite, 1971; Brattstrom 1979; Tracy et al., 1993; Bartelt and Peterson, 2005). Behavioural adjustments employed are often the selection of temperatures in available microhabitats, basking, and diel and seasonal cycles of activity (Huey, 1991; Hutchison and Dupré, 1992).

In species with a wide geographical range of distribution, latitudinal variation of local climate causes modifications of the annual activity cycle and may subsequently promote regionally different thermoregulatory strategies. The natterjack toad *Bufo*

calamita inhabits Western Europe from Sweden (58°N) to the southern tip of the Iberian Peninsula (38°N) and associated gradual changes of the activity cycle have been reported (reviews: Sinsch 1998, 2008). Significant trends (from north to south) are the contraction of the hibernation period from up to 8 months to less than 1 month, the parallel prolongation of the activity period to a maximum of about 6 months, and the increasing duration of aestivation periods in the Iberian Peninsula (Leskovar et al., 2006). These features qualify *B. calamita* as a model organism which may show adaptive modifications of behavioural thermoregulation along a latitudinal gradient. However, information on the influence of environmental temperature (t_e) on the behaviour and core temperature (t_b) of free-ranging toads is limited to two moist and temperate sites in Germany (50°N; Sinsch, 1998; Leskovar and Sinsch, 2001). Toads of these populations cope with the constraints of ambient temperature and of water availability by (1) being strictly nocturnal, (2) burrowing in moist sandy soils during day and prolonged dry periods, and (3) tolerating a voluntarily chosen t_b of 38 °C without harm. Nothing is known about the thermal ecology of natterjacks in habitats with hot, arid climate and soils which do not permit burrowing. This habitat type is common in the Iberian Peninsula and preliminary data on the ecology of populations inhabiting the semiarid region of the Ebro Valley (41°N) suggest that toads frequently leave hibernacula during mild winter periods, forage and grow (Miaud et al., 2000; Miaud and Sanuy, 2005; Sinsch et al., 2007). Nevertheless, available evidence is indirect and based on the skeletochronological analysis of annual bone growth patterns. To provide direct evidence for a local variation of thermal ecology from the pattern

* Corresponding author.

E-mail addresses: noromi@prodan.udl.cat (N. Oromí), dsanuy@prodan.udl.cat (D. Sanuy), sinsch@uni-koblenz.de (U. Sinsch).

observed in Germany, we monitored movements and t_b of free-ranging adult toads during the annual cycle using radio-tracking with temperature-sensitive transmitters. Aims of this study are (1) to describe the thermoregulatory behaviour and the range of t_b experienced by free-ranging *B. calamita* adults in the semiarid habitat of Mas de Melons, and (2) to analyse the temperature- and moisture-related shelter use during the dry summer period and during hibernation. The latitudinal variation of strategies employed to cope with environmental temperature and hydration stress is discussed.

2. Material and methods

2.1. Study area and local climate

Natterjack toads *Bufo* (= *Epidalea*) *calamita* were studied at Mas de Melons (41.51°N, 0.74°E, 240 m asl) in the valley of Ebro river (Lleida, Spain). The climate is continental-mediterranean with average summer temperatures of 24.7 °C in July and winter temperatures of 5.3 °C in January (Meteorological station of Lleida, period 1971–2000; Instituto Nacional de Meteorología). The area is considered semiarid with an average annual precipitation of 370 mm. The study was conducted in an agricultural area of 200 ha including fields of winter cereals and pasture (Fig. 1). Toads reproduce in permanent eutrophic cattle ponds or in ephemeral water bodies that form during heavy rainfall. Further details on the study site are given in Miaud et al. (2000), Miaud and Sanuy (2005), and Sinsch et al. (2007).

Local air temperatures (daily minimum t_{e_min} and maximum t_{e_max}) were provided by the meteorological station Castellidans, Servei Meteorològic de Catalunya (41.53°N, 0.754°E, 225 m asl) which is about 1 km distant from the study area. Air temperature t_{e_site} at the position of a toad was measured in the shade at about 1 m height using a digital thermometer (HD 9215; to the nearest 0.2 °C), as a surrogate for actual soil temperature. We did not measure temperature at the very place of the toad because radio-tracked individuals were often hidden in crevices or burrows at an unknown depth rendering measurements in the close vicinity impossible. Measurements were taken immediately after recording the core temperature of a toad. In 2008, we recorded additionally the temperature (logging interval: 30 min $t_{e_shelter}$)

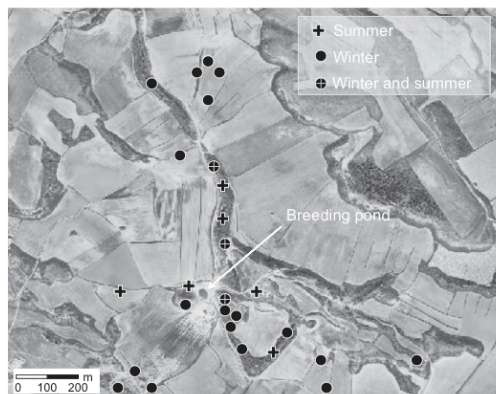


Fig. 1. Map of study area. The location of the 27 shelter sites which were used by the radio-tracked toads is indicated. Crosses refer to the daytime shelters during summer activity, dots to hibernacula. Modified from a map provided by the Institut Cartogràfic de Catalunya.

in an artificial toad shelter using a data logger (Escort Data Logging Systems; to the nearest 0.2 °C). The artificial shelter consisted in a cavity 7 cm below soil surface which was covered with rocks and simulated natural shelter sites in stone embankments.

2.2. Radio-tracking of toads

A total of 15 adults ($n=11$ males, $n=4$ females) were collected at Mas de Melons at March 24, 2006 ($n=5$), January 23, 2007 ($n=2$), April 6, 2007 ($n=1$), and January 5, 2008 ($n=7$) when they emerged from their daytime shelters following rainfall. Toads were transported to the laboratory, sexed, and body mass (to the nearest 0.1 g) and snout-vent length (to the nearest 0.1 mm) measured (Table 1). Each individual was anesthetized by immersion in 500 ml with 250 mg/L of ethyl 3-aminobenzoic methanesulfonate salt (MS-222). Temperature-sensitive radio transmitters (SOPT-2070; Wildlife Materials, Inc.) were implanted into the abdominal cavity through a latero-ventral longitudinal incision (<1.5 cm; Sinsch, 1989a). The wound was closed with 3–5 sutures using cotton thread. Following implantation toads were placed in a water-filled box (1 cm height) until they woke up. Individuals were maintained during 5–7 d in a terrarium (size: 30 cm \times 60 cm \times 45 cm) with access to water and fed *ad libitum* with cockroaches. When toads restored normal locomotion and feeding behaviour, they were released at the same site about 20 m east of the breeding pond shore and tracked for up to 3 months.

Prior to implantation, the radio transmitters were coated with paraffin which increased average mass to a total of 2.78 ± 0.07 g. In SOPT-2070 transmitters, an internal thermistor modulates the interval between signals transmitted depending on the ambient temperature. Each transmitter was calibrated in a water bath (range 0.1–37.0 °C) to obtain the empirical relationship between signal interval and temperature. Transmitter signal (frequency range: 150.025–150.430 MHz) was detected using a hand held scanner Albrecht AE 65 H. A device (self-manufactured at the University of Koblenz electronic lab) connected to the scanner measured the interval between two signals (to the nearest 1 ms) as the surrogate of transmitter temperature ($=t_b$). Intervals were converted to temperature using the regression models obtained before. In field, receiver and interval detector were fitted with a 3-element Yagi antenna which permitted the localization of individuals which were up to 50 m distant. Using a homing-in procedure we localized the shelter site of an individual to the nearest 1 m² and recorded position on a high resolution map. We distinguished three categories of shelter sites: rabbit hole, fissure in stone embankment, and crevice at the base of dry stone walls. Immediately following localization signal interval ($=t_b$) was measured. Position of toad and t_b were recorded daily during the monitoring period. At the days of low winter temperatures and of high summer temperatures, t_b of each individual was recorded up to five-times per day.

2.3. Statistical analyses

As toads were tracked during different seasons of different years, data were pooled to describe the annual variation of t_b . To infer seasonal thermoregulatory behaviour, data were classified according to summer and winter tracking periods (Table 1). The signal intervals of the radio transmitters were assigned to corresponding t_b by fitting transmitter-specific exponential models obtained by regression analyses. The relationship between t_b and corresponding t_{e_site} and $t_{e_shelter}$ was described by fitting 27 regression models. Description was based on the model

Table 1
Features of the radio-tracked *B. calamita* adults and of the tracking period.

Sex/ID	SVL (mm)	Mass (g)	Dates tracking period		Tracking days	Total distance moved (m)	Season
			Of release	Of last location			
Male 1	76.0	52.1	29/03	30/06/2006	65	372	Summer
Male 2	72.4	42.1	29/03	30/06/2006	65	309	Summer
Male 3	82.9	62.4	29/03	30/06/2006	65	436	Summer
Male 4	77.8	49.0	29/03	30/06/2006	80	300	Summer
Male 5	75.6	52.2	29/03	30/06/2006	80	56	Summer
Male 6	70.0	30.8	12/04	12/05/2007	24	576	Summer
Male 7	74.0	38.0	30/01	21/03/2007	67	1872	Winter
Female 1	80.0	56.5	30/01	21/03/2007	76	372	Winter
Male 8	70.0	31.8	09/01	30/03/2008	50	781	Winter
Female 2	66.0	30.4	09/01	30/03/2008	87	91	Winter
Male 9	68.0	25.8	09/01	30/03/2008	73	656	Winter
Male 10	60.0	21.8	09/01	30/03/2008	58	555	Winter
Female 3	71.0	41.7	09/01	30/03/2008	30	469	Winter
Male 11	75.0	44.9	09/01	30/03/2008	83	868	Winter
Female 4	74.0	54.7	09/01	30/03/2008	19	392	Winter

SVL: Snout-vent length.

providing the maximum R^2 . Distributions of temperature data were tested for normality. If data deviated significantly from normality, we used the Mann–Whitney–Wilcoxon u-test to compare medians and the Kolmogorov–Smirnov test to compare the shape of distributions. Significant level for all statistics was set at $\alpha=0.05$. All calculations were performed using procedures of program package Statgraphics Centurion, version XV.

3. Results

3.1. General features of thermal ecology at Mas de Melons

Toads were strictly nocturnal in the study area moving exclusively during nights following moderate to heavy rainfall. The daytime period was spent underground within shelters (see below). t_b of free-ranging natterjack toads ranged from a minimum of $+0.3$ °C during winter to a maximum of 32.2 °C during summer (Fig. 2A). In contrast, variation of t_a was -6.2 °C to 39.9 °C in 2006, -10.0 °C to 41.1 °C in 2007, and -4.6 °C to 37.2 °C in 2008. The pattern of annual variation of diel t_{e_min} and t_{e_max} and that of precipitation is exemplified for 2007 (Fig. 2B and C). Reproductive activity began on April 5 responding to the heavy rainfall on April 1 (39.8 mm/d) and consecutive days without freezing night frost. Aboveground activity continued until mid-June and ceased completely in response to the absence of precipitation. Foraging movements restarted mid-September when the autumnal rainfalls moistened soil again. In mid-November, the first night frosts triggered underground hibernation which continued with occasional short interruptions during warmer days until the beginning of the breeding period in May 12, 2008.

The linear regression model provided the best fit between t_b and t_{e_site} (slope 0.62 ± 0.02 , $P < 0.0001$; intercept 5.22 ± 0.38 °C, $P < 0.0001$; $n=540$). t_{e_site} accounted for 54.1% of total variance in t_b ($r=0.736$; $P < 0.0001$). t_{e_site} and $t_{e_shelter}$ were correlated significantly, but the regression model ($t_{e_shelter} = \exp[a + b * t_{e_site}]$) suggested a considerable attenuation of air temperature extremes by about 23% ($R^2=45.0\%$, $P < 0.0001$). Range of variation and median t_b recorded during summer and winter did not vary significantly between males and females (Mann–Whitney–Wilcoxon u-test, $P > 0.05$), therefore we pooled data for further analysis. Median t_b was significantly lower during winter (10.1 °C) than during summer (21.0 °C; Mann–Whitney–Wilcoxon u-test, $P < 0.0001$), but scatter was significantly larger during winter (Kolmogorov–Smirnov test, $P < 0.0001$; Fig. 3).

3.2. Shelter use and individual thermal performance

Daytime shelter during summer and hibernacula were located exclusively within the ruderal areas separating crop fields and bordering farm tracks (Fig. 1). The dry and argillaceous soil prevented the toads from actively burrowing. Instead they used existing cavities between soil and stones or rabbit holes. We recorded a total of 42 changes among a total of 27 different shelter sites corresponding to 2 rabbit holes, 15 fissures in stone embankment, and 10 crevices at the base of dry stone walls. The toads used 8 summer shelters (2 rabbit holes, 5 fissures, 2 crevices) and 21 hibernacula (2 rabbit holes, 11 fissures, 8 crevices). The only sites used as shelter during summer and winter were the rabbit holes and one fissure. Independent of season, all toads moved during the radio-tracking periods, from 56 m to 1872 m line-of-sight distance (Table 1).

During all tracking periods toads chose those shelter sites or hibernacula which provided a $t_{e_shelter}$ similar or exceeding t_{e_max} (Fig. 4). Depending on their position relative to ground surface, t_b showed either wide day-to-day fluctuations or a rather narrow range of variation. During summer, male 6 followed tightly any fluctuation of t_{e_max} (Fig. 4A), while male 4 stayed at a warm shelter in which air temperature fluctuations were attenuated (Fig. 4B). They achieved this type of behavioural t_b regulation by choosing and changing appropriate daytime shelters. During winter, the selective exploration of warm hibernacula continued and sometimes included a change of location by aboveground movements (Table 1). Male 9, for example, moved 555 m line-of-sight distance from a surface-close fissure to a deeper crevice (Fig. 4C). Other individuals such as female 3 stayed in the same hibernaculum which provided a reasonable attenuation of t_e fluctuation during the tracking period (Fig. 4D).

3.3. Individual models of behavioural thermoregulation

The average number of t_b measurements per individual was 36 (range 13–73) permitting the calculation of individual (linear) regression models to describe the influence of t_{e_site} on t_b (Table 2). Eight out of 15 slope estimates did not deviate significantly from zero suggesting independence of t_b from t_{e_site} . This type of behavioural thermoregulation dominated during summer and resulted in average t_b of 20 – 22 °C during summer and of 7 – 11 °C during winter (Fig. 5, Table 2). The remaining slope estimates which were significantly different from

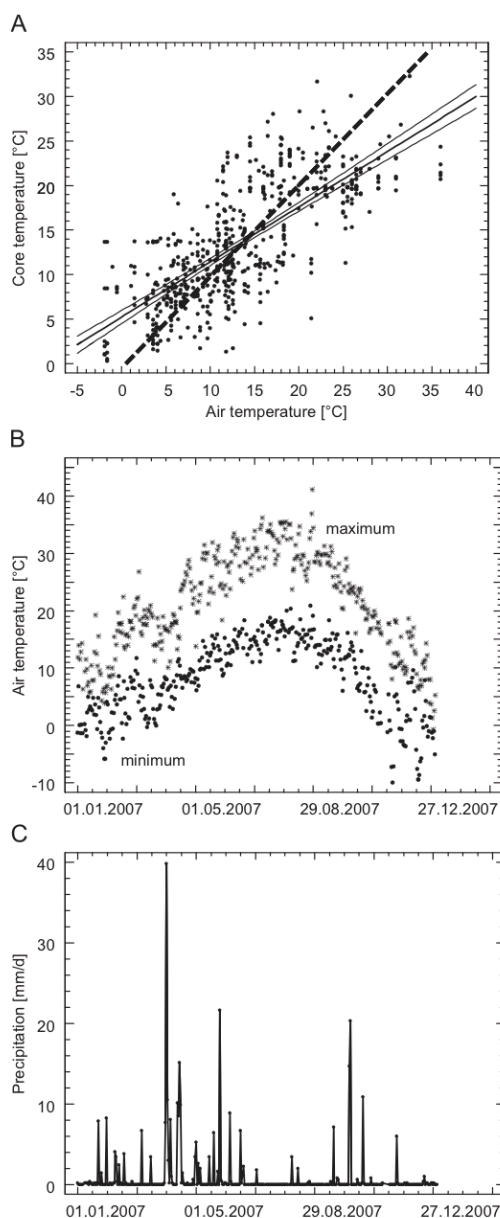


Fig. 2. (A) Effect of ambient air temperature (t_{e_site}) on the core temperature (t_b) of 15 radio-tracked *B. calamita* ($n=540$ observations). Each dot represents a single measurement. The regression line and the corresponding 95% confidence interval and the isotherm line (dotted) are also given (linear regression model). Seasonal variation of (B) minimum (dot, t_{e_min}) and maximum (asterisk, t_{e_max}) air temperature, and (C) daily precipitation in 2007.

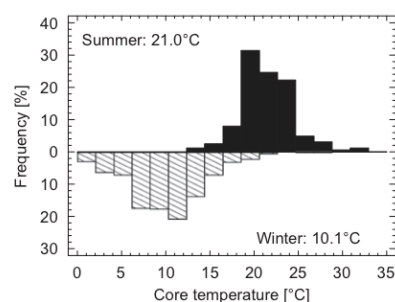


Fig. 3. Frequency histogram of core temperatures (t_b) measured in free-ranging *B. calamita* during summer ($n=162$ observations) and winter ($n=381$ observations). Numerical values refer to temperature medians. For details see Table 1.

zero varied between +0.19 and +0.76 suggesting the t_b increased parallel to t_{e_site} , but to lesser degree. However, the influence of t_{e_site} accounted for a maximum of 50.6% of the variation of t_b , mostly considerably less (Table 2). This type of conformity behaviour dominated during winter (Fig. 5).

4. Discussion

A common feature of the thermal ecology of terrestrial toads (formerly included in the genus *Bufo*, currently split in genera such as *Bufo sensu stricto*, *Epidalea*, *Pseudepidalea*, *Anaxyrus*, *Rhinella* and many more; Frost et al., 2006) is that t_b variation is an attenuated mirror of the considerably greater variation of t_e (e.g. Smits, 1984; Sinsch, 1989a; O'Connor and Tracy, 1992; Rausch et al., 2008; this study). This does not only hold true for hot summer temperatures which may exceed lethal limits in desert environments, but also for winter frosts which would kill individuals by crystallisation of body fluids, if not freeze-tolerant (Gelder et al., 1986; Storey and Storey, 1996; Leskovar and Sinsch, 2001). The strategies employed to reduce ambient temperature variation to a viable range of t_b are mainly based on the selection of appropriate microhabitats (Hutchison and Dupré, 1992; Schwarzkopf and Alford, 1996; Seebacher and Alford, 1999, 2002). The thermal biology of *B. calamita* in Spain and Germany also includes the attenuation of core temperatures as a general feature, but shelter selection was site-specifically different (this study; Sinsch, 1989b, 1998; Leskovar and Sinsch, 2001). Therefore, we first focus on the properties of local shelter microhabitats and then discuss the consequences for body temperature and its behavioural regulation.

4.1. Site-specific selection of shelter microhabitats

North of the Pyrenees natterjack toads inhabit predominantly flood plains and coastal dunes in which soil (mostly sand) permit burrowing, while this habitat type is scarce in the Iberian Peninsula and mainly restricted to the coastal areas (Sinsch, 2008). Daytime shelter, aestivation microhabitats and hibernacula of the northern populations are rarely lacunas below stones or wood debris and frequently small cavities dug into the sand, as exemplified in the populations inhabiting the valley of the River Rhine (Sinsch, 1989b, 1998; Leskovar and Sinsch, 2001). The self-dug cavities do not have connections to the ground surface because the channels collapse as soon as the toad digs deeper. The only difference among the seasonal shelter microhabitats is depth of cavities relative to ground surface ranging from a few

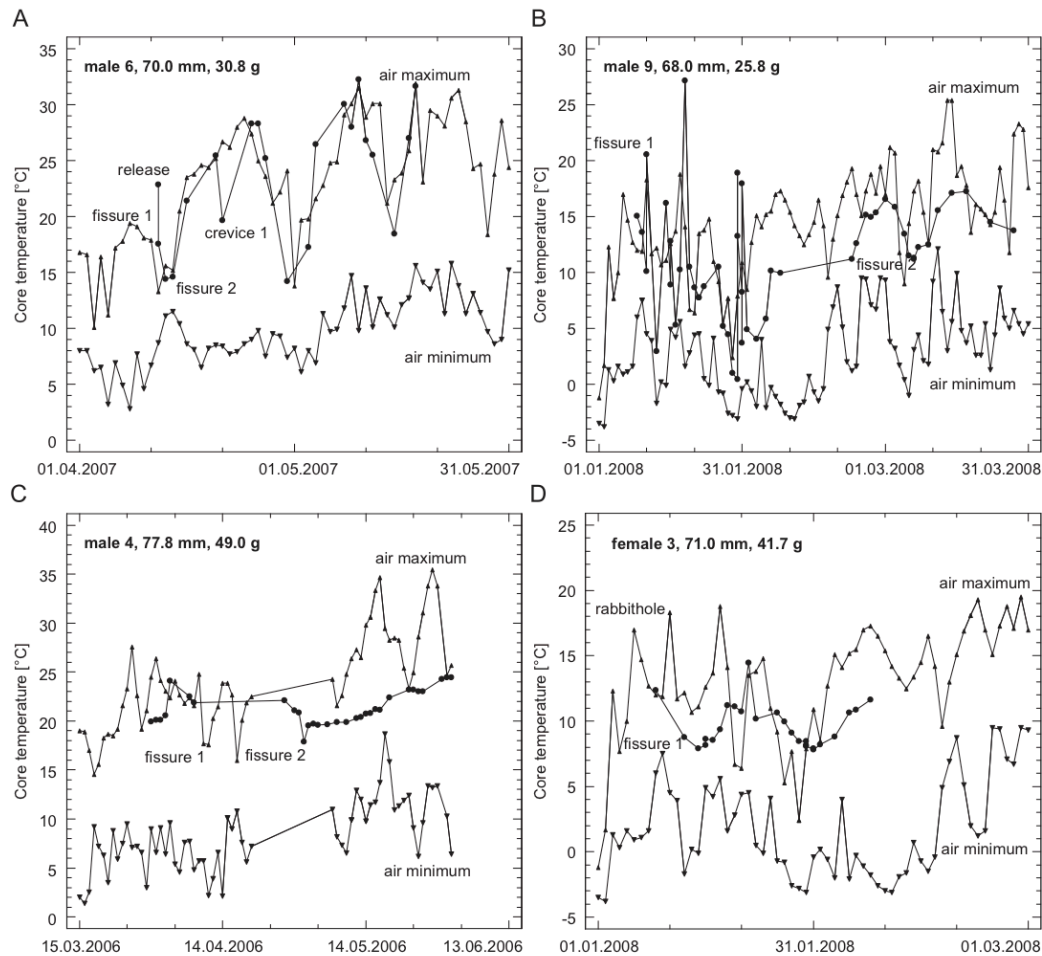


Fig. 4. Variation of t_b (bold line) and ambient air temperature (t_{e_min} , t_{e_max} ; thin lines) during summer (A, B) and winter (C, D). (A) Male 6, tracked in 2007, (B) Male 4, tracked in 2006, (C) Male 9, tracked in 2008, and (D) Female 3, tracked in 2008. Dots represent single measurements of a toad, triangles the daily minimum and maximum air temperature at 2 m height in the shade. Type of shelter and date of shelter change are also indicated.

centimetres to almost 2 m (Lindenthal et al., 1991; Golay, 1994). Experimental evidence suggests that during summer the toads follow primarily a soil moisture gradient to remain fully hydrated, while during winter frost avoidance seems to be the prevailing motivation as soil is water-saturated even close to the surface (Lindenthal et al., 1991; Sinsch, 1998).

In the semiarid climate of the Ebro valley the argillaceous soil forms a dense surface layer which is impenetrable for a burrowing toad. The only landscape structures offering protection against temperature and dehydration stress at the Mas de Melons are cavities in the embankments bordering fields and farm tracks. Rabbit burrows and cavities (fissures, crevices) between soil and stones or rock share the property to provide moist soil for osmotic water uptake and high air humidity which reduces evaporative water loss (Schwarzkopf and Alford, 1996). Our measurements within an artificial shelter demonstrate that the extremes of t_e are

attenuated within the shelter site chosen by the toads. Consequently, local daytime retreats combine the benefit of keeping water economy in balance with body temperatures which are close to t_{e_max} if they do not approach lethal limits (CTM=41 °C; Mathias, 1971). Movements within and among shelters indicate the attempt to optimize t_b by the selective use of moist microhabitat which additionally provide maximum ambient temperature.

4.2. Adaptive significance of site-specific behavioural thermoregulation

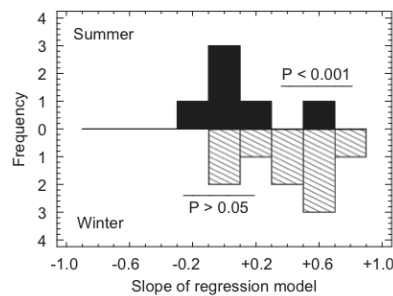
Selective microhabitat use enables ectotherms such as reptiles to maintain preferred t_b within a small range of variation throughout most of the day, as do garter snakes (thermal specialists; Huey and Hertz, 1984; Huey and Peterson, 1989).

Table 2

Individual linear regression models of the influence of local air temperature at the shelter site on the toads core temperature.

Sex/ID	Regression model		R ² (%)	Average core temperature (°C)	Season
	Intercept	Slope			
Male 1	23.9***	-0.09	10.0	22.0 ± 1.6	Summer
Male 2	24.6***	-0.16	13.1	21.2 ± 2.5	Summer
Male 3	17.3***	+0.15	17.1	20.7 ± 2.1	Summer
Male 4	23.3***	-0.09	10.3	21.4 ± 1.7	Summer
Male 5	21.8***	-0.06	3.1	20.6 ± 1.8	Summer
Male 6	9.7**	+0.68***	50.6	-	Summer
Male 7	7.9***	+0.19***	18.7	-	Winter
Female 1	4.9***	+0.55***	48.3	-	Winter
Male 8	2.4	+0.76***	49.4	-	Winter
Female 2	5.2***	+0.56***	36.1	-	Winter
Male 9	5.0***	+0.67***	42.5	-	Winter
Male 10	2.5*	+0.47***	31.8	-	Winter
Female 3	12.5***	-0.02	0.2	9.6 ± 1.7	Winter
Male 11	9.0***	+0.08	4.7	7.2 ± 4.5	Winter
Female 4	6.4	+0.37	14.4	11.0 ± 3.3	Winter

Levels of statistical significance: -P > 0.05, *P < 0.05, **P < 0.01, ***P < 0.001.

**Fig. 5.** Frequency histogram of the slopes of the linear regression models ($t_{\text{core-site}}$ vs. t_b) which were calculated for each radio-tracked individual. Data are classified for summer and winter, respectively, and for statistical significance (deviation from zero). For details see Table 2.

In contrast, temperature-selective behaviour of amphibians is often superposed by efforts to keep fully hydrated (Tracy et al., 1993). In *B. calamita*, t_b adjusted under controlled conditions ranges from 30 to 37 °C, if water is available, and decreases to 24–26 °C, if toads stay on dry sand (Strübing, 1954; Sinsch, 1998). In the field, *B. calamita* maintain t_b within the viable range by choosing appropriate microhabitats. Avoidance of t_b below 0 °C and above 38 °C and the absence of a narrow range of preferred t_b are thermoregulatory features of natterjacks independent of the habitat type. This pattern is consistent with that of thermal conformers which closely track ambient temperature. Interestingly, the same features are observed in the desert toad *Bufo punctatus* which might indicate that in the anurans of arid environments selection favours thermal generalist ("jack-of-all temperatures is a master of none"; Huey and Hertz, 1984; Rausch et al., 2008).

Do thermoregulatory traits differ between natterjacks inhabiting moist sand habitats and those of semiarid landscapes? Putting aside the primarily moisture-driven microhabitat selection, maximum t_b and preferred t_b range are about 4–6 °C lower at Mas de Melons during summer indicating that moist shelter are usually associated with relatively low temperatures. Thus, low and rather constant t_b at Mas de Melons does not reflect thermoregulatory efforts, but is simply the consequence of

hydro-selective behaviour. The superior level of summer t_b at the German sites is achieved by the toads' preference of sun-exposed embankments over shady ones, as both offer moist sand and therefore, result of thermoregulatory behaviour (Sinsch, 1989b, 1998). In conclusion, natterjacks display detectable behavioural efforts to increase summer t_b only in habitats which provide moist retreat sites of different temperatures.

During winter, temperature decreases and water availability increases throughout the geographical range of natterjacks suggesting that dehydration stress poses a minor problem to toads. In fact, scatter of t_b and frequency of shelter site change augmented at Mas de Melons reflecting the thermoregulatory effort to stay in frost-proof cavities. Still, minimum winter t_b was 3–4 °C lower than in hibernating toads in Germany which escape increasing frost intensity by burrowing deeper into the temperature-buffered sand. This type of thermoregulatory behaviour is not feasible in the dense soils at Mas de Melons and replaced by the alternative aboveground displacement during warmer winter nights. The same behaviour has been observed during a warm winter in Germany as well (Leskovar and Sinsch, 2001). However, these winter movements are rare foraging trips and do not contribute to thermoregulation because during frost periods even daytime air temperatures are too low to permit aboveground escape from freezing hibernacula.

In conclusion, *B. calamita* are thermal conformers with thermoregulatory behaviour restricted to the avoidance of upper and lower lethal temperatures. The avoidance behaviour differs site-specifically in response to the availability of moist retreat sites. Thus, observed t_b variation of toad mirrors primarily the thermal properties of those shelter sites which permit the maintenance of water balance. Natterjack toads do not represent an exception from the rule that the control of water balance outweighs the optimisation of core temperature in amphibians.

Acknowledgements

The permission for field studies and handling of the toads were issued by the Departament de Medi Ambient, Generalitat de Catalunya, Spain. Several students of the University of Lleida helped during radio-tracking. The comments of two anonymous reviewers helped to improve the manuscript.

References

- Bartelt, P.E., Peterson, C.R., 2005. Physically modeling operative temperatures and evaporation rates in amphibians. *J. Therm. Biol.* 30, 93–102.
- Brattstrom, B.H., 1963. A preliminary review of thermal requirements of amphibians. *Ecology* 44, 238–255.
- Brattstrom, B.H., 1979. Amphibian temperature regulation studies in the field and laboratory. *Am. Zool.* 19, 345–356.
- Frost, D.R., Grant, T., Faivovich, J., Bain, R.H., Haas, A., Haddad, C.F.B., De Sa, R.O., Channing, A., Wilkinson, M., Donnellan, S.C., Raxworthy, C.J., Campbell, J.A., Blotto, B.L., Moler, P., Drewes, R.C., Nussbaum, R.A., Lynch, J.D., Green, D.M., Wheeler, W.C., 2006. The amphibian tree of life. *Bull. Am. Mus. Nat. Hist.* 297, 1–370.
- Gelder, J.J. van, Olders, J.H.J., Bosch, J.W.G., Starmans, P.W., 1986. Behavior and body temperature of hibernating common toads *Bufo bufo*. *Holarctic Ecol.* 9, 225–228.
- Golay, N., 1994. Eine Methode zur äußerlichen Befestigung von Telemetriesendern an Kreuzkröten sowie erste Ergebnisse bei der Anwendung. *Ber. Landesamt Umwelts. Sachsen-Anhalt* 14, 44–48.
- Huey, R.B., 1991. Physiological consequences of habitat selection. *Am. Nat.* 137, S91–S115.
- Huey, R.B., Hertz, P.E., 1984. Is a jack-of-all-temperatures a master of none? *Evolution* 38, 441–444.
- Huey, R.B., Peterson, C.R., 1989. Hot rocks and not-so-hot rocks: retreat-site selection by garter snakes and its thermal consequences. *Ecology* 70, 931–944.
- Hutchison, V.H., Dupré, R.K., 1992. Thermoregulation. In: Feder, M.E., Burggren, W.W. (Eds.), *Environmental Physiology of the Amphibians*. The University of Chicago Press, pp. 206–249.
- Jørgensen, C.B., 1992. Growth and reproduction. In: Feder, M.E., Burggren, W.W. (Eds.), *Environmental Physiology of the Amphibians*. The University of Chicago Press, pp. 439–466.
- Leskovar, C., Sinsch, U., 2001. Hibernation behaviour of radiotracked natterjack toads *Bufo calamita* and green toads *Bufo viridis*. *Biota* 2 (Suppl.), 33–34.
- Leskovar, C., Oromí, N., Sanuy, D., Sinsch, U., 2006. Demographic life history traits of reproductive natterjack toads (*Bufo calamita*) vary between northern and southern latitudes. *Amphibia-Reptilia* 27, 365–375.
- Lillywhite, H.B., 1971. Thermal modulation of cutaneous mucus discharge as a determinant of evaporative water loss in the frog, *Rana catesbeiana*. *Z. F. Vergl. Physiol.* 73, 84–104.
- Lindenthal, E., Sinsch, U., Schneider, H., 1991. The behavioural and physiological ecology of estivation in natterjack toads (*Bufo calamita*). *Verh. Deut. Zool. Ges. (Tübingen)* 84, 318.
- Mathias, J.H., 1971. The comparative ecologies of two species of amphibia (*Bufo bufo* and *Bufo calamita*) on the Ainsdale sand dunes national nature reserve. Unpublished, Master-thesis, University of Manchester.
- Miaud, C., Sanuy, D., 2005. Terrestrial habitat preferences of the natterjack toad during and after the breeding season in a landscape of intensive agricultural activity. *Amphibia-Reptilia* 26, 359–366.
- Miaud, C., Sanuy, D., Avriplier, J.N., 2000. Terrestrial movements of the natterjack toad *Bufo calamita* (Amphibia, Anura) in a semi-arid, agricultural landscape. *Amphibia-Reptilia* 21, 357–369.
- O'Connor, M.P., Tracy, C.R., 1992. Thermoregulation by juvenile toads of *Bufo woodhousei* in the field and in the laboratory. *Copeia*, 865–876.
- Pinder, A.W., Storey, K.B., Ultsch, G.R., 1992. Estivation and hibernation. In: Feder, M.E., Burggren, W.W. (Eds.), *Environmental Physiology of the Amphibians*. The University of Chicago Press, pp. 250–276.
- Rausch, C.M., Starkweather, P.L., van Breukelen, F., 2008. One year in the life of *Bufo punctatus*: annual patterns of body temperature in a free-ranging desert anuran. *Naturwissenschaften* 95, 531–535.
- Schwarzkopf, L., Alford, R.A., 1996. Desiccation and shelter-site use in a tropical amphibian: comparing toads with physical models. *Funct. Ecol.* 10, 193–200.
- Seebacher, F., Alford, R.A., 1999. Movement and microhabitat use of a terrestrial amphibian (*Bufo marinus*) on a tropical island: seasonal variation and environmental correlates. *J. Herpetol.* 33, 208–214.
- Seebacher, F., Alford, R.A., 2002. Shelter microhabitats determine body temperature and dehydration rates of a terrestrial amphibian (*Bufo marinus*). *J. Herpetol.* 36, 69–75.
- Sinsch, U., 1989a. Behavioral thermoregulation of the Andean toad (*Bufo spinulosus*) at high-altitudes. *Oecologia* 80, 32–38.
- Sinsch, U., 1989b. Sommer- und Winterquartiere der Herpetofauna in Auskiesungen. *Salamandra* 25, 104–108.
- Sinsch, U., 1998. *Biologie und Ökologie der Kreuzkröte*. Laurenti Verlag.
- Sinsch, U., Oromí, N., Sanuy, D., 2007. Growth marks in natterjack toad (*Bufo calamita*) bones: histological correlates of hibernation and aestivation periods. *Herpet. J.* 17, 129–137.
- Sinsch, U., 2008. *Bufo calamita* Laurenti, 1768 - Kreuzkröte. In: Grossenbacher, K. (Ed.), *Handbuch der Amphibien und Reptilien Europas*. Aula Verlag, 5/1; 2008, pp. 339–413.
- Smits, A.W., 1984. Activity patterns and thermal biology of the toad *Bufo boreas halophilus*. *Copeia*, 689–696.
- Storey, K.B., Storey, J.M., 1996. Natural freezing survival in animals. *Ann. Rev. Ecol. Syst.* 27, 365–386.
- Strübing, H., 1954. Über Vorzugstemperaturen von Amphibien. *Z. Morph. Ökol. Tiere* 43, 357–386.
- Tracy, C.R., Christian, K.A., O'Connor, M.P., Tracy, C.R., 1993. Behavioral thermoregulation by *Bufo americanus*: the importance of the hydric environment. *Herpetologica* 49, 375–382.

Connectivity of amphibian populations: modelling the migratory range of radio- tracked natterjack toads

Submitted to: *Animal Conservation*

Authors: SINSCH, U.; OROMI, N.; MIAUD, C.; DENTON J.; SANUY, D.

The movement distances of natterjack adult toads recorded in the radio-tracking experience described in Chapter 1 was evaluated in order to analyse the variation of migratory range among different populations. In this chapter, radiotracked data from eight studies were included in a general analysis to model the movement distances of adult populations. We also qualified the influence of local habitat features, such latitude, altitude, precipitations, temperatures and substrate type on movement distances. These models allowed to estimate the size of buffer zones around breeding sites for the management of population, considering factors such as the maintenance of connectivity among local population or the decline of the endangered populations at geographical limit of distribution. With respect to metapopulations dynamics of natterjack toads, we predicted the spatial range of genetic cohesion and compare prediction with available evidence (see Chapter 5).

Author contribution: I have personally developed the concept of the paper with the direction of Prof. Drs Ulrich Sinsch and Delfi Sanuy. I performed the radiotracking and data analyse for population of Mas de Melons. I joined the data and made all the statistical and model analysis with Prof. Dr. Sinsch. I have written the paper in close collaboration with Prof. Dr. Sinsch and it was revised for the other authors for publication.

ABSTRACT

If populations are considered as the evolutionarily significant units of biological conservation, the delimitation of their spatial equivalent is crucial. In Amphibia, spatial organisation in metapopulations requires knowledge on the individual variation of migratory range within local populations to estimate the connectivity among neighbouring populations and thus, the size of metapopulation. The migratory behaviour of 143 adult *Bufo calamita* was monitored using radio telemetry at eight localities covering a latitudinal range of 13°. Aims of the study were to assess the geographic variation of migratory range and to model the relationship between movement distance and the proportion of adult populations. Migratory range was not sex-biased, but was three times lower in population inhabiting sandy areas than in those on clay soils (suboptimal shelter availability). For conservation management of local natterjack populations, we distinguish between the size of the core area (migratory range of 50% of the individuals) and that of genetic connectivity (the upper 5% of dispersers). Models obtained for “sand” populations (e.g. central Europe, UK) suggest that a minimum terrestrial buffer range of 600 m around the breeding site will be needed to keep the core area undisturbed. Connectivity among local populations is maintained at maximum distances of 2,250 m between the breeding ponds. Thus, the principal conservation problem in the UK is that most populations are isolated by distance and prone to local extinction, as recolonisation by dispersers from neighbouring sites is highly improbable. Corridors with low landscape resistance for dispersing toads do not seem to be required, because neither dry agricultural area with high mortality risks due to dehydration and predation in Spain nor urban and industrially used areas in Germany impede successful long-distance movements.

KEYWORDS: *Bufo calamita*; radio telemetry; dispersal model; local variability; metapopulation dynamics; buffer zone

INTRODUCTION

If populations are considered as the evolutionarily significant units of biological conservation, the delimitation of their spatial equivalent is crucial (e.g. Waples, 1995; Hey *et al.*, 2003; Schaefer, 2006). Understanding the functioning of populations requires delimiting subpopulations and estimating landscape connectivity (e.g. Waples & Gaggiotti, 2006; Baguette & Van Dyck, 2007; Ribeiro *et al.*, 2011). Landscape connectivity refers to functional (how dispersal is affected by landscape structure and elements) and structural connectivity (spatial configuration of habitat patches in the landscape, e.g. vicinity or presence of barriers, Taylor *et al.*, 1993). Dispersal is thus a key behavioural trait, constituting the focus of functional connectivity (Clobert *et al.*, 2001; Taylor *et al.*, 2006).

Among terrestrial vertebrates, amphibian's metapopulation ecology and conservation require reliable information on both functional and structural landscape connectivity (Safner *et al.*, 2011). The migratory range of an amphibian species determines its ability to disperse, i.e. to colonise or to re-colonise breeding sites, and to maintain genetic cohesion among neighbouring local populations (Sinsch, 1990; Smith & Green, 2005). Besides genetic estimates of connectivity (e.g. Waples & Gaggiotti, 2006), few extensive field studies report actual movement distances of a large number of marked individuals (e.g. *Rana luteiventris*, Funk *et al.*, 2005; *Bufo fowleri*, Smith & Green, 2006). A recent review compiling mark-recapture studies on 53 anuran species revealed that documented dispersal distance of 56 % of species was less than 1 km, but that of four species exceeded 10 km (Smith & Green, 2005). The longest documented movement of a marked amphibian amounted to 34 km in a subadult *Bufo fowleri* (Smith & Green, 2006), almost equalling the annual range expansion of 55 km of *Bufo marinus* in Australia (Phillips *et al.*, 2007). These impressive distances have modified our view on amphibians as slowly crawling, rather sedentary

ectotherms, but still estimates of dispersal distances include a considerable amount of uncertainty because most of them rely on chance recaptures of previously marked individuals. Dispersal distances do not seem to be sex-biased, but those of juveniles are larger (Funk *et al.*, 2005) or equal (Smith & Green, 2006). A more direct approach to estimate movement distances is radio-telemetric monitoring (White & Garrott, 1990). A Kernel density estimate for 13 temperate zone amphibians demonstrated that 50% of individuals stayed within a radius of 93 m around the breeding site (Rittenhouse & Semlitsch, 2007). However, it is currently unknown, whether conspecific populations of widely distributed species differ in their migratory behaviour in response to local environmental features and climate gradients.

The natterjack toad *Bufo calamita* is a well-suited model organism to analyse the inter- and intra-population variability of migratory range because it is one of the best studied temperate-zone anurans with respect to spatial ecology. Radio-tracking studies at eight localities cover a latitudinal range of 13° (Sinsch, 1988, 1992a; Denton & Beebee, 1993; Miaud *et al.*, 2000; Huste *et al.*, 2006; Oromi *et al.*, 2010; Sinsch & Leskovar, 2011). In this paper, we combine our data on radio-tracked adults: (1) to detect potential sex-specific differences in migratory range, (2) to model the relationship between movement distance and proportion of adult population, and (3) to quantify the influence of local habitat features such as latitude, altitude, temperature, precipitation and ground surface structure on movement distances. These data permit the analysis of habitat connectivity using combination of least-cost route analysis (with need estimate of species maximum dispersal ability, Adriaensen *et al.*, 2003) and graph-theoretic techniques (Chetkiewicz *et al.*, 2006; O'Brien *et al.*, 2006; Fall *et al.*, 2007). This is basic to estimate the size of buffer zones around breeding sites for the management of endangered populations at geographical limit of distribution (e.g. UK, Ireland, Estonia; Rannap *et al.*, 2007; Beebee, 2011), and the tolerable distance of stepping-stone habitats to maintain connectivity among local

populations (e.g. Stevens & Baguette, 2008). Moreover, we will be able to predict the spatial range of genetic cohesion and compare prediction with available evidence (Marangoni, 2006; Rowe & Beebee, 2007; Oromi *et al.*, in prep.).

Table 1. Geographic and climatic features of the study sites. Climate data used for modelling are the average of a 30-years period (1971-2000; sources: Meteorological Office (2008), UK; Deutscher Wetterdienst, Germany; Centre Inter-Départemental de Météo-France de Paris-Montsouris; Instituto Nacional de Meteorología, Spain). Abbreviations: E=Catalonia, Spain, F=France, D=Germany, UK=Great Britain.

Locality	Latitude [°N]	Altitude [m a.s.l.]	Mean July temperature [°C]	Annual precipitation [mm]	Ground surface/ Substrate
E: Mas de Melons	41.50	240	24.7	369	clay
E: Balaguer	41.75	220	24.7	369	clay
F: Paris	48.95	37	20.0	650	pasture
D: Urmitz	50.30	60	19.5	674	sand
D: St. Augustin	50.77	54	18.3	686	sand
UK: Woolmer	51.10	83	20.8	725	sand
UK: Birkdale	53.60	8	19.4	837	sand
UK: Dunnerholme	54.30	3	19.4	850	sand

MATERIAL AND METHODS

Study areas

A total of 143 adult natterjack toads *Bufo (=Epidalea) calamita* were radio-tracked at eight sites located in Spain, France, Germany and the UK between 1987 and 2008. Localities and climate features are summarized in Table 1. Detailed site descriptions have been published elsewhere: (1) agricultural areas in Balaguer and Mas de Melons (Miaud *et al.*, 2000, Oromi *et al.*, 2010), (2) urban park area in Paris (Huste *et al.*, 2006), (3) gravel and sand pits in Urmitz and St. Augustin (Leskovar & Sinsch, 2005; Sinsch, 1988, 1992a), (4) heath land at

Woolmer (Beebee *et al.*, 1982), and (5) coastal dunes near Birkdale and Dunnerholme (Denton & Beebee, 1993).

Radio-Telemetry procedure

Radio-tracking followed the same general procedure at all sites, but transmitter features and tracking devices differed locally. Adult toads were collected in the field, either at the breeding pond or in daytime shelter, and transported to the laboratory (Table 2). Toads were narcotized in MS 222 (ethyl-m-aminobenzoate methanesulfonate) or phenoxyethanol-solutions. In 130 toads, the transmitter was implanted into the abdominal cavities through a 1-cm lateral incision which was closed afterwards by 3-4 sutures, in the remaining 13 individuals (exclusively Balaguer) the transmitters were attached externally (Miaud *et al.*, 2000). The toads were put into fresh water to reverse the effects of the anaesthetic and recovered within 2 h displaying normal behaviour. After additional 2-24 h of recovery the toads were released at the capture site where they were found.

Transmitters were provided by distinct suppliers (Biotrack, Cambridge, UK: SSI and SPI; Custom Electronics, Urbana, Illinois, USA; Holohil System Ltd., Carp, Canada: BD2-GH; Sirtrack, New Zealand: single-stage; Wildlife Materials, Murphysboro, Illinois, USA: SOPI- and SOPT-2070). Mass of implanted transmitter package including battery and coating ranged between 1.75-3.6 g providing a battery life of 1-18 months, whereas external tags weighed 1.5-2.0 g. Receivers equipped with a three-element yagi antenna allowed for a maximum detection range of about 200 m which often decreased to less than 40 m depending on the shelter site of the toad.

Toads were located using a homing-in procedure (White & Garrott, 1990), usually at a daily basis. Depending on the original purpose of the telemetry study, some toads were located several times per day, others every week or two.

Table 2. Features of the radio-tracked toads. Duration of activity period is estimated as number of months in which several individuals made more than one surface movement during the years of tracking. Abbreviations: na=not available.

Locality	Males / Females	SVL range [mm]	Body mass range [g]	Tracking years	Activity period [month]
E: Mas de Melons	11 / 4	60 – 83	21.8 – 64.4	2006 – 2008	January – December
E: Balaguer	8 / 11	na	25.2 – 61.0	1997	January – December
F: Paris	13 / 3	69 – 75	30.0 – 44.0	2001	April – October
D: Urmitz	23 / 14	47 – 72	12.5 – 37.9	1999 – 2001	March – October
D: St. Augustin	21 / 12	56 – 71	19.2 – 33.6	1987 – 1989	April – October
UK: Woolmer	4 / 2	62 – 73	na	1988 – 1990	April – October
UK: Birkdale	5 / 7	56 – 73	na	1988 – 1990	April – October
UK: Dunnerholme	5 / 0	57 – 63	na	1988 – 1990	April – October
Total	90 / 53	47 – 83	12.5 – 64.4	1987 – 2008	

We quantified migratory behaviour estimating the following variables: (1) maximum daily movement distance as line-of-sight distance between the location at t_x and at t_{x+24h} ; (2) total movement distance as the accumulated line-of-sight distances between two successive locations during the whole tracking period, and (3) the frequency of days with migratory activity, calculated only for tracking periods exceeding 10 days data available for Urmitz, St. Augustin and Birkdale). Note that the two distance-related parameters describe exclusively movements which imply changes of shelter sites, whereas foraging surveys with

return to the same shelter are not included. Furthermore, toads rarely moved in straight lines and consequently, the recorded line-of-sight distances underestimate real migratory activity as well.

Models to estimate migratory range

Two models based on distinct empiric variables and assumptions on the migratory activity were used to estimate the spatial scale of migratory activity of toads around the breeding pond. Model 1 was based on the average daily movement distance of toads ($n = 123$) which were tracked at least 10 successive days. The potential annual movement distance (PAMD1) of these toads was calculated as the product of the empirically recorded average daily movement distance and the estimated number of days per year at which air temperature did not impede surface movements. Integrating long-term climate measurements and personal experience on the annual activity pattern of local populations, we assigned the following values of potential activity periods to the studied populations: 330 d to Balaguer and Mas de Melons, 210 d to Urmitz and 180 d to Paris, St. Augustin, Woolmer, Birkdale and Dunnerholme. PAMD1 is aimed to model the ordinary movement range of an individual as influenced by seasonal weather fluctuations.

Model 2 was based on the maximum daily movement distance recorded during the tracking period of each individual ($n = 124$). The potential annual movement distance (PAMD2) of these toads was calculated as the product of the empirically recorded maximum daily movement distance and the estimated number of days per year at which toads perform surface movements. The number of migration days was calculated as the product of the potential annual activity period and the frequency of days at which movements were recorded during an individual's tracking period. PAMD2 is aimed to model the largest possible movement range of an individual assuming optimal conditions throughout the activity period.

Resulting individual PAMD1- and PAMD2-values were ordered by magnitude to obtain a frequency distribution for the percentage of individuals which move distances of up to 15 km (50 m classes). The frequency distribution was compared with the prediction of a negative exponential function, $P = \exp(-D/k)$, where P is the probability that an individual moves the distance D or farther and k is equal to the mean dispersal distance (Ranius, 2006; Rink & Sinsch, 2007). Moreover, we tested 28 standard regression models to describe the PAMD frequency distributions. The model providing the best fit (maximum R^2) between PAMD distribution and prediction was chosen to calculate (1) the maximum movement distance of the 50 % less vagile individuals as a measure for the common migratory range, and (2) the minimum movement distance of the most vagile 5 % individuals as a measure for the disperser maintaining genetic connectivity among neighbouring breeding assemblages.

Statistical analyses

All variables were first tested for normality. As none of the variables conformed to normality, we used either non-parametric statistics to describe distributions or applied logarithmic transformation to normalise the data distribution. Pair wise comparisons of medians were performed using the Mann-Whitney u-test, multiple comparisons the Kruskal-Wallis test. Log-transformed total movement distances conformed to normality. Consequently, the potential effects of sex and ground surface (categorical factors) on this variable were estimated using a 2-factor ANCOVA with tracking period, latitude, altitude, July temperature and annual precipitation as covariates. Least square means and corresponding standard error were compared using a multiple range test with Bonferroni correction. Relationships between variables were assessed using the Pearson's product-moment correlation coefficient and the corresponding regression models. Significance level was set at $\alpha=0.05$. All calculations were based on the procedures of the program package STATGRAPHICS Centurion for Windows, version 15.0.

RESULTS

Field data on movement distances and movement frequency

The maximum distances which 124 radio-tracked toads were observed to move during one day (not available for Paris), i.e. during nocturnal activity, varied between 4.5 m and 686.5 m (Fig. 1A). As this parameter was not significantly related to the number of observations of migratory activity per individual (ANOVA, $F_{1,104}=0.65$, $P=0.428$; $R^2=0.6$ %), we evaluated its uncorrected variability between males and females and localities, respectively. Males and females did not differ significantly with respect to maximum daily movement distance (Mann-Whitney u-test, $P=0.437$). In contrast, there were significant differences among medians of the seven populations (Kruskal-Wallis test, $P<0.00001$; Fig. 1b).

The adults originating from Mas de Melons (Average rank: 95.5) and Balaguer (88.4) moved significantly farther than those from Urmitz (37.0) and Dunnerholme (22.0; pair wise comparison Mann-Whitney u-test, $P<0.00001$), whereas the other populations were intermediate between the extremes. The medians of movement frequency during the tracking period did not differ among the populations inhabiting Urmitz (5.9 %), St. Augustin (5.6 %) and Birkdale (6.0 %; Kruskal-Wallis test, $P = 0.831$). The overall median was 5.8 % ($n = 68$).

Documented total movement distances of 143 radio-tracked adults varied between 0 m and 4,411 m during 1 - 194 d of tracking (Fig. 2). Log-normalised total movement distance did not differ between males and females (ANCOVA, $F_{1,119}=0.47$, $P=0.495$), but co-varied significantly with log-normalised tracking period ($F_{1,119}=21.4$, $P<0.00001$), latitude ($F_{1,119}=19.3$, $P<0.00001$) and altitude ($F_{1,119}=26.1$, $P<0.00001$), whereas SVL (not available for Balaguer) did not co-vary significantly ($F_{1,102}=0.04$, $P=0.847$). Using the data set with body mass (not available for UK localities) as a co-variate instead of SVL, main effects remained

the same and body mass did not co-vary either (ANCOVA, $F_{1,115}=0.01$, $P=0.996$). Consequently, there is no evidence that the additional mass of the tracking package, varying between 4.5 % and 18.4 % of body mass, had a significant effect on the migratory behaviour of natterjack toads at any site.

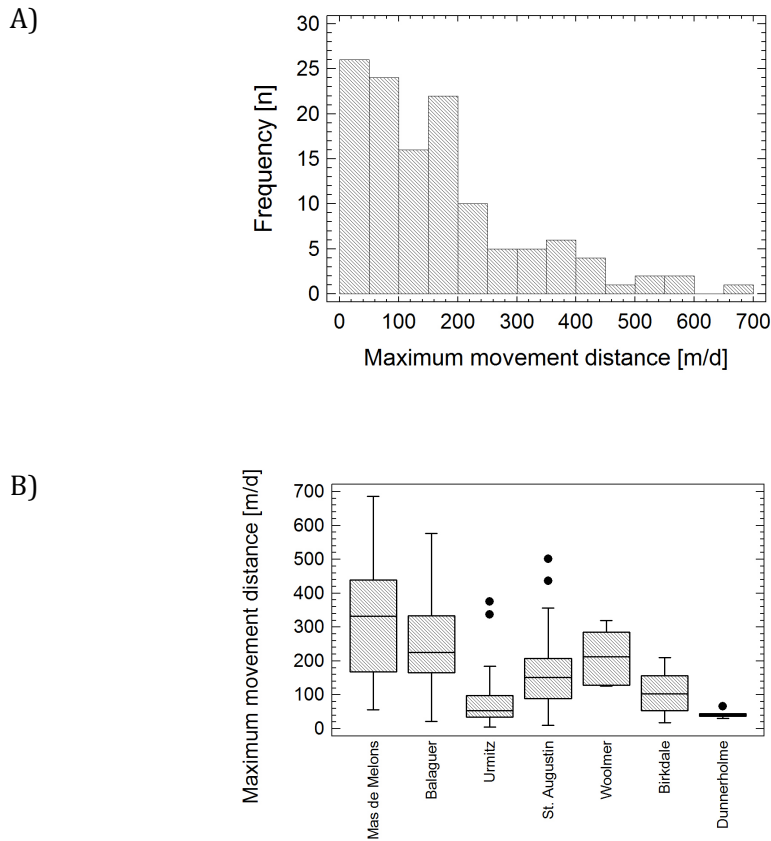


Figure 1. Maximum daily movement distances recorded during the tracking period of 124 individuals. (A) Frequency histogram. (B) Box-and whisker plot of local migratory distances. The horizontal bar indicates the median of distribution, the hatched area the inner 50 % of data, dots are outlier.

Modelling potential annual movement distances

The PAMD1 model was based on the average daily movement distance which a toad moved during its whole tracking period. The exclusion of 20 individuals with tracking periods of less than 10 days reduced the number of observations to 123. Analysis of co-variance considering sex and predominant surface soil as categorical factors and latitude, altitude, July temperature and annual precipitation as co-variables revealed that exclusively ground surface significantly affected log-normalised average daily movement distance (ANCOVA, $F_{1,122}=10.5$, $P=0.0001$). However, there was no significant difference (least square mean \pm standard error) between the populations living on sandy soil (log[distance m/d]: -0.17 ± 0.25) or pasture (-0.39 ± 0.47 ; $P>0.05$), whereas those inhabiting impenetrable clay soils moved significantly larger distances (2.79 ± 0.53 ; Multiple Range test, $P=0.018$).

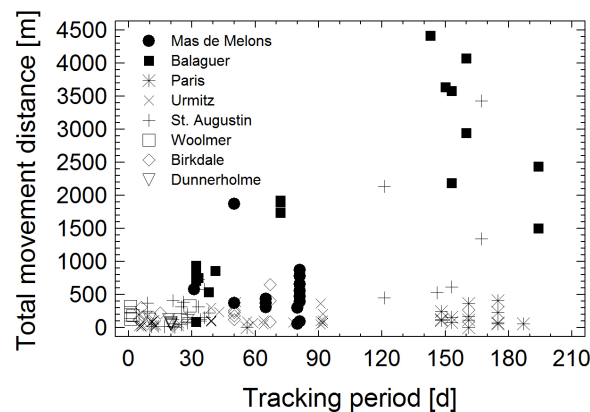


Figure 2. Total movement distance of 143 individuals during the tracking period at eight localities. Each symbol represents one individual.

Since migratory behaviour did not differ significantly between the populations inhabiting sandy soils and pasture, their movement data were pooled for the calculation of individual PAMD1 (SP data set, $n = 93$), whereas the

populations on clay soils were treated separately (C data set, $n = 34$). Modelling PAMD1 with a negative exponential function ($k_{SP} = 855.2$ m) yielded a highly significant correspondence of empiric frequency distribution and model predictions in SP data set (Pearson correlation coefficient $r = 0.998$; Fig. 3). In contrast, the empiric frequency distribution of the C data set was best described by a square root-x model (Frequency [%] = $125.66 - 1.09 * \text{Distance}^{-1/2}$; $r = 0.985$, Fig. 4). The PAMD1 models predict (1) that 50 % of adults will stay within a radius of 600 m (SP) and 4,800 m (C), respectively, around the breeding site, and (2) that 5 % will disperse farther than 2,550 m (SP) and 12,200 m (C), respectively.

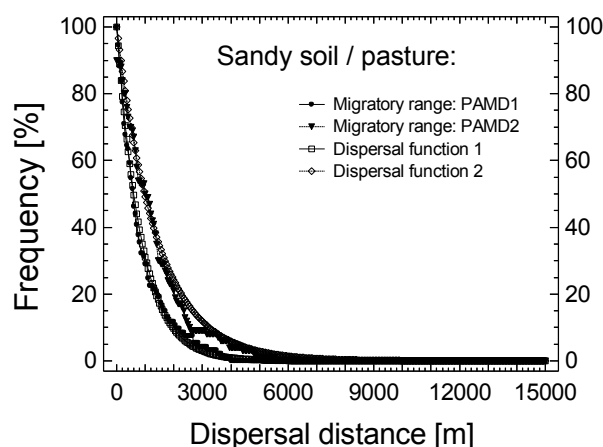


Figure 3. Frequency distribution of potential annual movement distance for natterjack toads inhabiting localities with predominantly sandy soils or pasture. Filled symbols (dots: PAMD1; triangles: PAMD2) show the empiric distributions, open symbols the corresponding dispersal models (negative exponential functions). For statistical details see text.

The PAMD2 model was based on the maximum daily movement distance which a toad was recorded to move during its whole tracking period. The exclusion of 19 individuals (3 did not move at all, data unavailable for the 16 individuals of the Paris population) reduced the number of observations to 124.

Based on the empiric movement frequency and the annual potential activity periods, we estimated the number days with migratory events as 20 d in Balaguer and Mas de Melons, 13 d in Urmitz and 11 d in St. Augustin, Woolmer, Birkdale and Dunnerholme. Movement data were pooled for the calculation of individual PAMD2 with respect to surface substrate (SP data set, $n = 90$; C data set, $n = 34$). Again, modelling PAMD2 with a negative exponential function ($k_{SP} = 1,407.7$ m) yielded a highly significant correspondence of empiric frequency distribution and model predictions in SP data set (Pearson correlation coefficient $r = 0.997$; Fig. 3). In contrast, the empiric frequency distribution of the C data set was best described by a square root-x model (Frequency [%] = $132.84 - 1.16 * \text{Distance}^{-1/2}$; $r = 0.979$, Fig. 4). The PAMD2 models predict (1) that 50 % of adults will stay within a radius of 1,150 m (SP) and 5,100 m (C), respectively, around the breeding site, and (2) that 5 % will disperse farther than 4,200 m (SP) and 12,150 m (C), respectively.

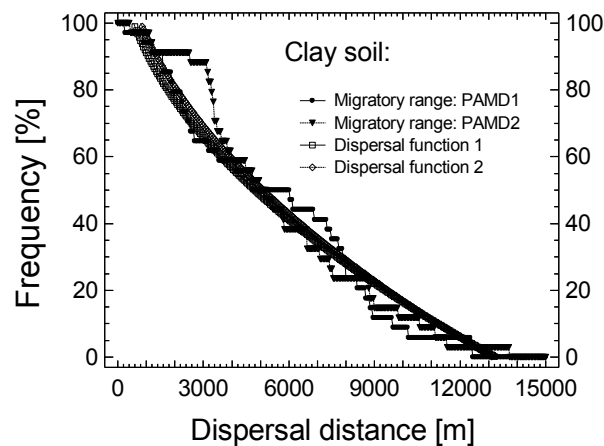


Figure 4. Frequency distribution of potential annual movement distance for natterjack toads inhabiting localities with predominantly clay soils. Filled symbols (dots: PAMD1; triangles: PAMD2) show the empiric distributions, open symbols the corresponding dispersal models (square root regression).

DISCUSSION

Habitat heterogeneity and landscape context is a major concern for amphibian conservation strategies (Werner *et al.*, 2009). The land covers which present variable resistances to ground-welling amphibian dispersal and the maximum distance they can move were used to infer movement costs and dispersal areas around breeding patches in frogs (Safner *et al.*, 2011) and toads (Joly *et al.*, 2003; Stevens *et al.*, 2004). The migratory range is thus a pivotal parameter for amphibian population functioning, but the quality of its estimate is not satisfactory (e.g. lost individuals in radio-tracking studies can be those with largest ranges leading to underestimate maximum ranges).

Our study provides evidence that the empirically determined maximum annual migratory range of adult natterjacks (4,441 m) is only exceeded by 11 out of 53 other anuran species previously studied and that the modelled annual movement distance of 5% of a population exceeds 12,200 m, placing natterjacks among the upper 10% of wide-ranging anuran species (Smith & Green, 2005). Reliability of model predictions on the migratory capacity of dispersing natterjacks is supported by microsatellite estimates of gene flow between neighbouring populations which suggest genetic cohesion of metapopulation systems along a geographic distance of about 100 km (Marangoni, 2006) and an altitudinal range of 1,500 m (Oromi *et al.*, in prep.). Therefore, we conclude that estimates on movement distances, activity period duration and movement frequencies underlying the models do not grossly deviate from reality. Before discussing implications of our model predictions for metapopulation dynamics and conservation management of natterjack toads, we critically evaluate model parameters to estimate an error margin for the predicted distances.

Validation of model parameter

The empiric data on maximum and on average daily movement distance were gathered using radio-tracked individuals, i.e. toads following a surgical intervention and carrying an additional mass of 1.75-3.6 g. As discussed before in several papers on radio-telemetry, there is no real control to detect potential effects on magnitude and frequency of movements because we are unable to follow toads without any type of tracking devices (e.g. Richards *et al.*, 1994; Rowley & Alford, 2007). However, our study clearly demonstrates that there is no graded effect on movement distance comparing toads with transmitter packages amounting to 4.5-18.4% of body mass. Therefore, the 5% (e.g. White & Garrott, 1990) and the 10% body mass rules (e.g. Richards *et al.*, 1994) proposed to minimise possible effects do not have a scientific base. If there is an effect, i.e. toads without transmitter packages move more than those radio-tracked, it does not make a difference using a light-weight or a heavy weight device within the described range. A serious disadvantage of the line-of-sight distance measure used is the fact that toads do not move in straight line from point A to point B (variation of straightness [0=circular path, 1=straight line]: 0.492-0.770) and that return movements to the same shelter (about 3-6 per month and individual in St. Augustin, equivalent to 13-163 m movement distance; Sinsch, unpubl. observ.) within temporary home ranges remain undetected (e.g. Sinsch, 1988, 1992b; Denton, 1991). In conclusion, the empiric distance parameters used certainly underestimate magnitudes of movements and affect the average daily distance (by about 100-150%) more than the maximum daily distance (by about 50-100%).

The duration of local activity period during which surface movements are possible actually differs between the years depending on climate variations, but our mostly long-term experience at the study sites suggests that deviations from the average used are in the range of ± 15 d at most. A major source of uncertainty is the estimated frequency of days with surface movements. The 6%

estimate is based on reliable data for only three localities, all of them in the humid northern range of distribution and exclusively with sandy soils. This estimate may be extended to the other northern populations as well, but it is doubtful whether or not they hold for the semiarid localities of northern Spain. Occasional observations rather suggest that in Spain movement frequency exceeds 6% due to demands of thermoregulatory behaviour and water balance (Oromi *et al.*, 2010). The magnitude of underestimation, however, remains obscure.

In summary, both models predict the lowest possible annual movement distances rather than average ones, and even doubling the predicted values would still fall into a reasonable range estimate.

Implications for metapopulation dynamics of natterjacks

There is considerable disagreement about sex- and age-specific differences in the migratory range of anurans. Two long-term studies exemplify the case: Smith and Green (2006) found that dispersal was neither sex-biased nor age-biased in *Bufo fowleri*, whereas in *Rana luteiventris* dispersal was greater in juveniles than in adults but not sex-biased (Funk *et al.* 2005). Radio-telemetric studies on another five anuran species did not support sex-biased dispersal (Rittenhouse & Semlitsch, 2007). In *Bufo calamita* a previous study claimed female-biased dispersal in a German population (Sinsch, 1992a), while sex-bias was absent in a Spanish population (Miaud *et al.*, 2000). Our present integrative study suggests that discrepancy was probably a matter of small sample size and that there is no sex-bias in movement distance. Moreover, movement capacity of juveniles (600 m/(1-4 d); Sinsch, 1997; Leskovar & Sinsch, 2005) and adults (686.6 m/d; this study) seem to be too similar to indicate a potential age-biased dispersal. The observed rescue effect (continuous immigration into sink populations) may therefore rely not only on dispersing juveniles, but also on adults (Sinsch, 1992a, 1997; Stevens *et al.*, 2004).

Variation of local migratory range was accounted for almost entirely by predominant surface substrate, distinguishing between clay and sand/pasture populations. Actual movement distances of radio-tracked toads and the two model predictions demonstrate that individuals inhabiting the clay-dominated localities of Mas de Melons and Balaguer move about three times more than those living in habitats that permit burrowing. Since PAMD1 and PAMD2 models do not differ in frequency-range predictions for the toads on impenetrable soil, we conclude that average migratory activity equals almost highest possible migratory activity. Connectivity of local populations by dispersers, a crucial parameter for the understanding of metapopulation dynamics (Stevens *et al.*, 2004), is much greater in Iberia than in populations north of the Pyrenees, indicating considerably larger metapopulation systems as also evidenced by the studies on gene flow (Marangoni, 2006; Oromi *et al.*, in prep.). In contrast, structural similarity of lowland habitats in central and northern Europe leads to similar magnitudes of migratory range despite considerable differences in local climate. All studied localities provide unconstrained access to moist shelter for diurnal or seasonal inactivity periods. Consequently, toads do not need frequent and far-ranging movements to keep water economy balanced. Food does not seem to represent a limiting resource either (e.g. Leskovar *et al.*, 2004). Modelling annual movement range of these populations shows remarkable differences between PAMD1 and PAMD2 which suggest that average movement distance is about half of the maximum movement distance. Connectivity of populations therefore, requires lower distances than in Spain, indicating an overall smaller size of metapopulation systems. However, we predict that populations inhabiting sites with suboptimal shelter availability will tend to have larger migratory range.

Estimated threshold distance for disperser refers to the upper 5% of migrating individuals which means 10-20 toads in the usually large German

populations and 1-5 in most Spanish ones. Considering that only a low percentage of adults actually reproduce (Beebee, 2009), we believe that the cut at 5% is a conservative measure which guarantees that among the dispersers reaching a neighbouring population is at least one individual per generation which mates successfully.

Implications for conservation management of local natterjack populations

For purposes of conservation and management of local amphibian populations, it is important to define: (1) terrestrial core habitats used by pond breeding species; and (2) the minimum distance to a neighbouring conspecific population to maintain connectivity (e.g. Semlitsch & Bodie, 2003). A generalized buffer zone of 30.5 m (= 100ft) is often used by wildlife managers, but barely has any scientific foundation (Goates *et al.*, 2007). Quantitative approaches based on maximum recorded dispersal distance (Semlitsch & Bodie, 2003) and Kernel density estimates of density (Rittenhouse & Semlitsch, 2007) rather suggest core habitat distances of 159 – 980 m are needed around the breeding site. We propose a measure based on the dispersal functions presented here. We consider the annual distance moved by 50 % of a local population as a reasonable size of core habitat including all microhabitats needed for reproduction, nutrition and hibernation. Applying this measure to *Bufo calamita* populations may help to scientifically found management measures at the northern edge of distribution where natterjacks have suffered severe declines mainly due to changed land use regimes (Banks *et al.*, 1994; Rannap *et al.*, 2007; McGrath & Lorenzen, 2010). Our analysis including data on three UK localities suggests that a minimum terrestrial buffer range of 600 m around the breeding sites will be needed to keep half of a population undisturbed. To maintain connectivity of neighbouring local populations within a metapopulation system distances should not exceed 2,250 m. However it is not yet clear whether dispersing natterjack toads require corridors with low landscape resistance for successful migrations. In Spain dry agricultural areas with high mortality risks

due to dehydration and predation do not impede long-distance movements and neither do urban and industrial areas in Germany. Still, most populations in the UK have to be considered isolated by distance and prone to local extinction, as a natural recolonisation by dispersers from neighbouring sites is highly improbable. Imperfect knowledge of animal dispersal is not limited to amphibian species, and the use of dispersal functions as described with the natterjack toad should be used in many other species to infer their dispersal abilities.

ACKNOWLEDGEMENTS

The permissions for field studies and handling of the toads were issued by the Departament de Medi Ambient, Generalitat de Catalunya, Spain, Préfecture de la Seine-Saint-Denis, CRNS, France, Regierungspräsident Köln, and Struktur- und Genehmigungsdirektion Nord, Koblenz, Germany. We thank the Nature Conservancy Council, UK, the Conseil Général de la Seine-Saint-Denis and Rhône-Alpes Région programme "AVENIR 1995-97", France, and the DFG (Si 391/1-4), Germany, for financial support. The Ministry of Defence, Sefton Borough Council, Dunnerholme Golf Course, the National Trust and the Royal Society for the Protection of Birds for permission to work on the UK study sites, the Untere Natur- und Landschaftsschutzbehörde Siegburg, Ordnungsamt and Umweltamt St. Augustin for the German study sites.

REFERENCES

- Adriaensen, F., Chardon, J.P., De Blust, G., Swinnen, E., Villalba, S., Gulinck, H. & Matthysen, E. (2003). The application of 'least-cost' modelling as a functional landscape model. *Landscape and Urban Planning* **64**, 233-247.
- Baguette, M. & Van Dyck, H. (2007). Landscape connectivity and animal behavior: functional grain as a key determinant for dispersal. *Landscape Ecol.* **22**, 1117-1129.

- Banks, B., Beebee, T. J. C. & Cooke, A. S. (1994). Conservation of the natterjack toad *Bufo calamita* in Britain over the period 1970-1990 in relation to site protection and other factors. *Biol. Conserv.* **67**, 111-118.
- Beebee, T.J.C. (2009). A comparison of single-sample effective population size estimators using empirical toad (*Bufo calamita*) population data: genetic compensation and size-genetic diversity correlations. *Mol. Ecol.* **18**, 4790-4797.
- Beebee, T.J.C. (2011). Modelling factors affecting population trends in an endangered amphibian. *J. Zool.* **284**, 1-8.
- Beebee, T.J.C., Bolwell, S., Buckley, J., Corbett, K.F., Griffin, J., Preston, M. & Webster, J. (1982). Observation and conservation of a relict population of the natterjack toad *Bufo calamita* (LAURENTI) in Southern England over the period 1972-1981. *Amphibia-Reptilia* **3**, 33-52.
- Chetkiewicz, C.L.B., Clair, C.C.S., Boyce, M.S. (2006). Corridors for conservation: Integrating pattern and process. *Annual Review of Ecology Evolution and Systematics* **37**, 317-342.
- Clobert, J., Danchin, E., Dhondt, A.A. & Nichols, J.D. (2001). *Dispersal*. Oxford University Press, Oxford.
- Denton, J.S. (1991). *The terrestrial ecology of the natterjack toad (Bufo calamita) and the common toad (Bufo bufo)*. PhD-thesis, University of Sussex.
- Denton, J.S. & Beebee, T.J.C. (1993). Density-related features of natterjack toad (*Bufo calamita*) populations in Britain. *J. Zool.* **229**, 105-119.
- Fall, A., Fortin, M.J., Manseau, M. & O'Brien, D. (2007). Spatial graphs: Principles and applications for habitat connectivity. *Ecosystems* **10**, 448-461.
- Funk, W.C., Greene, A.E., Corn, P.S. & Allendorf, F.W. (2005). High dispersal in a frog species suggests that it is vulnerable to habitat fragmentation. *Biol. Lett.* **1**, 13-16.
- Goates, M. C., Hatcha, K. A. & Eggett, D. L. (2007). The need to ground truth 30.5 m buffers: A case study of the boreal toad (*Bufo boreas*). *Biol. Conserv.* **138**, 474-483.

- Hey, J., Waples, R.S., Arnold, M.L., Butlin, R.K. & Harrison, R.G. (2003). Understanding and confronting species uncertainty in biology and conservation. *TREE* **18**, 597-603.
- Huste, A., Clobert, J. & Miaud, C. (2006). The movements and breeding site fidelity of the natterjack toad (*Bufo calamita*) in an urban park near Paris (France) with management recommendations. *Amphibia-Reptilia* **27**, 561-568.
- Joly P., Morand, C. & Cohas, A. (2003). Habitat fragmentation and amphibian conservation: building a tool for assessing landscape matrix. *C. R. Biol.* **326**: S132-S139.
- Leskovar, C., Wagner, T. & Sinsch, U. (2004). Saisonale Dynamik des Nahrungsspektrums syntoper Kreuz- (*Bufo calamita*) und Wechselkröten-Populationen (*B. viridis*) in einem rheinischen Auskiesungsgebiet. *Z. Feldherpetol.* **11**, 83-103.
- Leskovar, C. & Sinsch, U. (2005). Harmonic direction finding: A novel tool to monitor the dispersal of small-sized anurans. *Herpetol. J.* **15**, 173-180.
- Marangoni, F. (2006). *Variación clinal en el tamaño del cuerpo a escala microgeográfica en dos especies de anuros (Pelobates cultripes y Bufo calamita)*. PhD-thesis, Universidad de Sevilla.
- McGrath, A.L. & Lorenzen, K. (2010). Management history and climate as key factors driving natterjack toad population trends in Britain. *Anim. Conserv.* **13**, 483-494.
- Meteorological Office. (2008): Historic Station Data. Available at <http://www.metoffice.gov.uk/climate/uk/stationdata/index.html> (accessed on 30 April 2011).
- Miaud, C., Sanuy, D. & Avriillier, J.N. (2000). Terrestrial movements of the natterjack toad *Bufo calamita* (Amphibia, Anura) in a semi-arid, agricultural landscape. *Amphibia-Reptilia* **21**, 357-369.
- O'Brien, D., M. Manseau, Fall, A. & Fortin, M. J. (2006). Testing the importance of spatial configuration of winter habitat for woodland caribou: An application of graph theory. *Biol. Conserv.* **130**, 70-83.

- Oromi, N., Sanuy, D. & Sinsch, U. (2010). Thermal ecology of natterjack toads (*Bufo calamita*) in a semiarid landscape. *J. Therm. Biol.* **35**, 34-40.
- Phillips, B. L., Brown, G.P., Greenlees, M., Webb, J.K. & Shine, R. (2007). Rapid expansion of the cane toad (*Bufo marinus*) invasion front in tropical Australia. *Austral Ecol.* **32**, 169-176.
- Ranius, T. (2006). Measuring the dispersal of saproxylic insects: a key characteristic for their conservation. *Pop. Ecol.* **48**, 177-188.
- Rannap, R., Lõhmus, A. & Jakobson, K. (2007). Consequences of coastal meadow degradation: The case of the natterjack toad (*Bufo calamita*) in Estonia. *Wetlands* **27**, 390-398.
- Ribeiro, R., Carretero, M.A., Sillero, N., Alarcos, G., Ortiz-Santaliestra, M., Lizana, M. & Llorente, G. (2011). The pond network: can structural connectivity reflect on (amphibian) biodiversity patterns? *Landscape Ecol.* **26**, 673-682.
- Richards, S.J., Sinsch, U. & Alford, R.A. (1994). Radio-Tracking. In: *Standard Methods for Measuring Biological Diversity: Amphibians*. M. Donnelly, W. R. Heyer, R. F. Inger & R. W. McDiarmid, Smithsonian Institution Press: 155-158.
- Rink, M. & Sinsch, U. (2007). Radio-telemetric monitoring of dispersing stag beetles (*Lucanus cervus* L.): implications for conservation. *J. Zool.* **272**, 235-243.
- Rittenhouse, T.A.G. & Semlitsch, R.D. (2007). Distribution of amphibians in terrestrial habitat surrounding wetlands. *Wetlands* **27**, 153-161.
- Rowe, G. & Beebee, T.J.C. (2007). Defining population boundaries: use of three Bayesian approaches with microsatellite data from British natterjack toads (*Bufo calamita*). *Mol. Ecol.* **16**, 785-796.
- Rowley, J.J.L. & Alford, R.A. (2007). Techniques for tracking amphibians: The effects of tag attachment, and harmonic direction finding versus radio telemetry. *Amphibia-Reptilia* **28**, 367-376.

- Safner, T., Miaud, C., Gaggiotti, O., Decout, S., Rioux, D., Zundel, S., & Manel, S. (2011). Combining demography and genetic analysis to assess the population structure of an amphibian in a human-dominated landscape. *Conserv. Genetics* **12**,161-173.
- Schaefer, J.A. (2006). Towards maturation of the population concept. *Oikos* **112**, 236-240.
- Semlitsch, R.D. & Bodie, J.R. (2003). Biological criteria for buffer zones around wetlands and riparian habitats for amphibians and reptiles. *Conserv. Biol.* **17**, 1219-1228
- Sinsch, U. (1988). Temporal spacing of breeding activity in the natterjack toad, *Bufo calamita*. *Oecologia* **76**, 399-407.
- Sinsch, U. (1990). Migration and orientation in anuran amphibians. *Ethology Ecology & Evolution* **2**, 65-79.
- Sinsch, U. (1992a). Structure and dynamics of a natterjack toad (*Bufo calamita*) population. *Oecologia* **90**, 489-499.
- Sinsch, U. (1992b). Sex-biassed site fidelity and orientation behaviour in reproductive natterjack toads (*Bufo calamita*). *Ethol. Ecol. Evol.* **4**, 15-32.
- Sinsch, U. (1997). Postmetamorphic dispersal and recruitment of first breeders in a *Bufo calamita*-metapopulation. *Oecologia* **112**, 42-47.
- Sinsch, U. & Leskovar, C. (2011). Does thermoregulatory behaviour of green toads (*Bufo viridis*) constrain geographical range in the west? A comparison with the performance of syntopic natterjacks (*B. calamita*). *J. Therm. Biol.*, in press.
- Sinsch, U. & Seidel, D. (1995). Dynamics of local and temporal breeding assemblages in a *Bufo calamita* metapopulation. *Austral. J. Ecol.* **20**, 351-361.
- Smith, M.A. & Green, D.M. (2005). Dispersal and the metapopulation paradigm in amphibian ecology and conservation: are all amphibian populations metapopulations? *Ecography* **28**, 110-128.
- Smith, M.A. & Green, D.M. (2006). Sex, isolation and fidelity: unbiased long-distance dispersal in a terrestrial amphibian. *Ecography* **29**, 649-658.

- Stevens, V. M., Polus, E., Wesselingh, R.A., Schtickzelle, N. & Baguette, M. (2004). Quantifying functional connectivity: experimental evidence for patch-specific resistance in the Natterjack toad (*Bufo calamita*). *Landscape Ecol.* **19**, 829-842.
- Taylor, P.D., Fahrig, L. & Henein, K. (1993). Connectivity is a vital element of landscape structure. *Oikos* **68**, 571-573.
- Taylor, P.D., Fahrig, L. & With, K. (2006). Landscape connectivity: a return to basics. In: Crooks, K.R. & Sanjayan, M. (eds) *Connectivity conservation*. Cambridge University Press, Cambridge.
- Waples, R.S. (1995). Evolutionarily significant units and the conservation of biological diversity under the Endangered Species Act. In: *Evolution and the Aquatic Ecosystem: Defining Unique Units in Population Conservation* (Nielsen, J.L., ed.), pp. 8-27, American Fisheries Society.
- Waples, R.S. & Gaggiotti, O.E. (2006). What is a population? An empirical evaluation of some genetic methods for identifying the number of gene pools and their degree of connectivity. *Mol. Ecol.* **15**, 1419-1439.
- Werner, E.E., Relyea, R.A., Yurewicz, K.L., Skelly, D.K., & Davis, C.J. (2009). Comparative landscape dynamics of two anuran species: climate-driven interaction of local and regional processes. *Ecol. Monogr.* **79**:503-521.
- White, G.C. & Garrott, R.A. (1990). *Analysis of wildlife radio-tracking data*. San Diego, Academic Press.

Proximate mechanisms determining size variability in natterjack toads

Published in: *Journal of Zoology*, 281 (2010) 272-281.

Authors: SINSCH, U.; MARANGONI, F.; OROMI, N.; SANUY, D.; TEJEDO, M.

The Bergmann rule (1847) postulates that the body size of mammals and birds often increases along the latitudinal gradient, from south to north, and from low and high altitudes. However, there is a controversy in amphibians because the interaction between various mechanisms at the intra or interspecific level -as age at maturity, phenotypic plasticity or genetic diversity (Laugen et al., 2005)- can determine the variation in the size of species in a latitudinal or altitudinal gradient. In the Chapter 3, the variation of body size was analysed in a latitudinal gradient of *B. calamita* distribution. The interaction of multiple environmental factors during the first age of animal life determines the age at maturity, the adult size and the variation of size between the different populations.

Author contribution: In this paper we analysed five populations from different localities in a latitudinal gradient. I have done the sampling procedure and the skeletochronological analysis of two populations, Mas de Melons and Balaguer. In have colaboraetd in the statistical analysis and in the elaboration of the manuscript closely with the other authors.

Proximate mechanisms determining size variability in natterjack toads

U. Sinsch¹, F. Marangoni^{2*}, N. Oromi³, C. Leskovar¹, D. Sanuy² & M. Tejedo³

¹ Department of Biology, Institute of Integrated Sciences, University of Koblenz-Landau, Koblenz, Germany

² Department of Evolutionary Ecology, Estación Biológica de Doñana, CSIC, Sevilla, Spain

³ Escola Tècnica Superior Enginyeria Agrària, Departament Producció Animal (Fauna Silvestre), University of Lleida, Lleida, Spain

Keywords

amphibia; Anura; Bergmann's rule; life-history traits; age at maturity; longevity; potential reproductive lifespan; skeletochronology.

Correspondence

Ulrich Sinsch, Department of Biology, Institute of Integrated Sciences, University of Koblenz-Landau, Universitätsstr. 1, D-56070 Koblenz, Germany.
Email: sinsch@uni-koblenz.de

*Current address: FCEQyN, Universidad Nacional de Misiones, Consejo Nacional de Investigaciones Científicas y Técnicas, Félix de Azara 1552, 3300 Posadas, Argentina.

Editor: Tim Halliday

Received 15 December 2009; revised 17 February 2010; accepted 17 February 2010

doi:10.1111/j.1469-7998.2010.00702.x

Introduction

The body size of mammal and bird species often increases from a warm to a cool climate, that is from low to high latitudes and/or altitudes (Bergmann, 1847; Freckleton, Harvey & Pagel, 2003; Meiri & Dayan, 2003). This trend, known as Bergmann's rule, also holds for the intraspecific body size variation in the majority of endotherms (Ashton, 2002a; Meiri & Dayan, 2003). Ray (1960) first proposed that the latitudinal size variation of ectotherms follows the same trend. However, there is considerable controversy about the general presence and direction of latitudinal clines and the underlying mechanisms in amphibians at the intra- and interspecific level (Ashton, 2002b; Krizmanic, Vukov & Kalezić, 2005; Laugen *et al.*, 2005; Olalla-Tarraga & Rodríguez, 2007; Adams & Church, 2008; Cvetkovic *et al.*, 2009). *Plethodon* salamanders exemplify the case: body size was significantly related to the mean annual temperature in only 10 out of 40 species, three negatively and seven positively

Abstract

In the toad *Bufo calamita*, among-population variation of size follows roughly a converse Bergmann cline, but populations exist that do not fit this pattern. We propose that latitudinal body size variation is a byproduct of adaptive covariation among the life-history traits juvenile growth rate, longevity and lifetime fecundity. We choose five populations (two in Andalusia, two in Catalonia and one in Rhineland-Palatinate) representing a variation of adult size from 39 mm to 95 mm snout-vent length, a latitudinal gradient from 37 to 50° and an altitudinal gradient from sea level to 420 m. Skeletochronology was used to estimate the age-related life-history traits of 313 toads and their lifetime pattern of growth. At southern latitudes, toads matured and reproduced earlier than those at northern latitudes, but had a reduced potential reproductive lifespan due to lower longevity. Age-adjusted adult size depended mainly on the size achieved between metamorphosis and first hibernation or aestivation, which in turn was influenced by local factors. We propose that first-year size corresponds to the duration of the aboveground activity period, temperature during the activity period and the type of shelter sites and hibernacula available in the habitat. After attaining sexual maturity, the growth rates did not differ among populations. Interactions of multiple environmental factors during the first year of life determine age at maturity, adult size and size variation among populations. Local body size and potential reproductive lifespan covary to optimize lifetime fecundity throughout the geographical range. The presence of a small-sized population in southern Spain does not fit the pattern predicted by a converse Bergmann cline, but is compatible with the hypothesis that body size variation among *B. calamita* populations may be the evolutionary byproduct of optimized lifetime fecundity.

(Adams & Church, 2008). Moreover, regional body size clines may be present as in the frog *Rana temporaria* (Elmberg, 1991; Miaud, Guyétant & Elmberg, 1999), but taking into account larger latitudinal gradients, a concave pattern of variation arises as the result of interactions among age effects, phenotypic plasticity and genetic determination (Laugen *et al.*, 2005). Consequently, geographical size variation in amphibian species seems to have multiple causes, which may differ from the ecological, physiological and evolutionary explanations proposed for other tetrapods (e.g. heat balance hypothesis, migration ability hypothesis) and also between the interspecific and intraspecific level (Olalla-Tarraga & Rodríguez, 2007; Adams & Church, 2008; Gaston, 2008). Comprehensive case studies of the proximate causes of body size clines represent a further step deeper in understanding their evolution in amphibians and ultimately in vertebrates (Cvetkovic *et al.*, 2009).

The body size variation of temperate zone bufonid toads is well documented for *Bufo bufo*, *Bufo calamita* and

B. viridis in Europe, and for *B. hemiophys* and *B. woodhousii* in northern America (Nevo, 1972; Hemelaar, 1988; Kellner & Green, 1995; Castellano & Giacoma, 1998; Eaton *et al.*, 2005; Leskovar *et al.*, 2006; Cvetkovic *et al.*, 2009). All these case studies agree in that converse latitudinal Bergmann clines covering up to 3000 km characterize intraspecific body size variation. However, there is evidence of at least two populations at the limits of latitudinal distribution range that do not fit the general clinal pattern. In male *B. bufo* living at 64°N (northern range limit), the mean body size was considerably larger than in the more southern populations of Scandinavia (Cvetkovic *et al.*, 2009). Similarly, in the southernmost *B. calamita* population of the Iberian Peninsula, the mean body size was by far smaller than that of more northern populations in Spain (Leskovar *et al.*, 2006; Marangoni, Tejedo & Gomez-Mestre, 2008).

In this paper, we explore the causes of the (near) clinal body size variation in five populations of natterjack toads *Epidalea* (= *Bufo*) *calamita* (Laurenti, 1768) in the framework of the life-history theory. Fecundity increases with size and growth rate with ambient temperature, suggesting a complex interaction among local climate and trade-offs between delayed maturity (= increased body and clutch size) and longevity (Tejedo, 1992; Leskovar *et al.*, 2006; Sinsch, 2008). The populations studied in Spain and Germany represent a variation of adult size from 39 mm to 95 mm (snout–vent length, SVL) and an age range from 2 to 9 years of life, a latitudinal gradient from 37 to 50° and an altitudinal gradient from sea level to 420 m. As skeletochronological age determination also allows for the reconstruction of individual annual bone growth pattern, we were able to estimate the contribution of ontogenetic growth variation to body size at any age (Sinsch, Oromi & Sanuy, 2007b). The aims of this study are (1) to quantify the influence of climate and elevation gradients on the age-adjusted body size variation; (2) to estimate retrospectively the relative contribution of juvenile growth to adult body size; (3) to evaluate the hypothesis that the near-clinal body size variation is a byproduct of covariation between juvenile growth rate, longevity and lifetime fecundity.

Material and methods

Study areas

A total of 313 reproductive natterjack toads *E. calamita* were studied at five localities, which include four habitat types, coastal sand dunes (Reserva Biologica de Doñana, Andalusia, Spain), Mediterranean mountains (Navas, Andalusia, Spain), semi-arid arable plains (Balaguer and Mas de Melons, Catalonia, Spain) and flood plains of Central European streams (Urmitz, Rhineland-Palatinate, Germany). The habitat and local climate features, collection dates and numbers of reproductive adults sampled are summarized in Table 1; detailed descriptions of the localities are given in Leskovar *et al.* (2006), Sinsch *et al.* (2007b) and Marangoni *et al.* (2008). Climate data used for modelling are the average of a 30-year period (1971–2000) and refer to the meteorological stations of Reserva Biologica de Doñana (CSIC), and Lleida-Observatorio 2, Spain and Koblenz-Horchheim, Germany.

Sampling procedure and skeletochronological analysis

Following sunset, toads were collected at the local breeding ponds within 1–4 days of the spring reproduction period (Table 1). At the Urmitz study site, reproductive activity continues until August (Sinsch & Keltsch, 2002), and a second toad sample was collected within 12 days in June. The variation in the sex ratio is due to the fact that collecting surveys were not exhaustive and thus, samples represent a random sample of the local breeding assemblage. Toads were released again *in situ* following sex determination, measuring of snout–vent length (SVL, to the nearest mm) and toe-clipping (third toe of the right hind limb). The toes were stored in 70% ethanol at room temperature.

Laboratory protocols followed the standard methods of skeletochronology (e.g. Smirina, 1972). The samples collected in Urmitz, Balaguer and Mas de Melons were embedded in Historesin™ (Jung, Leica Microsystems Nussloch GmbH, Nussloch, Germany) and stained with

Table 1 Location, climate features and number of toads collected

Locality	Coordinates	Altitude (m a.s.l.)	Air temperature January/July (°C)	Annual precipitation (mm)	Date of collection	Males/females
Reserva Biologica	37°00'44"N 06°30'13"W	24	10.6/24.5	585	14.–15.III. 2002	32/27
Navas	37°47'28"N 06°04'41"W	420	9.6/25.7	640	3.–6.III. 2002	26/28
Mas de Melons	41°30'50"N 00°43'30"E	240	5.3/24.7	369	21.–23.IV. 2004	43/4
Balaguer	41°46'30"N 00°46'50"E	220	5.3/24.7	369	1.IV. 2004	34/14
Urmitz (spring)	50°21'43"N 07°35'48"E	60	2.7/19.5	674	19.–23.IV. 2000	29/25
Urmitz (summer)	50°21'43"N 07°35'48"E	60	2.7/19.5	674	4.–17.VI. 2000	25/26

cresylviolet (details in Sinsch, di Tada & Martino, 2001). Diaphysis was cross sectioned at 10 μm using a Jung RM2055 rotation microtome. The samples collected in Andalusia were embedded in Tissue-Tek (Sakura Finetek, Europe B.V., Alphen aan den Rijn, the Netherlands) and stained with Ehrlich's hematoxylin (details in Tejedo, Reques & Esteban, 1997). Diaphysis was cross sectioned at 16 μm using a cryomicrotome (Microm International GmbH, Walldorf, Germany). A previous study demonstrated that the differences in staining procedure did not interfere with the detectability of LAGs (Sinsch *et al.*, 2007a). Cross sections were examined light microscopically at magnifications of $\times 200$ using an Olympus BX 50 (Olympus Europa GmbH, Hamburg, Germany) or Leica DME (Leica Microsystems Ventricle GmbH, Wetzlar, Germany). High-resolution cameras Olympus DP20 (Olympus Europa GmbH) and Sony SSC-DC50AP (Sony Deutschland, Köln, Germany) were used to take digital images from those diaphysis sections in which the size of the medullar cavity was at its minimum and that of bone at its maximum.

One digital image per individual was selected for further analysis. Image files were transferred to UTHSCSA ImageTool, version 3.0, and calibrated using a standard micrometer. In a first step of analysis, we recorded the presence/absence of the line of metamorphosis (LM) and of lines of arrested growth (LAGs). The number of LAGs visible in each cross section was assessed by the first author and independently by one or two co-authors. In those toads with no remnant of the LM, we checked for the potential resorption of LAGs using the method of Hemelaar (1985). In a second step, we distinguished annual growth marks (LAGs *sensu stricto*) from non-annual ones (irregular interruptions of the hibernation inactivity periods), using the method described in Sinsch *et al.* (2007b). The age of maturity was defined as the lowest age recorded in a reproductive toad of a given population. Finally, we measured the area of periosteal bone between two neighboring LAGs to the nearest μm^2 in those cross sections in which the bone section was complete, to obtain an objective measure for the annual bone growth. In cases of multiple line formation within one year (common in the samples of Catalonia, Sinsch *et al.*, 2007b), the outermost line of a group was chosen as the final growth mark of a year. The dataset obtained to model the age-size relationship included the categorical variables locality (coded 1–5), spring/summer cohort (coded 1/2), sex (male = 1, female = 2), presence/absence of LM (coded 1/0), presence/absence of non-annual growth marks (coded 1/0) and the quantitative variables age (number of LAGs) and SVL (mm) with $N = 313$ individual toads. The dataset used to analyze the growth pattern of periosteal bone included additionally the quantitative variables total bone area (μm^2) and up to eight areas of yearly bone growth (= growth rate) depending on the age of the toad. The number of observations was lower ($N = 280$) because 33 incomplete bone sections allowed age determination, but not area measurement.

Statistical analyses

All variables were first tested for normality. Multifactorial analyses of covariance (ANCOVA, type III sums of squares)

were used to assess the effects of age (covariate), cohort and locality on SVL and total bone area, respectively. Multiple comparisons of the least square means were based on 95% Bonferroni confidence intervals. The age-size relationship was modelled using a general linear regression model (GLM). We tested a total of 22 variable transformations to linearize the relationship. The double-reciprocal model provided the best fit (maximum R^2). The one-factor regression model of age (1/LAG) and size (1/SVL) was optimized using a Box-Cox transformation and complemented by successively introducing sex and locality (categorical variables). Variables were added to the model, if their corresponding coefficients were significantly different from zero (ANOVA), that is they contributed to the amount of variance explained. Further GLMs were calculated to fit the bone area-size relationship and the age-bone growth rate relationship. All GLM estimates are expressed as mean and corresponding standard error. Significance level was set at $\alpha = 0.05$. All calculations were based on the procedures of the program package STATGRAPHICS Centurion, version XV (STATPOINT Inc., Warrenton, VA, USA).

Results

The area between the outermost LAG and the edge of the bone from the Urmitz population was usually very small in the individuals collected during spring and considerably larger in those collected in June, indicating that toads resume growth at the beginning of the local spring breeding period. The proportion of individuals in which the LM was at least partially visible in the phalange bone sections varied significantly among the localities (ANCOVA, $F_{4,301} = 66.7$, $P < 0.00001$; proportion adjusted for age as covariate, $F_{1,301} = 3.3$, $P = 0.0714$), ranging from 2.3% in Urmitz (least square mean), over 12.9 and 14.4% in Balaguer and Mas de Melons, respectively, 43.3% in Navas and 88.8% in Reserva Biologica de Doñana. Resorption of the inner LAG was detected in only one out of 313 individuals (Urmitz site). The number of individuals showing non-annual growth marks also varied locally. The proportion was low in Navas (13.0%), Urmitz (21.6%) and Reserva Biologica de Doñana (22.1%), suggesting almost uninterrupted hibernation or aestivation, and high in Balaguer (75.0%) and Mas de Melons (80.8%), suggesting short-term growth periods interrupting hibernation (Sinsch *et al.*, 2007b; Orómi, Sanuy & Sinsch, 2010). Local age distributions differed significantly from each other (Kolmogorov-Smirnov test, all paired comparisons $P < 0.00001$; Fig. 1). Minimum age at maturity was one LAG in the Reserva Biologica de Doñana ($n = 20$, 33.9% of all individuals) and at Navas ($n = 1$, 1.9%), while in all northern populations, maturity was attained at a minimum of two LAGs (Balaguer $n = 11$, 22.9%; Mas de Melons $n = 3$, 6.4%; Urmitz spring cohort $n = 2$, 3.7%; Urmitz summer cohort $n = 3$, 5.9%). At Navas, most first breeders pertained to the two LAG class ($n = 23$, 40.7%).

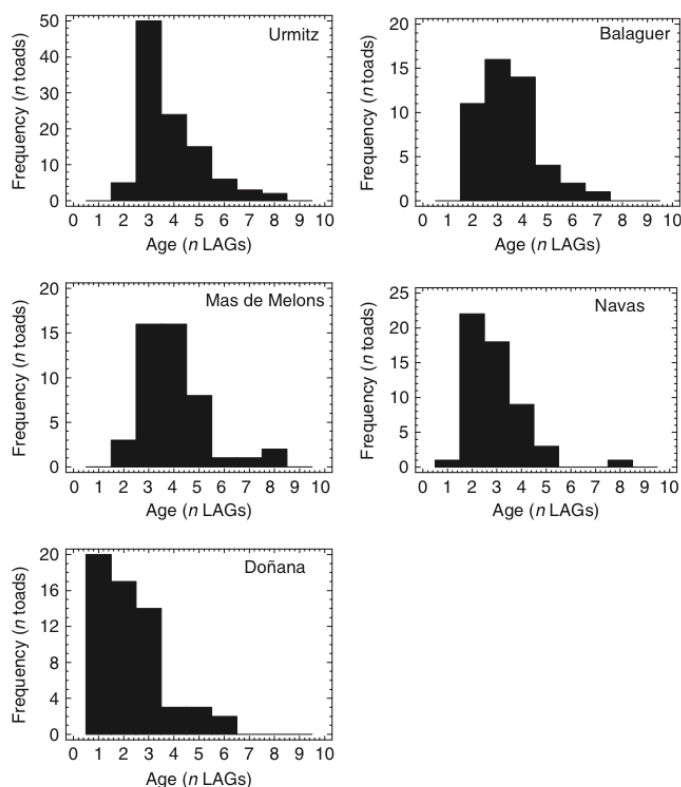


Figure 1 Age distribution of reproductive toads at the five localities studied.

Local size variation predicted by age and sex

At the Urmitz study site, SVL of adults did not differ between males and females (multifactorial ANCOVA, $F_{1,104} = 3.0$, $P = 0.0875$) and between spring/summer breeding individuals ($F_{1,104} = 1.7$, $P = 0.1965$), but significantly influenced by the covariate age ($F_{1,104} = 1687$, $P < 0.0001$). At the neighboring localities Balaguer and Mas de Melons, SVL of adults was neither influenced by sex (multifactorial ANCOVA, $F_{1,94} = 3.8$, $P = 0.0553$) nor by locality ($F_{1,94} = 0.01$, $P = 0.9139$), but covaried significantly with age ($F_{1,94} = 5.4$, $P = 0.0224$). Consequently, we reduced the six subsets of data (Table 1) to four by pooling those of the spring and summer cohorts at Urmitz and those of Balaguer and Mas de Melons, respectively.

The multifactorial ANCOVA revealed that the age-adjusted least square means of SVL (covariate age: $F_{1,312} = 90.8$, $P < 0.0001$) differed considerably among the localities ($F_{3,312} = 256.9$, $P < 0.0001$), Balaguer/Mas de Melons (79.0 mm), Navas (69.4 mm), Reserva Biológica de Doñana (56.5 mm) and Urmitz (56.5 mm), but not between sexes ($F_{1,312} = 3.6$, $P = 0.0601$). However, multiple range comparison revealed that there were only three size-groups

because the means of the small-sized populations did not vary among each other ($P < 0.05$). Quantitative modelling of size variability by GLM explained 72.7% of SVL variance by the combined effects of locality, sex and age (Table 2A, Fig. 2). Age accounted for only 13.4% of the variance, sex for 0.3%, whereas local differences explained 59.0%. The annual increase of size in adults did not differ significantly among localities (ANOVA, $P = 0.1081$), but the intercepts of the model did (ANOVA, $P < 0.0001$). In summary, the age- and gender-dependent body size variation of adults was low within each locality, and the major source of variation among the localities was the size at which the mostly two LAGs-old individuals became reproductive, that is the size achieved during the juvenile stage and then population divergence in adult size is a function of variation in juvenile growth rates.

Patterns of annual bone growth

At the Urmitz study site, the annual bone growth rate of toads that reproduced during spring differed significantly from that of individuals that reproduced during summer at the first (multifactor ANCOVA; $F_{1,100} = 6.9$, $P = 0.0102$)

Table 2 GLM models to predict size (SVL) (A) or by phalange bone size (C) by age and local factors

Model coefficient	Estimate ± standard error	Wilk's lambda	F-value	Significance	Contribution to R ²
<i>(A) Box-Cox transformation applied power: -1.36311</i>					
Constant	0.98698 ± 0.000234				
1/age	0.003416 ± 0.000543	0.8858	39.4	<i>P</i> < 0.00001	13.4%
Locality					
1/4	0.002129 ± 0.000157	0.3157	221.8	<i>P</i> < 0.00001	59.0%
2/4	-0.003407 ± 0.0001628				
3/4	-0.00076 ± 0.000181				
Sex	0.000207 ± 0.000096	0.9850	4.7	<i>P</i> = 0.0316	0.3%
Total R ² (adjusted for degrees of freedom)					72.7%
<i>(B) Box-Cox transformation applied power: 0.193897</i>					
Constant	0.893239 ± 0.000197				
1/bone area	783.34 ± 33.10	0.3261	560.2	<i>P</i> < 0.00001	80.0%
Region	0.000674 ± 0.000118	0.8926	32.6	<i>P</i> < 0.00001	1.1%
Total R ² (adjusted for degrees of freedom)					81.1%

Best fit (maximum R²): double reciprocal model. Model A differs from B by the introduction of local habitat and climate features as categorical or quantitative variables. Numerical range of variables: age, 1–8 LAGs; sex, male = 1, female = -1; locality, 1 = Urmitz, 2 = Balaguer/Mas de Melons, 3 = Navas, 4 = Reserva Biologica de Doñana; region: 1 = Urmitz/Navas/Reserva Biologica de Doñana, 2 = Balaguer/Mas de Melons.

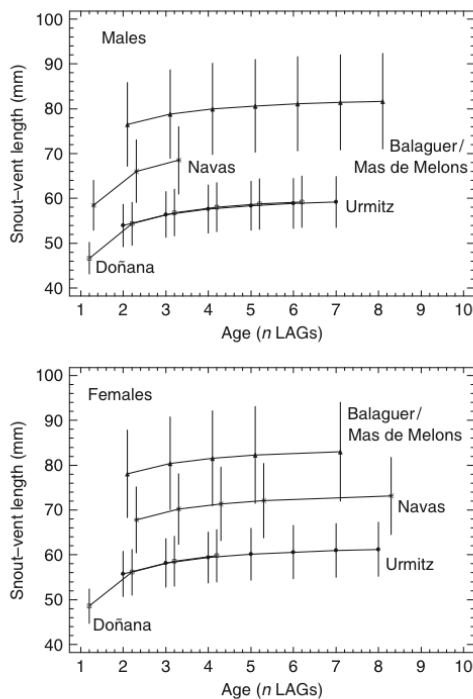


Figure 2 Age-size relationship for the studied populations (generalized linear regression models). (a) Males; (b) Females. Symbols represent the SVL estimates (least square means) and bars show the corresponding 95% confidence interval.

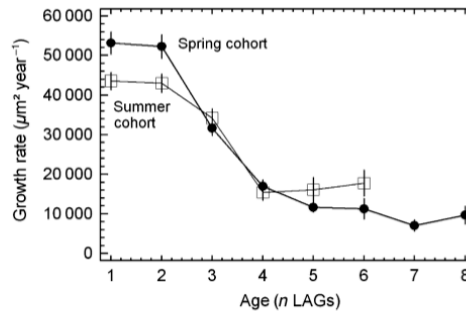


Figure 3 Annual growth rate of periosteal bone of toads reproducing in spring (*N* = 52) or in summer (*N* = 49) at the Urmitz study site. Data are given as least square means and the corresponding 95% confidence interval. Statistical details are given in the text.

and second year of life ($F_{1,100} = 5.2$, $P = 0.0252$), respectively, whereas sex did not influence the growth rate in any year ($F_{1,100}$, $P > 0.05$; Fig. 3). Age at the time of capture (multifactor ANCOVA; $F_{1,100}$, $P > 0.05$) did not covary with the annual bone growth rate, suggesting that periosteal bone does not condense with increasing age in natterjack toads. Growth rates were significantly smaller in the summer cohort (least square means: 43 272 μm^2 during the first year; 43 362 μm^2 during the second year) than in the spring cohort (53 435 μm^2 and 51 581 μm^2 , respectively). Consequently, we used only the spring cohort of Urmitz for further comparisons with the Spanish populations, which consist exclusively of spring breeders.

At any year of life, the average bone growth rate varied significantly among localities, but not between males and

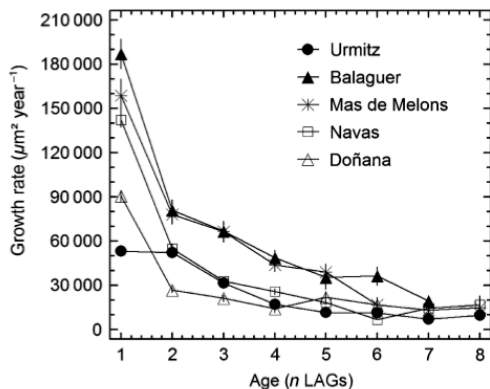


Figure 4 Least square means and the corresponding 95% confidence interval for the lifetime growth pattern of periosteal bone in five localities. Statistical details are given in the text.

females and independent of age at capture, as revealed by multifactorial ANCOVAs (Fig. 4). During the first year of life (ANCOVA; $F_{4,230} = 84.2$, $P < 0.0001$), bone growth differed significantly among all the localities (multiple range test; $P < 0.05$): Balaguer (least square mean: $191\,925 \pm 6701 \mu\text{m}^2$), Mas de Melons ($168\,196 \pm 7696 \mu\text{m}^2$), Navas ($138\,456 \pm 5365 \mu\text{m}^2$), Reserva Biologica de Doñana ($83\,117 \pm 5471 \mu\text{m}^2$) and Urmitz ($61\,100 \pm 5749 \mu\text{m}^2$). During the second year of life (ANCOVA; $F_{4,206} = 22.6$, $P < 0.0001$), bone grew most in the Catalonian populations forming a homogeneous group (multiple range test; $P < 0.05$), followed by a group formed by the Navas and Urmitz populations, and by the Doñana population. During the subsequent years of life, Urmitz and the Andalusian populations formed a homogeneous group with respect to annual bone growth (multiple range test; $P < 0.05$), whereas the Catalonian population continued to exhibit significantly greater growth rates.

Retrospective size prediction by bone area

The GLM of the bone area–adult size relationship explained 81.1% of SVL variance by total bone area as quantitative variable and of region as a categorical variable, which led to a small, but significant contribution due to the difference among the Catalonian populations on the one hand and those of Andalusia and Urmitz on the other (Table 2B, Fig. 5). The variables sex and age did not increase R^2 significantly. The GLM, therefore, allows for a retrospective estimation of SVL at any stage of the lifetime of a natterjack toad calculating the corresponding SVL to the bone area bordered by each LAG.

The translation of bone growth pattern into annual SVL gain revealed significant differences among all the populations with respect to SVL achieved before the formation of the first LAG and between the populations of the Iberian Peninsula and those from Germany for the second and third

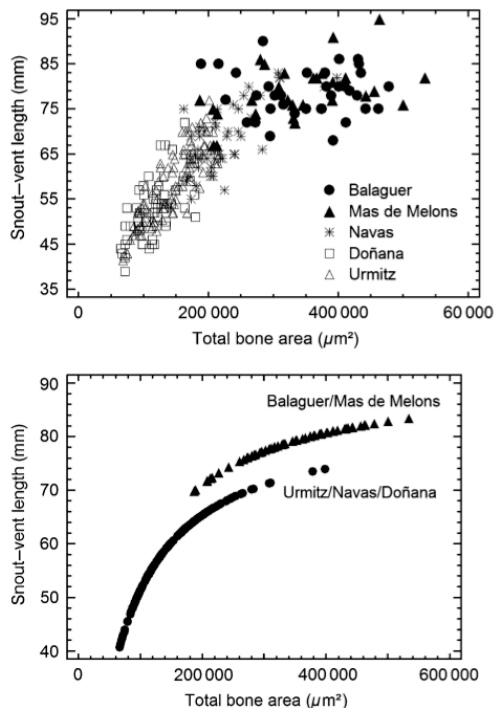


Figure 5 Bone size–SVL relationship. (a) Scatter plot of 280 individuals; (b) GLM model for two groups of localities; coefficients are given in Table 2, and symbols represent individual estimates.

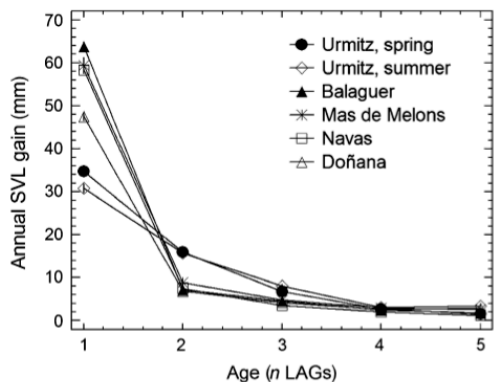


Figure 6 Least square means of annual SVL gain and the corresponding 95% confidence interval estimated from the bone growth pattern of toads originating from five localities. At the Urmitz site, the toads breeding in spring are distinguished from those breeding during summer.

LAGs (Fig. 6). Except for the first year of life, Spanish populations did not differ in their growth pattern (Fig. 6). While the average first year SVL varied between 47.5 mm (Reserva Biologica de Doñana) and 63.7 mm (Balaguer), second year SVL gain declined sharply at all localities to 7.4 ± 1.2 mm and annual SVL gain remained unchanged at 2.5 ± 1.0 mm from the fourth year onwards (Fig. 6). The first year size at the German locality differed significantly between the members of the spring and summer cohorts and the average SVL was about 14 mm smaller than that of the small-sized Spanish population in Doñana (Table 3). Following the first year of life, the growth pattern of the two cohorts was undistinguishable, but second and third year SVL gain (15.9 mm and 7.4 mm) was significantly greater than in the Spanish populations (ANOVA, $P < 0.001$). From the fourth year onwards, the growth pattern of all populations converged to a homogeneous annual SVL gain of about 2.5 mm.

Discussion

Size variations among conspecific amphibian populations exposed to environmental gradients are probably driven by different proximate causes than those mentioned to explain latitudinal size clines at the interspecific level in Amphibia (Olalla-Tarraga & Rodriguez, 2007; Gaston, 2008; Cvetkovic *et al.*, 2009). The broad-scale geographical pattern of body size for the European amphibian fauna seems to be compatible with a Bergmann cline, if the data analysis is based on the maximum SVL of each anuran species (Olalla-Tarraga & Rodriguez, 2007). However, the validity of this approach is doubtful because it neglects the intraspecific size variation in species such as *B. bufo*, *B. calamita* and *R. temporaria*, which follows roughly a converse Bergmann cline (Miaud *et al.*, 1999; Laugen *et al.*, 2005; Leskovar *et al.*, 2006; Cvetkovic *et al.*, 2009; this study). Basic to a better understanding of size variation in Amphibia as a group is consequently the analysis of the proximate causes of intraspecific size variation in the framework of the life-history theory (Angilletta *et al.*, 2004; Adams & Church, 2008). The large adult size obtained by delaying sexual maturity provides the benefit of increased clutch size, but may be associated with costs such as increased juvenile and adult mortality. The life-history theory predicts that delayed

maturity and associated size gain should be selected for if lifetime fecundity is increased.

The lifetime growth pattern of European toads includes a short aquatic larval period in which usually less than 20% of the adult size is attained and a terrestrial period with indeterminate growth that declines at sexual maturity (Werner, 1986; Hemelaar, 1988). In *B. calamita*, metamorph size ranges from 4 to 12 mm SVL, covarying with larval density and interspecific competition in the breeding pond (Goater, 1994; Gomez-Mestre & Tejedo, 2002; Sinsch, 2008). The populations studied do not depart from this rule, with the average metamorph size ranging from 6 to 9 mm (Sinsch, Höfer & Keltsch, 1999; N. Oromi, M. Tejedo & F. Marangoni, unpub. obs.). Thus, body size variation is mainly a consequence of the terrestrial growth period. Unlike in *B. viridis*, local genetic adaptations appear to be a negligible source of size variation in *B. calamita* because the populations in Andalusia do not exhibit significant genetic divergence but close to maximum differences in adult size (Castellano & Giacoma, 1998; Marangoni, 2006; this study).

Environmental causes of phenotypic size plasticity

There are several potential determinants of phenotypic plasticity in the adult body size of amphibians, for example age and associated variation of growth rate, food availability and temperature-dependent limitations of annual growth periods (e.g. Jørgensen, 1992; Gramapurohit, Shanbhag & Saidapur, 2004). The GLM model describing adult growth of natterjacks indicates that the annual growth rates are similar (about 2.5 mm year^{-1}) in all populations and thus independent of latitude. The independence of the growth rate from altitude, however, refers exclusively to the studied lowland range of up to 420 m a.s.l., while natterjacks inhabit altitudes of up to 2540 m a.s.l. in the Sierra Nevada, Spain (Sinsch, 2008). Preliminary data indeed suggest the presence of a converse Bergmann cline in populations of the Sierra de Gredos, Spain (Oromi, study in progress).

In contrast, the intercepts of the GLM, that is the size of the first breeders, do distinguish among the populations, suggesting that the environmental impact on subadult terrestrial growth period is the primary source of size variation among natterjack populations. More precisely, it is the first-year growth that yields the difference, as

Table 3 Estimated SVL variation at the end of the first terrestrial growth period (one LAG) among the studied toad population

Locality	Average SVL (\pm SE) (mm)	Duration of first year aboveground feeding activity (month)	Duration of first year inactivity period (month)	Feeding/growth during aestivation/hibernation
Reserva Biologica de Doñana	47.5 ± 1.0	4–6	c. 4 (aestivation)	No
Navas	58.3 ± 1.0	4–6	c. 4 (aestivation)	Unknown
Mas de Melons	59.7 ± 1.4	4–6	c. 4 (aestivation + hibernation)	Yes
Balaguer	63.7 ± 1.2	4–6	c. 4 (aestivation + hibernation)	Yes
Urmitz (spring)	34.8 ± 1.1	3–4	c. 6 (hibernation)	No
Urmitz (summer)	30.8 ± 1.1	2–3	c. 6 (hibernation)	No

All SVL estimates differ significantly from each other (ANOVA, multiple-range test; $P < 0.05$).

demonstrated by the retrospective SVL estimation. Within eventual determinants of juvenile growth, food availability does not seem to constrain the terrestrial growth of natterjacks at any site studied so far (review: Sinsch, 2008; Urmitz; Leskovar, Wagner & Sinsch, 2004). Therefore, we first concentrate on the environmental factors modulating and limiting the duration and intensity of the first-year growth period, and then focus on the subsequent geographical variation of life-history traits such as age and size at maturity and their evolutionary significance.

In the temperate zone, the growth rates vary seasonally, determined by the varying environmental temperatures (i.e. duration of activity period) and food supply (Jørgensen, 1992). The growth rates of metamorphs at Urmitz, Germany, provide the test case, as temperature and the non-limiting food supply are identical for the spring and summer cohort, but the latter has a shorter activity period before hibernation, resulting in a *c.* 4 mm lower SVL (Sinsch *et al.*, 1999; Leskovar *et al.*, 2004; Table 3). The subsequent inactivity period of juveniles burrowed in sand does not allow further growth partially due to inherent growth rhythms demonstrated under controlled conditions and the absence of food in the hibernacula (Leskovar & Sinsch, 2001). Thus, the length of the activity period explains entirely the first-year size variation between the members of the spring and summer cohorts in Germany.

Although adults of the Urmitz and the Doñana population are similar-sized, first-year individuals grew about 12 mm larger in Spain, demonstrating different lifetime growth regimes at the two sites. The most likely cause for the faster metamorph growth is ambient temperature, which allows higher metabolic rates and better food utilization in Spain (Jørgensen, 1992). The duration of the aboveground activity period appears to be similar, in Germany ended by hibernation, in Andalusia by aestivation (Table 3). As inactive individuals are burrowed in the sandy soil at both sites, we do not expect the Doñana toads to feed and to grow during aestivation, but this hypothesis remains to be proven. If correct, size variation between first-year individuals of the Urmitz and the Doñana population is a direct temperature effect, which is compensated for adult size by the increased second-year growth of the still immature toads in Urmitz.

The first-year individuals of the remaining three Spanish populations were another 12–15 mm larger than the Doñana toads, although the local climate is similar, but moderately colder during winter. Consequently, the duration of aboveground activity is also similar, but the microhabitats used for aestivation (all localities) and hibernation (Balaguer/Mas de Melons) are rabbit holes, rock fissures and crevices because the absence of sandy soils prevents the toads from actively burrowing as in Urmitz and Doñana (Oromi *et al.*, 2010). Radio-telemetric monitoring of adults at Mas de Melons and several short bone growth periods during winter suggest that the shelter microclimate allows locomotor activity including foraging underground (Sinsch *et al.*, 2007b; Oromi *et al.*, 2010). Similar information is not yet available for the Navas site. Still, it seems reasonable to assume that the different microhabitats used for hibernation

and/or aestivation are the proximate causes for the size difference of juveniles. Cavity systems also provide shelter for invertebrate prey, while the small-volume burrows in sand without connection to the ground surface do not. Thus, the period that toads spend underground can be considered as an extension of the feeding period in Balaguer and Mas de Melons, and additional growth is achieved in comparison with the burrowed soil of the Doñana population. However, field reciprocal transplant experiments on the Spanish spadefoot toad, *Pelobates cultripes*, between Doñana and Navas environments show reduced growth in the sandy environment for all the populations, thus suggesting a role of environmentally induced plasticity in the growth rates across environments that may explain the unexpected lower growth of the Doñana populations (M. Tejedo & F. Marangoni, unpubl. data). Reduced size has also been observed in other amphibian species inhabiting the Doñana region (Diaz-Paniagua, Mateo & Andreu, 1996; Diaz-Paniagua & Mateo, 1999).

In summary, the geographical body size variation in *B. calamita* populations is probably the result of interactions among latitudinal temperature effects, microhabitat choice for hibernation/aestivation and habitat constraints to growth determining a pattern to some extent compatible to a converse Bergmann cline. This is in agreement with the latitudinal trend observed in *B. bufo* (Cvetkovic *et al.*, 2009) and supports the idea that amphibians do not follow Bergmann's rule (Ashton, 2002b; Adams & Church, 2008). The proximate causes identified act mainly on juveniles during their first year of life, which represents the sensitive period for fast growth in anurans (Jørgensen, 1992).

Fitness consequences of body size variation on lifetime fecundity

The youngest first breeders were 1-year-old individuals (about one-third of the Doñana population), indicating that the threshold size for maturity is about 40 mm SVL in *B. calamita*. This size is reached and often considerably passed in all populations from the Iberian Peninsula during the first year of life, suggesting that natterjacks are sexually mature at the beginning of the second year. The sharp decline of bone growth during the second year is another indicator of previous sexual maturation (Hemelaar, 1988). Still, there was an increasing tendency from south to north to postpone first reproduction to the third (two LAGs) or the fourth year (three LAGs). The benefit is an SVL increase of 7–10 mm on average, allowing for a significantly larger clutch size than in first breeders at the minimum SVL (Tejedo, 1992). In Germany, first-year growth is too low to attain sexual maturity, and therefore, the growth rate remains high during the second year in which individuals pass the threshold size similar to the growth pattern in *B. bufo* (Hemelaar, 1988). Again, most adults postpone reproduction for at least 1 year.

In natterjacks, size at first reproduction and age at maturity are not tightly coupled. Delayed reproduction may be selected for if fitness increases by the SVL-associated

fecundity gain and lifetime fecundity is optimized, that is the product of clutch size and potential reproductive lifespan (longevity – age at first reproduction). The potential reproductive lifespan of females decreases from north to south: 6–5 years (spring/summer cohort) in Urmitz to 3–4 years in Catalonia and Andalusia (Leskovar *et al.*, 2006; this study). Because the average clutch size follows a converse gradient, lifetime fecundity is probably similar in most of the geographical range (Sinsch, 2008). The southernmost Doñana population is the exception from the rule, with small clutches similar to those in Germany and a low potential reproductive lifespan as in the rest of the Iberian Peninsula. Considering the harsh environmental condition at this site and short longevity, selection should favor early reproduction at small SVL (Marangoni, 2006; Marangoni *et al.*, 2008). Applying a life-history perspective, latitudinal body size variation among *B. calamita* populations, and perhaps also that of other amphibians species, is the evolutionary byproduct of optimized lifetime fecundity.

Acknowledgments

The permissions for field studies and handling of the toads were issued by the Struktur und Genehmigungsbehörde Nord, Koblenz, Germany, the Departament de Medi Ambient, Generalitat de Catalunya and the Consejería de Medio Ambiente de la Junta de Andalucía and the Reserva Biológica de Doñana, Spain. This work was supported by the grant CGL2004-01872/BOS from Dirección General de Investigación Científica y Técnica conceded to M. Tejedo. T.J.C. Beebe and an anonymous reviewer provided useful comments on an earlier draft of the paper.

References

- Adams, D.C. & Church, J.O. (2008). Amphibians do not follow Bergmann's rule. *Evolution* **62**, 413–420.
- Angilletta, M.J., Niewiarowski, P.H., Dunham, A.E., Leaché, A.D. & Porter, W.P. (2004). Bergmann's clines in ectotherms: illustrating a life-history perspective with sceloporine lizards. *Am. Nat.* **164**, E168–E183.
- Ashton, K.G. (2002a). Patterns of within-species body size variation of birds: strong evidence for Bergmann's rule. *Global Ecol. Biogeogr.* **11**, 505–523.
- Ashton, K.G. (2002b). Do amphibians follow Bergmann's rule? *Can. J. Zool.* **80**, 708–716.
- Bergmann, C. (1847). Über die Verhältnisse der Wärmeökonomie der Thiere zu ihrer Grösse. *Göttinger Studien* **1**, 595–708.
- Castellano, S. & Giacoma, C. (1998). Morphological variation of the green toad, *Bufo viridis*, in Italy: a test of causation. *J. Herpetol.* **32**, 540–550.
- Cvetkovic, D., Tomasevic, N., Ficetola, G.F., Crnobrnja-Isailovic, J. & Miaud, C. (2009). Bergmann's rule in amphibians: combining demographic and ecological parameters to explain body size variation among populations in the common toad *Bufo bufo*. *J. Zool. Syst. Evol. Res.* **47**, 171–180.
- Diaz-Paniagua, C. & Mateo, J.A. (1999). Geographic variation in body size and life-history traits in Bosca's newt (*Triturus boscai*). *Herpetol. J.* **9**, 21–27.
- Diaz-Paniagua, C., Mateo, J.A. & Andreu, A.C. (1996). Age and size structure of populations of small marbled newts (*Triturus marmoratus pygmaeus*) from Doñana National Park (SW Spain). A case of dwarfism among dwarfs. *J. Zool.* **239**, 83–92.
- Eaton, B., Paszkowski, C.A., Kristensen, K. & Hiltz, M. (2005). Life-history variation among populations of Canadian Toads in Alberta, Canada. *Can. J. Zool.* **83**, 1421–1430.
- Elmberg, J. (1991). Ovarian cyclicity and fecundity in boreal common frogs *Rana temporaria* L. along a climatic gradient. *Funct. Ecol.* **5**, 340–350.
- Freckleton, R.P., Harvey, P.H. & Pagel, M. (2003). Bergmann's rule and body size in mammals. *Am. Nat.* **161**, 821–825.
- Gaston, K.J. (2008). Ecogeographical rules: elements of a synthesis. *J. Biogeogr.* **35**, 483–500.
- Goater, C.P. (1994). Growth and survival of postmetamorphic toads: interactions among larval history, density, and parasitism. *Ecology* **75**, 2264–2274.
- Gomez-Mestre, I. & Tejedo, M. (2002). Geographic variation in asymmetric competition: a case study with two larval anuran species. *Ecology* **83**, 2102–2111.
- Gramapurohit, N.P., Shanbhag, B.A. & Saidapur, S.K. (2004). Growth, sexual maturation and body size dimorphism in the Indian bullfrog, *Hoplobatrachus tigerinus* (Daudin). *Herpetologica* **60**, 414–419.
- Hemelaar, A.M.S. (1985). An improved method to estimate the number of year rings resorbed in phalanges of *Bufo bufo* (L.) and its application to populations from different latitudes and altitudes. *Amphibia-Reptilia* **6**, 323–342.
- Hemelaar, A.M.S. (1988). Age, growth and other population characteristics of *Bufo bufo* from different latitudes and altitudes. *J. Herpetol.* **22**, 369–388.
- Jørgensen, C.B. (1992). Growth and reproduction. In *Environmental physiology of the amphibians*: 439–466. Feder, M.E. & Burggren, W.W. (Eds). Chicago: The University of Chicago Press.
- Kellner, A. & Green, D.M. (1995). Age structure and age at maturity in Fowler's toads, *Bufo woodhousii fowleri*, at their northern range limit. *J. Herpetol.* **29**, 485–489.
- Krizmanic, I., Vukov, T.D. & Kalezić, M.L. (2005). Bergmann's rule is size-related in European newts (*Triturus*). *Herpetol. J.* **15**, 205–206.
- Laugen, A.T., Laurila, A., Jonsson, K.I., Soderman, F. & Merila, J. (2005). Do common frogs (*Rana temporaria*) follow Bergmann's rule? *Evol. Ecol. Res.* **7**, 717–731.
- Leskovar, C., Oromi, N., Sanuy, D. & Sinsch, U. (2006). Demographic life history traits of reproductive natterjack toads (*Bufo calamita*) vary between northern and southern latitudes. *Amphibia-Reptilia* **27**, 365–375.

- Leskovar, C. & Sinsch, U. (2001). Hibernation behaviour of radiotracked natterjack toads *Bufo calamita* and green toads *Bufo viridis*. *Biota (Suppl.)* **2**, 33–34.
- Leskovar, C., Wagner, T. & Sinsch, U. (2004). Saisonale Dynamik des Nahrungsspektrums syntoper Kreuz- (*Bufo calamita*) und Wechselkröten-Populationen (*B. viridis*) in einem rheinischen Auskiesungsgebiet. *Z. Feldherpetol.* **11**, 83–103.
- Marangoni, F. (2006). *Variación clinal en el tamaño del cuerpo a escala microgeográfica en dos especies de anuros (Pelobates cultripes y Bufo calamita)*. PhD Thesis, Universidad de Sevilla, Sevilla.
- Marangoni, F., Tejedo, M. & Gomez-Mestre, I. (2008). Extreme reduction in body size and reproductive output associated with sandy substrates in two anuran species. *Amphibia-Reptilia* **29**, 541–553.
- Meiri, S. & Dayan, T. (2003). On the validity of Bergmann's rule. *J. Biogeogr.* **30**, 331–351.
- Miaud, C., Guyétant, R. & Elmberg, J. (1999). Variations in life-history traits in the common frog *Rana temporaria* (Amphibia:Anura): a literature review and new data from the French Alps. *J. Zool.* **249**, 61–73.
- Nevo, E. (1972). Climatic adaptation in size of the green-toad, *Bufo viridis*. *Isr. J. Med. Sci.* **8**, 1010 (abstract).
- Olalla-Tarraga, M.A. & Rodriguez, M.A. (2007). Energy and interspecific body size patterns of amphibian faunas in Europe and North America: anurans follow Bergmann's rule, urodeles its converse. *Global Ecol. Biogeogr.* **16**, 606–617.
- Oromi, N., Sanuy, D. & Sinsch, U. (2010). Thermal ecology of natterjack toads (*Bufo calamita*) in a semi-arid landscape. *J. Therm. Biol.* **35**, 34–40.
- Ray, C. (1960). The application of Bergmann's rule and Allen's rule to the poikilotherms. *J. Morphol.* **106**, 85–109.
- Sinsch, U. (2008). *Bufo calamita* Laurenti, 1768 – Kreuzkröte. In *Handbuch der Amphibien und Reptilien Europas*: 339–413. Grossenbacher, K. (Ed.). Wiesbaden: Aula Verlag.
- Sinsch, U., Höfer, S. & Keltsch, M. (1999). Syntopes Vorkommen von *B. calamita*, *B. viridis* und *Bufo bufo* in einem rheinischen Auskiesungsgebiet. *Z. Feldherpetol.* **6**, 43–64.
- Sinsch, U. & Keltsch, M. (2002). Die Fekundität von Kreuzkröten (*Bufo calamita*) und Wechselkröten (*B. viridis*) in einem rheinischen Auskiesungsgebiet. *Z. Feldherpetol.* **9**, 1–15.
- Sinsch, U., Leskovar, C., Drobig, A., König, A. & Grosse, W.R. (2007a). Life history traits in green toad (*Bufo viridis*) population: indicators of habitat quality. *Can. J. Zool.* **85**, 665–673.
- Sinsch, U., Oromi, N. & Sanuy, D. (2007b). Growth marks in natterjack toad (*Bufo calamita*) bones: histological correlates of hibernation and aestivation periods. *Herpetol. J.* **17**, 129–137.
- Sinsch, U., di Tada, I.E. & Martino, A.L. (2001). Longevity, demography and sex-specific growth of the Pampa de Achala Toad, *Bufo achalensis* CEI, 1972. *Stud. Neotrop. Fauna and Environ.* **36**, 95–104.
- Smirina, E.M. (1972). Annual layers in bones of *Rana temporaria*. *Zoologicheskyy Zh.* **51**, 1529–1534.
- Tejedo, M. (1992). Effects of body size and timing of reproduction on reproductive success in female natterjack toads (*Bufo calamita*). *J. Zool.* **228**, 545–555.
- Tejedo, M., Reques, R. & Esteban, M. (1997). Actual and osteochronological estimated age of natterjack toads (*Bufo calamita*). *Herpetol. J.* **7**, 81–82.
- Werner, E.E. (1986). Amphibian metamorphosis: growth rate, predation risk, and the optimal size at transformation. *Am. Nat.* **128**, 319–341.

Altitudinal variation of demographic life-history traits does not mimic latitudinal variation in natterjack toads (*Bufo calamita*)

Accepted in: *Zoology*

Authors: OROMÍ, N.; SANUY, D.; SINSCH, U.

The life history traits of *Bufo calamita* have been studied in a latitudinal gradient. Leskovar et al. (2006) found that the age at maturity, longevity and potential reproductive lifespan of females increased along the south-north gradient of species distribution. In contrast, the size of females were bigger in southern than in northern populations. These differences suggested a trade-off between the two traits -potential reproductive lifespan and size- that optimizes the interpopulation fecundity, leading finally to the same biological efficiency. The Chapter 4 shows the study of demographic life history traits of *B. calamita* in an altitudinal gradient of the Sierra de Gredos, in the Central System of the Iberian Peninsula. The differences between populations seem to be due to the active period in the different altitudes.

Author contribution: First authorship reflects that I was the main contributor to the paper. I have done the sampling and the skeletochronological analysis of all populations. I have written the paper in close collaboration with Prof. Drs Ulrich Sinsch and Delfi Sanuy.

ABSTRACT

In anuran amphibians, age- and size-related life-history traits vary along latitudinal and altitudinal gradients. We test the hypothesis that altitudinal and latitudinal effects cause similar responses by assessing demographic life-history traits in nine *B. calamita* populations inhabiting elevations from sea level to 2,270 m. Skeletochronologically determined age at maturity and longevity increased at elevation exceeding 2,000 m, but female potential reproductive lifespan (PRLS) did not increase with altitude, as it did in northern latitudes. Integrating available evidence, lifetime fecundity of natterjacks decreases at the upper altitudinal range because PRLS is about the same as in lowland populations but females are smaller. In contrast, small size of northern females was compensated for by increased PRLS which minimised latitudinal variation of lifetime fecundity. This study provides evidence that altitudinal effects on life-history traits do not mimic latitudinal effects. Life history trait variation along the altitudinal gradient seems to respond directly to the contraction of the annual activity. As there is no evidence for increasing mortality in highland populations, reduced lifetime fecundity may be the ultimate reason for the natterjacks' inability to colonise elevation exceeding 2,500 m.

KEYWORDS:

Amphibia, Anura, phenotypic plasticity, life-history traits, age at maturity, longevity, potential reproductive lifespan, skeletochronology.

INTRODUCTION

Life-history traits such as age at maturity, longevity and larval growth rates show a significant co-variation with altitude and latitude in many amphibian species (Morrison and Hero 2003; Marquis and Miaud 2008). Multiple proximate causes such as genetic adaptation and/or phenotypic plasticity may underlie this pattern (Roff 2001; Sultan and Spencer 2002). Divergent selection among local environments and restricted gene flow can cause populations to evolve in response to specific ecological conditions, leading to local adaptation. Phenotypic plasticity is an evolutionary factor that can promote or retard genetic change (Paenke et al. 2007; Marquis and Miaud 2008). In anuran amphibians, conspecific populations along a wide altitudinal range vary in the several life-history traits, but except for *Rana sylvatica* (Berven 1982a, b) it remains mostly unclear, whether variation is based on genetic adaptation or phenotypic plasticity.

Typical demographic life-history traits affected are age at maturity and longevity. Delayed sexual maturation at high elevations is a common response in most species (*Pleurodema thaul*: Iturra-Cid et al. 2010), often associated with a subsequent increase of longevity (*Amolops mantzorum*: Liao and Lu 2010a; *Bufo bufo*: Hemelaar 1988; *Hyla annectans*: Liao and Lu 2010b; *Nanorana parkeri*: Ma et al. 2009a; *Rana sylvatica*: Berven 1982; *R. temporaria*: Ryser 1996; Miaud et al. 1999; *R. swinhoana*: Lai et al. 2005; *R. chensinensis*: Lu et al. 2006; Ma et al. 2009b). Corresponding effects on body size seem to differ among species (Morrison and Hero 2003). A positive correlation between size and altitude was detected in *Bufo bufo* (Hemelaar 1988), *Rana chensinensis* (Lu et al. 2006; Ma et al. 2009a), *R. sauteri* (Lai et al. 2003), *R. swinhoana* (Lai et al. 2005), *Pleurodema thaul* (Iturra-Cid et al. 2010), a negative correlation in *Rana temporaria* (Ryser 1996, but see Kozłowska 1971 for a positive correlation and Elmberg 1991 for no correlation), *R. muscosa* (Matthews and Miaud 2007), *R. nigromaculata* (Liao

et al. 2010), and *Nanorana parkeri* (Ma et al. 2009b), whereas no correlation was detected in *Amolops mantzorum* (Liao and Lu 2010a) and *Hyla annectans* (Liao and Lu 2010b). Available evidence suggests that altitudinal effects on age and size are selected for independently and that anuran species differ considerably in magnitude of response. As life-history theory predicts that latitudinal and altitudinal effects cause similar demographic responses in amphibians (Morrison and Hero 2003), proximate mechanisms modulating latitudinal variation of life-history traits age and size may determine altitudinal variability as well. To our knowledge this prediction has not been tested in a single species covering the complete latitudinal and altitudinal range.

We aim to test the hypothesis of common proximate mechanisms of age and size variation along geographical gradients in the natterjack toad (*Bufo calamita*). This anuran qualifies as a model organism because of its wide latitudinal (36° - 58°) and altitudinal range (sea level to 2,540 m) and well-understood latitudinal variability of age and size at lowland localities (Leskovar et al. 2006; Sinsch 2008; Sinsch et al. 2010). In males, age at maturity and longevity showed a slight, but insignificant tendency to increase with latitude, whereas longevity and subsequently potential reproductive lifespan (PRLS) augmented in females (Leskovar et al. 2006). In contrast, the pattern of associated size variability was not compatible with a latitudinal cline but suggests optimisation of lifetime fecundity by a counter gradient selection of female size and PRLS (Sinsch et al. 2010). High rates of gene flow over large distances (Marangoni 2006; Oromi et al. in prep.) may suggest that age-adjusted size variation to the environmental constraints is more probably due to phenotypic plasticity of a “general purpose genotype” than to local genetic adaptation, but putatively neutral genetic differentiation amongst wild populations often confounds phenotypic and genetic variation (e.g. Pujol et al. 2008, Richter-Boix et al. 2010, Lind et al. 2011). If response of life-history traits to altitudinal environmental variation mimics that along the latitudinal gradient, we predict (1) that female PRLS will increase because the delayed age at

maturity will be outweighed by a stronger increase of longevity, and (2) that size will decrease concurrently because of the contraction of the annual growth period. The interaction of these trends would optimise lifetime fecundity at high-elevation sites. We test these specific predictions by analysing trait variation in nine populations which inhabit the Sierra de Gredos and neighbouring lowland sites in Spain along an elevation range from 10 m to 2,270 m.

MATERIAL AND METHODS

Populations and study sites

A total of 252 reproductive natterjack toads *Bufo (=Epidalea) calamita* were studied at seven localities at 10 m to 2,270 m altitude in Spain. Habitat and local climate features, collection dates and numbers of reproductive adults sampled are summarized in Table 1. Five populations (Navaluenga = NL, La Dehesa del Barraco = DB, La Cedrera = CE, Cavadores = CA and Navasomera = NS) are situated in the Sierra de Gredos (Central Iberian System). The breeding sites of *B. calamita* in these localities are humid meadows and ponds at the lower sites, and glacial lagoons at the high-montane sites. Two populations (Palamós = PA, El Pinós = PI) were located outside the Sierra de Gredos (Table 1). The PA breeding sites were ephemeral ponds in a dune system 1 km distant from the Mediterranean coast. El Pinós is situated in the Sierra del Reclot 3.8 km distant from the Mediterranean coast, and toads reproduced in ephemeral water bodies or in artificial ponds. To complement analysis of altitudinal variation, we included data on two populations inhabiting the Ebro valley (Balaguer = BA, Mas de Melons = MM; Table 1), published in Leskovar et al. (2006) and Sinsch et al. (2007). Climate variables used were minimum and maximum annual air temperature, average annual air temperature, and precipitation [mm/year]. Temperature (15-year averages) and precipitation data (20-year averages) for the nine study areas were derived from the “Atlas climático digital de la Península Ibérica” (Ninyerola et al. 2005), except for precipitation data (30-year

average) for BA and MM (Meteorological station of Lleida, details in Leskovar *et al.*, 2006). Substrate of study areas was classified either as “rock”, i.e. predominantly bare rock with crevices and little vegetation, or “clay”, i.e. fined-grained minerals forming near impermeable soils, or “sand”, i.e. coarse-grained soil with more than 50 % of material retained by a 0.075 mm sieve.

Table 1. Location of the nine populations studied, local climate features and number of toads collected.

Locality	Coordinates	Altitude [m a.s.l.]	Mean temperature (Min-Max) [°C]	Precipitation [mm]	Substrate type	Date of collection	male/female
Palamós	41°51'49"N 03°07'59"W	10	15 (+11 - 20)	844	sand	March 2006	35/12
Balaguer (Leskovar et al. 2006)	41°46'30"N 00°46'50"W	220	14 (+8 - 21)	369	clay	April 2004	34/14
Mas de Melons (Leskovar et al. 2006)	41°30'50"N 00°43'30"W	240	14(+8 - 20)	369	clay	April 2004	43/4
Navaluenga	40°24'57"N 04°40'47"W	752	13 (+7 - 20)	735	rock	April 2008	23/21
El Pinós	38°22'44"N 00°58'40"W	760	15 (+9 - 20)	313	clay	April 2007	14/14
Dehesa del Barraco	40°27'53"N 4°38'12"W	920	13 (+7 - 19)	676	rock	March 2010	25/5
La Cedrera	40°30'33"N 4°39'16"W	1472	10 (+3 - 16)	652	rock	March 2010	31/14
Cavadores	40°16'19"N 5°15'12"W	2100	7 (0 - 14)	1172	rock	May 2009	27/0
Navasomera	40°15'07"N 5°15'49"W	2270	6 (-1 - 13)	923	rock	June 2008	28/3

Sampling procedure and skeletochronological age estimation

Toads were collected at the local breeding ponds during the spring reproduction period of 2004-2010 (Table 1). The variation in sex ratio is due to the fact that collecting surveys were not exhaustive and thus, samples represent a random sample of the local breeding assemblage. Toads were released *in situ* following sex determination, measuring of snout-vent length (SVL, to the nearest mm) and toe-clipping (3rd toe of the right hind limb). The toes were stored in 70% ethanol at room temperature.

Laboratory protocols for skeletochronology followed the procedure described in Tejedo et al. (1997). The samples were decalcified in 3% nitric acid for at least 20 min. Cross sections (16 μm) were prepared using a freezing microtome (MICROM HM 50 N) and stained with Ehrlich's hematoxyline (Sierra de Gredos populations) or cresylviolet (PA, PI). Cross sections were examined light microscopically for the presence of growth marks (strongly stained lines in the periosteal bone) at magnifications of 400x using an OLYMPUS BX 50. We selected diaphysis sections in which the size of the medullar cavity was at its minimum and that of periosteal bone at its maximum. We distinguished three types of growth marks: (1) line of metamorphosis (mark produced in response to the physiological changes during metamorphosis), (2) annual growth marks (= Lines of Arrested Growth, LAGs, indicators of hibernation), and (3) non-annual faint lines (indicators of occasional interruptions of hibernal inactivity periods). The number of LAGs (annual growth marks) was assessed by the first author and independently by one or two co-authors to estimate age as the number of completed hibernations. Non-annual growth marks were distinguished from annual one using the method described in Sinsch et al. (2007) to calculate the percentage of individuals per population which showed the histological equivalents of interrupted hibernation. The age at maturity was defined as the age of the youngest reproductive individual recorded at each locality, whereas the median age indicated the age class which usually included the greatest number of individuals. The median age was calculated as an

integrative measure of lifespan. Longevity was assessed as the maximum number of LAGs counted in an individual of each population. Potential reproductive lifespan (PRLS) was calculated as the difference between longevity and minimum age at maturity.

Statistical analysis

All variables were first tested for normality. The influence of altitude on the percentage of multiple lines per population and on local age variation was estimated by fitting data in 29 regression models. We chose the reciprocal-Y squared-X model for multiple lines prediction and the squared-x model for age prediction because they provided the best fit, i.e. maximum R^2 . As age and SVL distributions were significantly skewed, data were normalised by $\log(10)$ -transformation. A factorial analysis of log-normalized age and size data and the corresponding environmental factors were implemented to obtain a small number of statistically independent factors, which account for most of the variation of original variables. An orthogonal VARIMAX rotation was performed to maximize the sum of the variances of the squared loadings, i.e. rotation procedure approximates factor loadings of individual variables either close to zero or to \pm one. Potential sexual dimorphism with respect to altitude-adjusted, demographic life-history traits was tested for by analyses of covariance (ANCOVA, type III sums of squares). Multifactorial ANCOVA were used to assess environmental effects on log-normalized SVL and age. Categorical factors were sex and substrate type. As covariates we considered the continuous variables latitude, altitude, minimum temperature, maximum temperature, average temperature, and precipitation. Age (number of LAGs) was used as an additional covariate for SVL. As one population (CA) was exclusively represented by males, we excluded CA from all ANCOVAs including sex as fixed factor. Multiple comparisons of the least square means were based on 95% Bonferroni confidence intervals. Significance level was set at $\alpha = 0.05$. All calculations were performed using the procedures of the program package STATGRAPHICS Centurion, version XV (STATPOINT Inc.).

RESULTS

Replacement of periosteal bone by endosteal one, i.e. potential resorption of the first LAG, did not interfere with age determination in 138 individuals studied because the line of metamorphosis was still visible in the cross sections. In the remaining individuals we considered prolonged periosteal bone growth before the deposition of the first visible LAG as an indicator of the first-year activity period in which bone growth rate is at its maximum (Sinsch et al. 2010). Multiple line formation was common in all studied populations ranging from 35.5 % in NS to 85.7 % in PI. Excluding the only site with sandy substrate (CA, 40.7 %), altitude correlated significantly with the frequency of multiple lines explaining 95.4 % of variance in percentage (regression model: multiple lines [%] = $1/(0.0116231 + 0.00000003 * \text{Altitude [m]}^2)$; $r = 0.977$, $F_{1,7} = 126.6$, $P < 0.001$; Fig. 1). The number of individuals interrupting hibernation decreased with altitude.

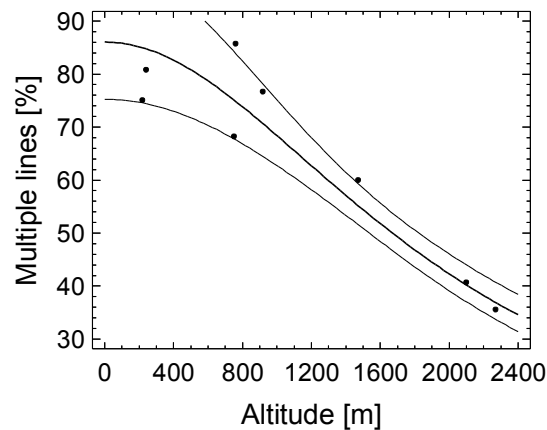


Figure 1. Altitudinal variation of the frequency of multiple lines in eight populations inhabiting localities with rock and clay substrate. Dots represent each population, and the bold line indicates the regression model and its 95 % confidence range (thin lines). For statistical details see text.

Factorial analysis of a data set comprising age, size, and six environmental variables revealed that age and size loaded different factors (Table 2). The set of

eight variables included two factors with an eigenvalue > 1 which accounted for 78.0% of variance. Factor 1 represented SVL and associated environmental variables, mainly substrate type and precipitation. Factor 2 represented age and was additionally loaded by altitude and temperature variables. Consequently, we focussed further analysis on age structure and size separately.

Table 2. Factorial analysis of age and size variation and environmental variability, given as factor loading matrix after orthogonal VARIMAX rotation.

	<i>Factor 1</i>	<i>Factor 2</i>	<i>Estimated Communality</i>
Eigenvalue	5.64	1.38	
Variance	62.7%	15.3 %	
log10(SVL)	0.919	-0.032	0.845
log10(LAGs)	-0.056	-0.688	0.478
Altitude	-0.500	-0.847	0.968
Latitude	-0.075	0.547	0.305
Average temperature	0.570	0.783	0.938
Minimum temperature	0.468	0.834	0.915
Maximum temperature	0.628	0.730	0.927
Precipitation	-0.890	-0.218	0.840
Substrate type	0.845	0.297	0.803

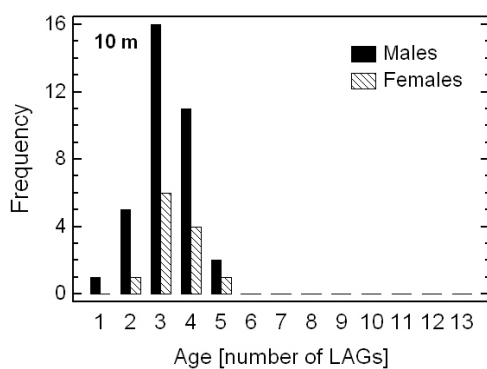
Age structure

Demographic life-history traits of natterjack toads varied substantially among localities and gender (Table 3, Fig. 2). The earliest age at which sexual maturity can be attained ranged from one LAG (male, PA) to five LAGs (male, female, NS), maximum lifespan was 12 LAGs (male, NS). Age at maturity and age median did not differ between males and females (multifactorial ANCOVA, $F_{1,15} = 2.43$, $P = 0.143$; $F_{1,15} = 0.03$, $P = 0.870$), but increased significantly with altitude ($F_{1,15} = 28.35$, $P < 0.001$; $F_{1,15} = 8.86$, $P = 0.011$). Altitude explained 35.7% of variance in age (regression model: Age [LAGs] = 3.32 + 0.0000055 * Altitude [m]², $r = 0.508$, $F_{1,346} = 191.8$, $P < 0.001$; Fig. 3A). In contrast, longevity and PRLS did neither vary sex-specifically (multifactorial ANCOVA, $F_{1,15} = 0.10$, $P = 0.757$; $F_{1,15} = 0.06$, $P = 0.810$) nor were significantly affected by altitude ($F_{1,15} = 2.57$, $P = 0.133$; $F_{1,15} = 0.06$, $P = 0.813$).

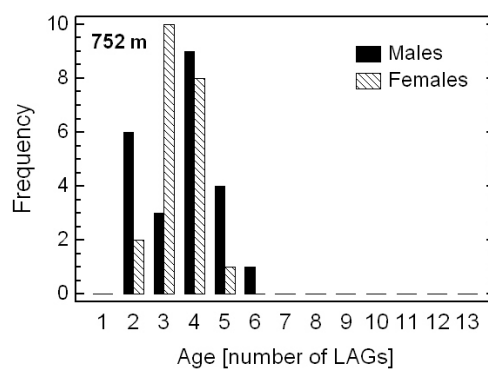
Table 3. Life-history traits observed in nine populations along an altitudinal gradient .

Locality	Sex	AM [LAGs]	Median Age [year]	SVL [mm; mean±SE]	Longevity [LAGs]	PRLS [year]
Palamós	Males	1	3	61.6 ± 0.8	5	4
	Females	2	3	59.4 ± 1.6	5	3
Balaguer (Leskovar et al. 2006)	Males	2	4	79.1 ± 0.8	7	5
	Females	2	3	80.1 ± 1.4	5	3
Mas de Melons (Leskovar et al. 2006)	Males	2	4	79.0 ± 0.7	8	6
	Females	3	4	85.5 ± 2.7	7	4
Navaluenga	Males	2	4	63.5 ± 1.0	6	4
	Females	2	3	66.1 ± 1.2	5	3
El Pinós	Males	2	3	78.6 ± 1.3	4	2
	Females	3	4	83.0 ± 1.4	8	5
Dehesa del Barraco	Males	2	3	65.5 ± 1.0	5	3
	Females	3	3	67.0 ± 2.4	5	2
La Cedrera	Males	2	3	63.9 ± 0.9	6	4
	Females	3	3	66.5 ± 1.4	6	3
Cavadores	Males	2	6	62.6 ± 0.9	9	7
	Females	-	-	-	-	-
Navasomera	Males	5	7	58.8 ± 0.9	12	7
	Females	5	6	60.3 ± 3.5	6	1

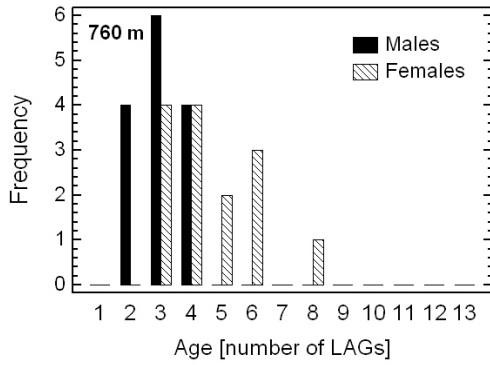
A)



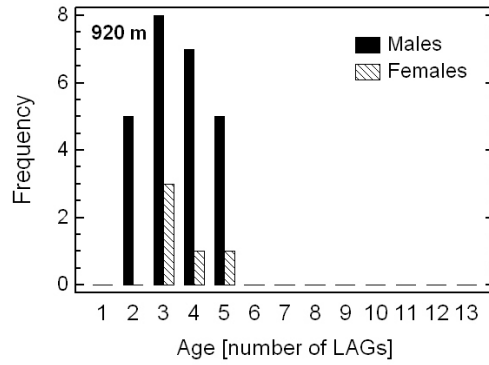
B)



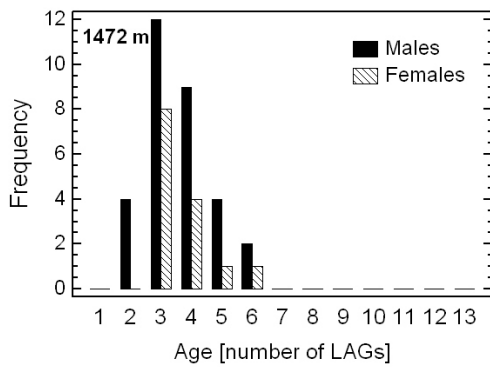
C)



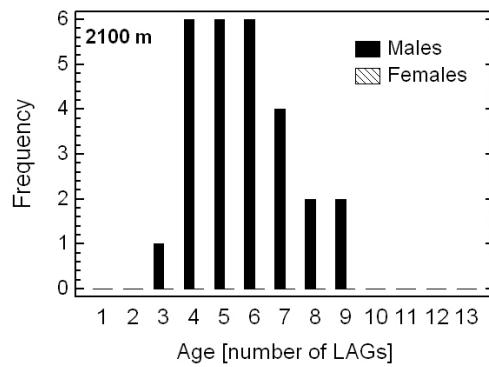
D)



E)



F)



G)

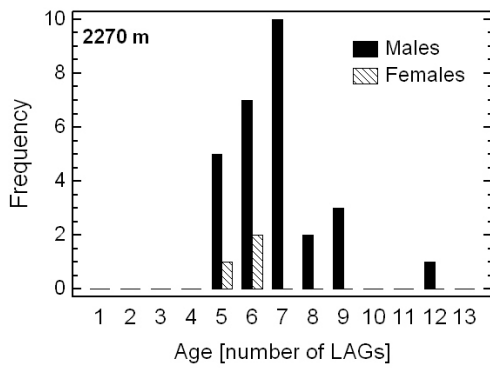


Figure 2. Sex-specific age structure of seven *B. calamita* populations. (A) Palamós, (B) Navaluenga, (C) El Pinós, (D) Dehesa del Barraco, (E) La Cedrera, (F) Cavadores, and (G) Navasomera. For Details see Table 1.

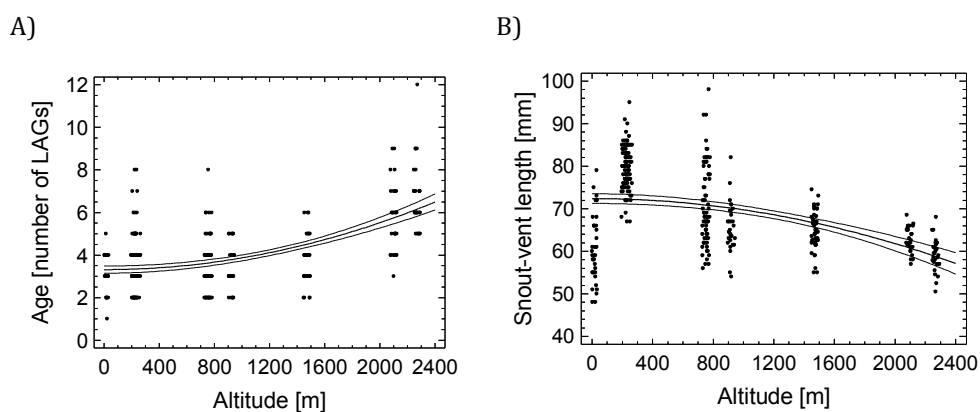


Figure 3. Altitudinal variation of local (A) age and (B) size distribution. Each dot represents an individual; dots are slightly jittered to visualize the number of same-aged or same-sized toads. Regression model is presented analogous to Fig. 1. For statistical details see text.

Log-normalized age distribution of toads did not differ significantly between males and females, but among the three substrate type (ANCOVA, Table 4A). Multiple range comparison showed that the PA toads living on sandy substrate (LS mean \pm SE: 0.191 ± 0.096) were significantly younger on average than those on rock (0.465 ± 0.021 ; $P < 0.05$) which in turn were younger than those on clay (0.830 ± 0.044 , $P < 0.05$). There was no detectable interaction between the fixed factors sex and substrate. Among covariates, only maximum temperature and precipitation were significantly related to age, whereas altitude and latitude did not co-vary with age (Table 4A). Thus, the influence of altitude on age detected in the univariate correlation analysis is consequence of the systematic change of temperature and rainfall with altitude.

Table 4: Multifactorial ANCOVAs for (A) Age as log₁₀(LAGs), and (B) Size as log₁₀(SVL).

(A)

<i>Source</i>	<i>Square sum</i>	<i>df</i>	<i>Mean sum of squares</i>	<i>F-ratio</i>	<i>p-values</i>
Covariates:					
Altitude	0.0042	1	0.0042	0.24	0.625
Latitude	0.0098	1	0.0098	0.56	0.455
Precipitation	0.5921	1	0.5921	34.01	< 0.001
Minimum Temperature	0.0155	1	0.0155	0.89	0.3467
Maximum Temperature	0.1360	1	0.1360	7.81	0.006
Main effects					
Sex	0.0260	1	0.0260	1.50	0.222
Substrate	0.7609	2	0.3804	11.32	<0.001
Interactions					
Sex x Substrate	0.0301	2	0.0151	0.86	0.422
Residuals	5.3804	309	0.0174		
Total (corrected)	7.9232	319			

(B)

<i>Source</i>	<i>Square sum</i>	<i>df</i>	<i>Mean sum of squares</i>	<i>F-ratio</i>	<i>p-values</i>
Covariates:					
Age [log ₁₀ (LAGs)]	0.0144	1	0.0144	13.51	<0.001
Altitude	9.1895E-6	1	9.1895E-6	0.01	0.926
Latitude	9.4533E-7	1	9.4533E-7	0.00	0.976

Precipitation	0.0002	1	0.0002	0.18	0.674
Average Temperature	2.6235E-5	1	2.6235E-5	0.02	0.876
Minimum Temperature	4.0004E-5	1	4.0004E-5	0.04	0.847
Main effects					
Sex	0.0008	1	0.0008	0.80	0.373
Substrate	0.0078	2	0.0039	3.65	0.027
Interactions					
Sex x Substrate	0.0081	2	0.0040	3.79	0.024
Residuals	0.3284	308	0.0011		
Total (corrected)	1.2169	319			

Age-Size relationship

Altitude explained 22.0% of variance in size, if not adjusted for age (regression model: $SVL [mm] = 72.38 - 0.0000026 * Altitude [m]^2$, $r = 0.469$, $F_{1,346} = 97.4$, $P < 0.001$; negative Bergmann cline, Fig. 3B). Comparison of local SVL distributions revealed that there were three populations including exceptionally large-sized adults (BA SVL range: 68 - 90 mm, MM 67 - 95 mm, PI 72 - 98 mm) and six populations with a normal size range of adults (PA 48 - 79 mm, NL 56 - 73 mm, DB 54 - 82 mm, CE 55 - 75 mm, CA 57 - 69 mm, NS 51 - 68 mm; Leskovar et al. 2006, Sinsch et al. 2010). The occurrence of very large individuals was restricted to populations inhabiting areas with predominantly clay substrate.

Log-normalized SVL distribution of toads did not differ significantly between males and females, but among the three substrate type (ANCOVA, Table 4B). Multiple range comparison showed that the PA toads living on sandy substrate (LS mean \pm SE: 1.763 ± 0.054) were significantly smaller on average than those on rock (1.845 ± 0.088 ; $P < 0.05$) and those on clay (1.851 ± 0.088 , $P < 0.05$). There was a significant interaction between the fixed factors sex and substrate

with females being slightly larger than males on rock and clay substrates (Table 4B). Among the covariates considered, exclusively log-normalized age influenced significantly SVL, whereas altitude, latitude, precipitation and temperature did not co-vary with SVL (Table 4B). Thus, the influence of altitude on size detected in the univariate correlation analysis is consequence of the systematic change in age and ground substrate with altitude.

DISCUSSION

Age- and size-related life history traits in *Bufo calamita* show a significant co-variation with either altitude (this study) or latitude (Leskovar et al. 2006; Sinsch et al. 2010), as predicted by life history theory. Consequently, natterjack toads provide the unique opportunity to test the prediction that latitudinal and altitudinal effects cause similar responses (Morrison and Hero 2003). Our study provides evidence that age-related altitudinal effects were distinct from latitudinal ones, affecting males and females in the same manner (delayed age at maturity, increased longevity, unchanged PRLS), whereas latitudinal effects were notable only in females (unchanged age at maturity, increased longevity, increased PRLS). Size, in contrast, decreased roughly towards north and in highland populations, but showed a wide variability in Iberian lowland populations. Thus, the size reduction towards north is indeed a reliable predictor of altitudinal size variation, whereas age-related traits were independently selected for and follow distinct latitudinal and altitudinal patterns.

Proximate causes of the observed variation in age- and size related life-history traits may be genetic differentiation or phenotypic plasticity or a combination of both (Berven 1982a, b, Roff 2001, Marquis and Miaud 2008). Microsatellite variation among the populations in the Sierra de Gredos (NL, DB, CE, CA, NS) has been assessed in a parallel study (Oromi et al. in prep.). Despite 1,500 m difference of elevation between NL and NS microsatellites demonstrate

a high gene flow and little support for a local genetic differentiation. Significant gene flow among seven natterjack populations has also been detected along a 100 km cline including an altitudinal range of 400 m (southern Spain; Marangoni 2006). Both studies agree in that there is little evidence for genetic isolation by distance or altitude to promote local modifications of the gene pool. However, in the absence of common garden experiments neither the microsatellite studies nor our data set presented here allow to distinguish, whether or not despite of gene flow adaption contributes significantly to the variation of life-history traits in *B. calamita* (Pujol et al. 2008, Richter-Boix et al. 2010, Lind et al. 2011). Local variability of age structure and size distribution of natterjacks along the studied altitudinal gradient follows different response patterns and will be discussed separately.

Altitudinal variation of adult age structure

Notable modification of local age structure was detected exclusively in the populations at more than 2,000 m elevation and consisted in an increased age at maturity and longevity, leaving unaffected PRLS. In Alpine populations of *B. bufo* (Hemelaar 1988; Schabetsberger et al. 2000) and *R. temporaria* (Ryser, 1996; Miaud *et al.*, 1999) maximum longevity of 12-15 years (= LAGs) was about the same as in *B. calamita*, probably representing a threshold age of European anurans under these harsh conditions. Similar trends in demographic life-history traits have been found in other bufonid and ranid species (Berven 1982a, b; Lai et al. 2005; Lu et al. 2006; Ma et al. 2009a) suggesting a generalised response of anurans to high-elevation conditions. We conclude that a compensatory increase of female PRLS to counteract the decrease in size and therefore of annual fecundity fails due to the altitudinal constraint of longevity, unlike the situation in northern latitudes.

As previously proposed for the altitudinal variation in *B. bufo* (Hemelaar 1988) and *R. temporaria* (Miaud et al. 1999), we hypothesise that the proximate mechanism promoting delayed maturity and increased longevity is the

contraction of annual activity period during which individuals may feed and grow and are exposed to predation. Bone growth pattern of natterjacks supports this hypothesis because the high incidence of multiple LAG-formations in populations at elevations below 2,000 m indicates frequent short growth periods during the winter months (Sinsch et al. 2007) and thus, a prolonged activity period. Only at the upper range of altitudinal distributions winter becomes too severe for foraging on the surface and annual activity period decreases notably. An elevation of about 2,500 m in the Iberian Peninsula seems to represent the thermal equivalent of the -5 °C isotherm in January which describes the northern and eastern range limit at sea level (Sinsch 2008). At higher elevations and farther north and east annual activity period becomes probably too short to allow for a successful larval development plus the storage of sufficient fat and glycogen reserves of juveniles to survive winter (Sinsch and Leskovar 2011).

Altitudinal variation of size

Multiple causes such as age, food availability and temperature may underlie geographical variation of adult size in anurans (Jørgensen 1992; Gramapurohit et al. 2004). In natterjack toads, growth during the first year of life determines adult size and subsequently size variation among population along a latitudinal gradient (Sinsch et al. 2010). Different first year growth rates are proximately caused by the duration of surface activity period which constrains access to food, whereas there is no indication that food availability itself may limit growth (e.g. Leskovar et al. 2004). Considering the contraction of annual activity period at high elevations we hypothesise that parallel size decrease is caused by the reduced terrestrial feeding period between metamorphosis and first hibernation. In contrast, frequent multiple annual LAG-formations indicates that metamorphs may grow potentially during their complete first terrestrial year, if inhabiting elevations below 2,000 m in Spain (Sinsch et al. 2007; this study). Radio-tracked adults of the MM population were also shown to forage on surface during the winter months (Oromi et al. 2010).

While decrease of size with increasing altitude and/or latitude is a common feature of natterjack populations (Leskovar et al. 2006; Sinsch 2008), there is considerable size variation among lowland populations in the Iberian Peninsula which is not related to latitude and/or altitude (Marangoni 2006; Sinsch et al. 2010; this study). Climatic constraints of annual activity period are similar in these populations, but still, age-adjusted SVL may differ by up to 30 mm. The only obvious factor distinguishing localities which allow for very large adults is the predominant type of surface soil. All currently known populations (Table 1) with large-sized toads inhabit semiarid localities (220 – 760 m asl) with clay soils which form a dense surface layer that do not permit active burrowing (Oromi et al. 2010). Therefore, daytime shelter and hibernacula are mammal burrows and cavities in stone embankments which keep the toads in (attenuated) contact with ambient air temperatures. Stimuli to leave shelter are core temperatures exceeding 4°C and/or dehydration (Oromi et al. 2010; Sinsch and Leskovar 2011). Hence, migratory activity associated with foraging and subsequent growth of *B. calamita* is superior at these sites than in those with predominantly sandy soils (Sinsch et al. 2007, 2011). Rock habitats represent a basically similar ground structure offering rock fissures and crevices as the only shelter type for toads. Radio-tracking data from toads in rock habitats are not yet available but we hypothesize that the toads' behaviour will rather resemble that of their conspecifics in clay habitats than that of toads in sand habitats. ANCOVA of size variation supports the similarity of clay and rock habitats (Table 4B). Toads burrowed deeply into moist sandy soil act as thermal conformers with underground soil temperature and do not experience dehydration stress during winter or summer (Sinsch and Leskovar 2011). Therefore, they rarely leave hibernacula, as evidenced by a minor frequency of multiple annual LAGs (Sinsch et al. 2010). In conclusion, shelter choice makes the difference and promotes distinct growth patterns in localities depending on the ground surface structure.

Consequences of age and size variation on the viability of populations

Age- and size-related life-history traits of *B. calamita* seem to respond independently and directly to the contraction of the annual activity period along a south-north axis or an altitudinal gradient (Leskovar et al. 2006; Sinsch et al. 2010; this study). The local soil condition and subsequent shelter choice for hibernation or aestivation further affect the magnitude of surface activity and thus indirectly size variation. It remains currently open, whether observed variability of the life-history traits studied is mainly due to the phenotypic plasticity of a “general purpose genotype”, or like in *Rana sylvatica* (Berven 1982a, b) to a combination of genetic adaptation and phenotypic plasticity. However, females respond more sensitively to environmental constraints than males in both species. Age at maturity and longevity and therefore PRLS of females are crucial for the long-term viability of populations because together with size they determine lifetime fecundity. Integrating available evidence, lifetime fecundity of natterjacks decreases at the upper altitudinal range because PRLS is about the same as in lowland populations but females are smaller. As there is no evidence for increased mortality in highland populations, reduced lifetime fecundity may be the ultimate reason for the natterjacks’ inability to colonise elevation exceeding 2,500 m, and probably to expand geographical range further north.

ACKNOWLEDGEMENTS

The permissions for field studies and handling of the toads were issued by the Departament de Medi Ambient, Generalitat de Catalunya and the Consejería de Medio Ambiente de la Junta de Castilla y León. The project was financed by the Ministerio de Ciencia e Innovación, Gobierno de España, and the Diputació de Lleida.

REFERENCES

- Berven, K.A., 1982a. The genetic basis of altitudinal variation in the Wood Frog *Rana sylvatica*. I. An experimental analysis of life history traits. *Evolution* 36, 962–983.
- Berven, K.A., 1982b. The genetic basis of altitudinal variation in the Wood Frog *Rana sylvatica*. II. An experimental analysis of larval development. *Oecologia* 52, 360–369.
- Elmberg, J., 1991. Ovarian cyclicity and fecundity in boreal common frogs *Rana temporaria* L. along a climatic gradient. *Funct. Ecol.* 5, 340–350.
- Gramapurohit, N.P., Shanbhag, B.A., Saidapur, S.K., 2004. Growth, sexual maturation and body size dimorphism in the Indian bullfrog, *Hoplobatrachus tigerinus* (Daudin). *Herpetologica* 60, 414–419.
- Hemelaar, A., 1988. Age, growth and other population characteristics of *Bufo bufo* from different latitudes and altitudes. *J. Herpetol.* 22, 369–388.
- Iturra-Cid, I., Ortiz, J.C., Ibarzüengoytia, N.R., 2010. Age, size, and growth of the Chilean Frog *Pleurodema thaul* (Anura: Leiuperidae): Latitudinal and altitudinal effects. *Copeia*, 609–617.
- Jørgensen, C.B., 1992. Growth and reproduction. In: Feder, M.E., Burggren, W.W. (Eds.), *Environmental physiology of the amphibians*. The University of Chicago Press, Chicago, pp. 439–466.
- Kozłowska, M., 1971. Differences in the reproductive biology of mountain and lowland common frogs. *Acta biol. Cracov.* 14, 17–32.
- Lai, S.J., Kam, Y.C., Lin, Y.S., 2003. Elevational variation in reproductive and life history traits of Sauter's frog *Rana sauteri* Boulenger, 1909 in Taiwan. *Zool. Stud.* 42, 193–202.
- Lai, Y.C., Lee, T.H., Kam, Y.C., 2005. A skeletochronological study on a subtropical, riparian ranid (*Rana swinhoana*) from different elevations in Taiwan. *Zool. Sci.* 22, 653–658.

- Leskovar, C., Oromi, N., Sanuy, D., Sinsch, U., 2006. Demographic life history traits of reproductive natterjack toads (*Bufo calamita*) vary between northern and southern latitudes. *Amphibia-Reptilia* 27, 365-375.
- Leskovar, C., Wagner, T., Sinsch, U., 2004. Saisonale Dynamik des Nahrungsspektrums syntoper Kreuz- (*Bufo calamita*) und Wechselkröten-Populationen (*B. viridis*) in einem rheinischen Auskiesungsgebiet. *Z. Feldherpetol.* 11, 83-103.
- Liao, W.B., Lu, X., 2010a. A skeletochronological estimation of age and body size by the Sichuan torrent frog (*Amolops mantzorum*) between two populations at different altitudes. *Animal Biology* 60, 479-489.
- Liao, W.B., Lu, X., 2010b. Age structure and body size of the Chuanxi Tree Frog *Hyla annectans chuanxiensis* from two different elevations in Sichuan (China). *Zool. Anz.* 248, 255-263.
- Liao, W.B., Zhou, C.Q., Yang, Z.S., Hu, J.C., Lu X., 2010. Age, size and growth in two populations of the dark-spotted frog *Rana nigromaculata* at different altitudes in southwestern China. *Herpetol. J.* 20, 77-82.
- Lind, M. I., Ingvarsson, P.K., Johansson, H., Hall, D., Johansson, F., (2011). Gene flow and selection on phenotypic plasticity in an island system of *Rana temporaria*. *Evolution* 65, 684-697.
- Lu, X., Li, B., Liang, J.J., 2006. Comparative demography of a temperate anuran, *Rana chensinensis*, along a relatively fine elevational gradient. *Can. J. Zool.* 84, 1789-1795.
- Ma, X., Lu X., Merilä, J., 2009a. Altitudinal decline of body size in a Tibetan frog. *J. Zool.* 279, 364-371.
- Ma, X., Tong, L.N., Lu, X., 2009b. Variation of body size, age structure and growth of a temperate frog, *Rana chensinensis*, over an elevational gradient in northern China. *Amphibia-Reptilia* 30, 111-117.
- Marangoni, F., 2006. Variación clinal en el tamaño del cuerpo a escala microgeográfica en dos especies de anuros (*Pelobates cultripes* y *Bufo calamita*). PhD Thesis, University of Seville, Sevilla.

- Marquis, O., Miaud, C., 2008. Variation in UV sensitivity among common frog *Rana temporaria* populations along an altitudinal gradient. *Zoology* 111, 309-317.
- Matthews, K.R., Miaud, C., 2007. A skeletochronological study of the longevity and age structure of the mountain yellow-legged frog, *Rana muscosa*, in the Sierra Nevada, California. *Copeia*, 986-993.
- Miaud, C., Guyétant, R., Elmberg, J., 1999. Variations in life-history traits in the common frog *Rana temporaria* (Amphibia: Anura): a literature review and new data from the French Alps. *J. Zool.* 249, 61-73.
- Morrison, C., Hero, J.M., 2003. Geographic variation in life history characteristics of amphibian: a review. *J. Anim. Ecol.* 72, 270-279.
- Ninyerola, M., Pons, X., Roure, J.M., 2005. Atlas Climático Digital de la Península Ibérica. Metodología y aplicaciones en bioclimatología y geobotánica. Universidad Autónoma de Barcelona, Bellaterra.
- Oromi, N., Sanuy, D., Sinsch, U., 2010. Thermal ecology of natterjack toads (*Bufo calamita*) in a semi-arid landscape. *J. Therm. Biol.* 35, 34-40.
- Price T.D., Qvarnström, A., Irwin D.E., 2003. The role of phenotypic plasticity in driving genetic evolution. *Proc. R. Soc. Lond. B* 270, 1433-1440.
- Pujol, B., Wilson, A.J., Ross, R.I.C., Pannell, J.R., 2008. Are $Q_{ST}-F_{ST}$ comparisons for natural populations meaningful? *Molecular Ecology* 17, 4782-4785.
- Richter-Boix, A., Teplitsky, C., Rogell, B., Laurila, A., 2010. Local selection modifies phenotypic divergence among *Rana temporaria* populations in the presence of gene flow. *Mol. Ecol.* 19, 716-731.
- Roff, D.A., 2001. Life History Evolution. Sinauer Associates, Sunderland, MA.
- Ryser, J., 1996. Comparative life histories of a low and a high-elevation population of the common frog *Rana temporaria*. *Amphibia-Reptilia* 17, 183-195.

- Schabetsberger, R., Langer, H., Jersabek, D., Goldschmid, A. 2000. On age structure and longevity in two populations of *Bufo bufo* (Linnaeus, 1758), at high altitude breeding sites in Austria (Anura: Bufonidae). *Herpetozoa* 13, 187-191.
- Sinsch, U., 2008. *Bufo calamita* Laurenti, 1768 - Kreuzkröte. In: Grossenbacher, K. (ed.) *Handbuch der Amphibien und Reptilien Europas*. Aula Verlag. 5/1, Wiesbaden, pp. 339-413
- Sinsch, U., Leskovar, C., 2011. Does thermoregulatory behaviour of green toads (*Bufo viridis*) constrain geographical range in the west? A comparison with the performance of syntopic natterjacks (*B. calamita*). *J. Therm. Biol.* 36, 346-354.
- Sinsch, U., Marangoni, F., Oromi, N., Leskovar, C., Sanuy, D., Tejedo, M., 2010. Proximate mechanisms determining size variability in natterjack toads. *J. Zool.* 4, 272-281.
- Sinsch, U., Oromi, N., Sanuy, D., 2007. Growth marks in natterjack toad (*Bufo calamita*) bones: histological correlates of hibernation and aestivation periods. *Herpetol. J.* 17, 129-137.
- Sinsch, U., Oromi, N., Miaud, C., Denton, J., Sanuy, D., 2011. Connectivity of local *Bufo calamita* populations: modelling the migratory range of radio-tracked toads. *Anim. Conserv.* (submitted).
- Sultan, S.E., Spencer, H.G., 2002. Metapopulation structure favors plasticity over local adaptation. *Am. Nat.* 160, 271-283.
- Tejedo, M., Reques, R., Esteban, M., 1997. Actual and osteochronological estimated age of natterjack toads (*Bufo calamita*). *Herpetol. J.* 7, 81-82.

Genetic variability of geographical populations of the natterjack toad (*Bufo calamita*)

In preparation

Authors: OROMI, N.; RICHTER-BOIX, A; SANUY, D; FIBLA, J.

The variation of suitable habitats across altitudinal and latitudinal gradients can influence on the dispersal capacities of individuals that ultimately can cause differentiation among population. In fact, different selective pressures acting on local environments can promote genetic differentiation, which may derive in a local adaptation. However, the historical events experienced by a population can also drive local differentiation. In this last chapter, genetic variation of *Bufo calamita* among a global European distribution was analysed using polymorphic microsatellite markers. Genetic variation was estimated using diversity parameters, such as mean number of alleles per locus and expected heterozygosity, obtained from our results and those published in the literature in order to analyse the genetic differences among populations in an altitudinal gradient, and to evaluate the consequences of mountains as barriers across European distribution.

Author contribution: First authorship reflects that I was the main contributor to the paper. I have done the sampling and obtained the genetic data of all populations. I have analysed the data and written the paper with Dr. Joan

Fibla. Drs Alex Richter-Boix and Delfi Sanuy contributed to in the analysis interpretation and the elaboration of the manuscript.

ABSTRACT

Across altitudinal and latitudinal gradients, the proportion of suitable habitats varies, influencing the individual dispersal that ultimately can produce differentiation among population. The natterjack toad (*Bufo calamita*) is distributed across a wide geographical range that qualifies the species as interesting for a geographic analysis of its genetic variability. Five populations of *Bufo calamita* in the Sierra de Gredos (Spain) has been studied in an altitudinal gradient ranging from 750 to 2270 m asl using microsatellite markers. In addition, we analysed the latitudinal genetic variation of *B. calamita* within a global European distribution by using genetic diversity parameters (mean number of alleles per locus; M_a and expected heterozygosity; H_E) obtained from our results and those published in the literature. The low level of genetic differentiation found between populations of *B. calamita* (F_{st} ranging from 0.0115 to 0.1018) and the decreases in genetic diversity with altitudinal range (M_a from 13.6 to 8.3, H_E from 0.82 to 0.74) can be interpreted by the combined effects of discontinuous habitat, produced mainly by the high slopes barriers, and geographical distance. In the latitudinal gradient, genetic diversity decreases from south to north as a consequence of historical species colonization. We conclude that the genetic variability of *B. calamita* along its wide altitudinal and latitudinal geographic distribution mainly reflects the colonization history of the species after the last glacial period

KEYWORDS

Bufo calamita, microsatellite markers, genetic diversity, altitudinal and latitudinal gradient.

INTRODUCTION

Genetic variation is required for the evolution of populations in response to environmental changes (Reed & Frankham 2003). Environmental factors such as altitude, topography and glacial history may influence genetic variation. Across altitudinal and latitudinal gradients, the proportion of suitable habitats varies, influencing the individual dispersal that ultimately can produce differentiation among population (Palo et al. 2003; Stéphanie et al. 2003). In fact, different selective pressures acting on local environments across altitudinal gradients can promote genetic differentiation, which may derive from a local adaptation (Slatkin 1987; Palo et al. 2003). However, the historical events experienced by a population can also drive local differentiation. In species with a wide geographic distribution and a glacial colonization history, it can be difficult to discriminate between genetic diversity that results from postglacial colonization patterns and genetic differentiation that results from habitat influences (e.g. local selective pressures, recent habitat fragmentation, Allentoft et al. 2009). As these two processes act at very different spatial and temporal scales, they can have different effects on genetic diversity and fitness (Swindell & Bouzat 2006).

In amphibians, metapopulation structures generally have a high gene flow that can preclude complete differentiation between populations over large geographical distances (Brede & Beebee 2004). In fact, the migratory range of species determines its capacity to maintain the genetic cohesion within local populations that favours the persistence of the species in its distribution range (Smith & Green 2005). Thus, the dispersal range, the population size and the genetic relationships between individuals are essential to understanding the evolution of a species (Petit et al. 2001). In this sense, the study of genetic variability is important to determine the levels of genetic differentiation among populations at both geographic and altitudinal distance scales. Genetic

differentiation between populations was positively correlated with geographic distance in several amphibian studies (e.g. Palo et al. 2004; Arioli et al. 2010) at a large scale. The impact of altitudinal gradient on dispersal and gene flow seems to differ between species. For example, genetic variation was negatively correlated with altitude in the frog *Rana luteiventris* (Funk et al. 2005) and in the salamander *Ambystoma macrodactylum* (Giordano et al. 2007) whereas no correlation was detected in *R. chensinensis* (Zhan et al. 2009). Genetic differentiation by geographical (isolation by distance) or altitudinal distance (a combination of isolation by distance and isolation by geographic barriers) evolves over time and arises from the balance of local genetic drift within populations and dispersal of individuals between populations.

The natterjack toad (*Bufo calamita*) is distributed across a wide geographical range (Sinsch 2008) that qualifies the species as interesting for a geographic analysis of its genetic variability. Previous studies of geographic genetic differentiation using polymorphic microsatellite *loci* in *B. calamita* in lowland populations (at 0 to 400 m asl) of the southern Iberian peninsula found no genetic differentiation between populations separated by more than 100 Km (Marangoni 2006). However, species distribution studies covering a broad latitudinal range show a negative correlation between genetic variation and distance from the Iberian Peninsula, which is the Pleistocene glacial refuge from which all extant populations are derived (Beebee & Rowe 2000). In contrast, the altitudinal effect in *B. calamita* genetic variability has not yet been assessed.

The present study analyses the impact of both altitude and geographical distance, in an effort to expand what is known about *B. calamita* genetic variability. We used expected heterozygosity and allelic richness as components of genetic diversity. Some authors consider that allelic richness is an important measure of genetic diversity and a relevance key in conservation programmes (Petit et al. 1998; Simianer, 2005; Foulley & Ollivier, 2006). Allelic diversity is particularly important from a long-term perspective, because the limit of

selection response is mainly determined by the initial number of alleles regardless of the allelic frequencies (Hill & Rasbash 1986) and, because it reflects better past fluctuations in population size. As the maximum altitudinal range of natterjacks distribution is in the mountains of the Iberian Peninsula (at 2,400 in the Sierra de Gredos and 2,540 in the Sierra Nevada; (Sinsch 2008) we chose five populations inhabiting the Sierra de Gredos. In these mountains, the *B. calamita* distribution ranges from 750 to 2270 m asl. The study aimed to (i) characterize the genetic diversity of each population (ii) analyse the genetic differences among populations in an altitudinal gradient, (iii) analyse if mountains constitute a natural barriers for *B. calamita* affecting genetic diversity among populations across an altitudinal gradient, and (iv) evaluate the consequences of mountains as barriers across European distribution, studying its latitudinal genetic variation using genetic diversity parameters obtained from published studies.

MATERIAL AND METHODS

Study sites and population sampling

A total of five populations of natterjack toads (*Bufo calamita*) were studied on the north side of the Sierra de Gredos (Central Iberian System, Spain), following the altitudinal gradient of the mountain: Navaluenga, Nal750; La Dehesa del Barraco, Deh920; La Cedrera, LaC1470; Cavadores, Cav2100; and Navasomera, Nas2300 (Table 1). The climate is Mediterranean with an average mean temperatures range from 6 to 12 °C, with a range between 0 and 2 °C during the coldest months (December, January and February) and 20 to 22 °C during the hottest months (July and August) depending on the altitude (Ninyerola et al. 2005). Precipitation ranges from 1000 mm to >2000 mm (Palacios et al. 2003). The breeding sites of *B. calamita* in the Sierra de Gredos are humid meadows and ponds at lower sites, and glacial lagoons at higher altitudes.

Table 1. Population description.

Population	Coordinates	Altitude [m]	Date of collection	male/female
Navaluenga	40°24'57.33"N 4°40'47.45"W	752	April 2008	32/21
Dehesa del Barraco	40°27'44,53"N 4°38'12,37"W	920	March 2010	39/11
La Cedrera	40°30'33,52"N 4°39'16,41"W	1472	March 2010	35/16
Cavadores	40°16'19.61"N 5°15'12.94"W	2100	May 2009	31/1
Navasomera	40°15'07.31"N 5°15'49.47"W	2270	June 2008	36/5

Toads were captured during nighttime and released *in situ* following sex determination, based on measuring the snout-vent length (SVL). The DNA was extracted from the first phalange of a toe, clipped using the Chelex100 protocol described in (Walsh et al. 1991).

Microsatellite analysis

Genetic analysis was based on the study of eight microsatellite *loci* previously described by Rowe et al. 1997 (*Bcalμ1*, *Bcalμ2*, *Bcalμ3*, *Bcalμ4*, *Bcalμ5*, *Bcalμ6*, *Bcalμ7*) and Rowe & Burke 2001 (*Bcalμ10*). Following the methodology used by (Gomez-Mestre & Tejedo 2004) genotypes at microsatellite polymorphism were determined by polymerase chain reaction (PCR) amplification using fluorescence labelled primers. Briefly, the oligo forward of each set of primers was stained by one of the four FAM, VIC, NED and PET fluorochromes. PCR reaction for each set of primers contained: 2.5 mM MgCl₂, 0.1 mM BSA, 0.25 mM dNTPs, Taq DNA polymerase 1 U, forward and reverse primers 0.25 μM each, and DNA 2 μl (50-150 ng) in 20 μl of PCR buffer 1x. The PCR amplification was done by first denaturing at 94 °C for 5 min, followed by 40 cycles of 1 min at 94

°C, 1 min at 62 °C and 1 min at 72 °C. A final extension step was done by incubating samples during 10 min at 72 °C. Amplified products were resolved by capillary gel electrophoresis on a Genetic Analyser 3130 (ABI-PRISM, Applied Biosystems) using POP-7 polymer. Allele sizes were determined by GeneScan-500 LIZ standard marker (ABI-PRISM, Applied Biosystems). Genotype calls were obtained by GeneMapper Software Version 4.0.

Data analysis

Allele and genotype frequencies, number of alleles per locus, mean number of alleles per locus (M_a), and expected (H_E) and observed (H_o) heterozygosity were estimated using the Micosatellite Toolkit (Park 2001). Allelic richness (AR) was obtained for each population using FSTAT 2.9.3 (Goudet 2001). M_a , AR and H_E were used as indicators of genetic diversity. The influence of altitude on the H_E , AR and M_a was estimated by fitting data to several regression models. We chose the double reciprocal model for H_E , S-curve model for AR and reciprocal-X model for M_a predictions because they provided the best fit, defined as maximum R^2 . The analysis was performed using the statistical package STATGRAPHICS Plus 5.0. Genotype frequencies were tested for conformity to Hardy-Weinberg equilibrium by GENEPOP 3.4 (Raymond & Rousset 1995) using the Markov chain method with 10000 permutations. This package was also used to estimate the fixation F_{st} and R_{st} and to evaluate marker-to-marker genotypic disequilibrium adjusting for Bonferroni correction. Genetic differentiation was estimated using the D_{est} (Jost, 2008) with the SMOGD software (Crawford, 2010).

A molecular analysis of variance (nested AMOVA) was done using ARLEQUIN 3.1 (Excoffier et al. 2006) to compare genetic diversity between and within populations as well as between altitude groups. We considered three groups altitudinal groups: i) less than 1000 m: Nal750 and Deh920, ii) between 1000 and 2000 m: LaC1470 and iii) more than 2000 m: Cav2100 and Nas2300. The geographical and altitudinal pattern of genetic variation was analysed by a

partial Mantel test (10000 permutations) carried out between genetic distance matrices (based on F_{st}) and geographical and altitudinal distance matrices using the PASSAGE v2. Software (Rosenberg & Anderson 2011). A third constant matrix was used in both test (geographical matrix for F_{st} and altitude correlation; altitude matrix for F_{st} and geographical correlation) for accurate the estimation of Mantel test statistics.

Latitudinal genetic variation

In addition to data obtained in the present study, *B. calamita* genetic diversity data was compiled from literature (Table 2). In total, 34 populations from different localities of natterjack toad distribution were included in the data analysis. The mean number of alleles per locus (M_a) and the expected heterozygosity (H_E) were available for all populations and used as estimators of genetic diversity. The influence of latitude on the M_a and on the H_E was estimated by fitting data in two-regression models: the lineal model and the exponential model, which represent to predefined hypothesis. The lineal model is expected when genetic diversity decrease constantly along the latitudinal gradient following an isolation by distance pattern. However, the exponential model is expected in the presence of barriers bursting the continuous decrease of genetic diversity from the glacial refuge across the latitudinal gradient. We also analysed the data separately between the populations from Spain (glacial refuge) and those past the Pyrenees (post glacial colonization) in order to evaluate the expansion of the specie. The analysis was performed using the statistical package STATGRAPHICS Plus 5.0.

Table 2. Geographical variation of genetic diversity represented as Mean number of alleles per locus (M_a), expected (H_E) and observed (H_o) heterozygosity in *Bufo calamita* populations. Microsatellite loci were described by Rowe *et al.* 1997 (*Bcalμ1*, *Bcalμ2*, *Bcalμ3*, *Bcalμ4*, *Bcalμ5*, *Bcalμ6*, *Bcalμ7*), Rowe *et al.* 2000 (*Bcalμ10*, *Bcalμ11*).

Population	M_a	H_E	H_o	Latitude [°N]	Altitude [m]	References	Loci
Abalarío (Spain)	12.50	0.80	0.62	36.06	63	Gómez-Mestre & Tejedo 2004	<i>Bcalμ1</i> , <i>Bcalμ2</i> , <i>Bcalμ3</i> , <i>Bcalμ4</i> , <i>Bcalμ5</i> , <i>Bcalμ7</i> , <i>Bcalμ10</i> , <i>Bcalμ11</i>
RBD (Spain)	12.75	0.81	0.62	37.00	24	Marangoni 2006	<i>Bcalμ1</i> , <i>Bcalμ2</i> , <i>Bcalμ3</i> , <i>Bcalμ4</i> , <i>Bcalμ5</i> , <i>Bcalμ7</i> , <i>Bcalμ10</i> , <i>Bcalμ11</i>
Bodegones (Spain)	12.37	0.80	0.52	37.13	32	Marangoni 2006	<i>Bcalμ1</i> , <i>Bcalμ2</i> , <i>Bcalμ3</i> , <i>Bcalμ4</i> , <i>Bcalμ5</i> , <i>Bcalμ7</i> , <i>Bcalμ10</i> , <i>Bcalμ11</i>
Juncosilla (Spain)	12.00	0.80	0.61	37.17	20	Marangoni 2006	<i>Bcalμ1</i> , <i>Bcalμ2</i> , <i>Bcalμ3</i> , <i>Bcalμ4</i> , <i>Bcalμ5</i> , <i>Bcalμ7</i> , <i>Bcalμ10</i> , <i>Bcalμ11</i>
Toba (Spain)	7.50	0.63	0.53	37.19	950	Gómez-Mestre & Tejedo 2004	<i>Bcalμ1</i> , <i>Bcalμ2</i> , <i>Bcalμ3</i> , <i>Bcalμ4</i> , <i>Bcalμ5</i> , <i>Bcalμ7</i> , <i>Bcalμ10</i> , <i>Bcalμ11</i>
Pedreso (Spain)	11.20	0.82	0.59	37.40	395	Marangoni 2006	<i>Bcalμ1</i> , <i>Bcalμ2</i> , <i>Bcalμ3</i> , <i>Bcalμ4</i> , <i>Bcalμ5</i> , <i>Bcalμ7</i> , <i>Bcalμ10</i> , <i>Bcalμ11</i>
Navas (Spain)	13.00	0.79	0.54	37.40	420	Marangoni 2006	<i>Bcalμ1</i> , <i>Bcalμ2</i> , <i>Bcalμ3</i> , <i>Bcalμ4</i> , <i>Bcalμ5</i> , <i>Bcalμ7</i> , <i>Bcalμ10</i> , <i>Bcalμ11</i>
Sanlúcar (Spain)	9.75	0.76	0.47	37.40	34	Marangoni 2006	<i>Bcalμ1</i> , <i>Bcalμ2</i> , <i>Bcalμ3</i> , <i>Bcalμ4</i> , <i>Bcalμ5</i> , <i>Bcalμ7</i> , <i>Bcalμ10</i> , <i>Bcalμ11</i>
Taraje (Spain)	9.38	0.66	0.63	38.04	420	Gómez-Mestre & Tejedo 2004	<i>Bcalμ1</i> , <i>Bcalμ2</i> , <i>Bcalμ3</i> , <i>Bcalμ4</i> , <i>Bcalμ5</i> , <i>Bcalμ7</i> , <i>Bcalμ10</i> , <i>Bcalμ11</i>
Navasomera (Spain)	8.37	0.74	0.69	40.15	2270	This study	<i>Bcalμ1</i> , <i>Bcalμ2</i> , <i>Bcalμ3</i> , <i>Bcalμ4</i> , <i>Bcalμ5</i> , <i>Bcalμ7</i> , <i>Bcalμ10</i> , <i>Bcalμ11</i>
Cavadores (Spain)	9.15	0.72	0.67	40.16	2100	This study	<i>Bcalμ1</i> , <i>Bcalμ2</i> , <i>Bcalμ3</i> , <i>Bcalμ4</i> , <i>Bcalμ5</i> , <i>Bcalμ7</i> , <i>Bcalμ10</i> , <i>Bcalμ11</i>
Navaluenga (Spain)	13.62	0.82	0.82	40.20	750	This study	<i>Bcalμ1</i> , <i>Bcalμ2</i> , <i>Bcalμ3</i> , <i>Bcalμ4</i> , <i>Bcalμ5</i> , <i>Bcalμ7</i> , <i>Bcalμ10</i> , <i>Bcalμ11</i>
La Dehesa del Barraco (Spain)	11.80	0.77	0.75	40.20	920	This study	<i>Bcalμ1</i> , <i>Bcalμ2</i> , <i>Bcalμ3</i> , <i>Bcalμ4</i> , <i>Bcalμ5</i> , <i>Bcalμ7</i> , <i>Bcalμ10</i> , <i>Bcalμ11</i>
La Cedrera (Spain)	10.50	0.75	0.79	40.30	1470	This study	<i>Bcalμ1</i> , <i>Bcalμ2</i> , <i>Bcalμ3</i> , <i>Bcalμ4</i> , <i>Bcalμ5</i> , <i>Bcalμ7</i> , <i>Bcalμ10</i> , <i>Bcalμ11</i>
Mas de Melons (Spain)	11.38	0.85	0.81	41.30	240	Oromí, Fibla & Sanuy unpublished	<i>Bcalμ1</i> , <i>Bcalμ2</i> , <i>Bcalμ3</i> , <i>Bcalμ4</i> , <i>Bcalμ5</i> , <i>Bcalμ7</i> , <i>Bcalμ10</i> , <i>Bcalμ11</i>
Preixens (Spain)	12.00	0.82	0.82	41.47	326	Oromí, Fibla & Sanuy unpublished	<i>Bcalμ1</i> , <i>Bcalμ2</i> , <i>Bcalμ3</i> , <i>Bcalμ4</i> , <i>Bcalμ5</i> , <i>Bcalμ7</i> , <i>Bcalμ10</i> , <i>Bcalμ11</i>

Batchouta (Spain)	9.80	0.79	0.7	43.14	700	Oromí, Nicieza & Tejedo unpublished	Bcal μ 1, Bcal μ 2, Bcal μ 3, Bcal μ 4, Bcal μ 5, Bcal μ 7, Bcal μ 10, Bcal μ 11
Brittany (France)	4.38	0.49	0.35	47.57	0	Beebee & Rowe 2000	Bcal μ 1, Bcal μ 2, Bcal μ 3, Bcal μ 4, Bcal μ 5, Bcal μ 6, Bcal μ 7, Bcal μ 8
Boulogne (France)	3.88	0.48	0.45	49.39	0	Beebee & Rowe 2000	Bcal μ 1, Bcal μ 2, Bcal μ 3, Bcal μ 4, Bcal μ 5, Bcal μ 6, Bcal μ 7, Bcal μ 8
Ernzen (Luxemburg)	3.90	0.58	0.63	49.48	300	Frantz <i>et al.</i> 2009	Bcal μ 1, Bcal μ 3, Bcal μ 4, Bcal μ 5, Bcal μ 6, Bcal μ 8, Bcal μ 9, Bcal μ 11, Buca2, Buca6
Steinfort (Luxemburg)	3.80	0.57	0.58	49.48	300	Frantz <i>et al.</i> 2009	Bcal μ 1, Bcal μ 3, Bcal μ 4, Bcal μ 5, Bcal μ 6, Bcal μ 8, Bcal μ 9, Bcal μ 11, Buca2, Buca6
FLC (Belgium)	3.67	0.65	0.65	50.05	160	Stevens <i>et al.</i> 2006	Bcal μ 1, Bcal μ 2, Bcal μ 5, Bcal μ 6, Bcal μ 7, Bcal μ 10
MAR (Belgium)	3.83	0.45	0.45	50.05	150	Stevens <i>et al.</i> 2006	Bcal μ 1, Bcal μ 2, Bcal μ 5, Bcal μ 6, Bcal μ 7, Bcal μ 10
MER (Belgium)	3.67	0.48	0.48	50.05	220	Stevens <i>et al.</i> 2006	Bcal μ 1, Bcal μ 2, Bcal μ 5, Bcal μ 6, Bcal μ 7, Bcal μ 10
ROM (Belgium)	3.83	0.57	0.57	50.05	180	Stevens <i>et al.</i> 2006	Bcal μ 1, Bcal μ 2, Bcal μ 5, Bcal μ 6, Bcal μ 7, Bcal μ 10
Kerry (Ireland)	2.38	0.34	0.33	51.52	0	Beebee & Rowe 2000	Bcal μ 1, Bcal μ 2, Bcal μ 3, Bcal μ 4, Bcal μ 5, Bcal μ 6, Bcal μ 7, Bcal μ 8
Poland	2.00	0.24	0.28	52.06	0	Beebee & Rowe 2000	Bcal μ 1, Bcal μ 2, Bcal μ 3, Bcal μ 4, Bcal μ 5, Bcal μ 6, Bcal μ 7, Bcal μ 8
Ooy-Polder (Holland)	5.13	0.52	0.46	52.29	0	Beebee & Rowe 2000	Bcal μ 1, Bcal μ 2, Bcal μ 3, Bcal μ 4, Bcal μ 5, Bcal μ 6, Bcal μ 7, Bcal μ 8
E/SE (England)	2.50	0.35	0.3	52.35	0	Beebee & Rowe 2000	Bcal μ 1, Bcal μ 2, Bcal μ 3, Bcal μ 4, Bcal μ 5, Bcal μ 6, Bcal μ 7, Bcal μ 8
Texel (Holland)	2.63	0.36	0.43	53.08	0	Beebee & Rowe 2000	Bcal μ 1, Bcal μ 2, Bcal μ 3, Bcal μ 4, Bcal μ 5, Bcal μ 6, Bcal μ 7, Bcal μ 8
Merseyside (England)	2.63	0.29	0.29	53.44	0	Beebee & Rowe 2000	Bcal μ 1, Bcal μ 2, Bcal μ 3, Bcal μ 4, Bcal μ 5, Bcal μ 6, Bcal μ 7, Bcal μ 8
Cumbria (England)	3.75	0.39	0.34	55.10	0	Beebee & Rowe 2000	Bcal μ 1, Bcal μ 2, Bcal μ 3, Bcal μ 4, Bcal μ 5, Bcal μ 6, Bcal μ 7, Bcal μ 8
Denmark	3.90	0.30		55.22	0	Beebee & Rowe 2000	Bcal μ 1, Bcal μ 2, Bcal μ 3, Bcal μ 4, Bcal μ 5, Bcal μ 6, Bcal μ 7, Bcal μ 8
Sweden	1.63	0.20	0.14	58.41	293	Beebee & Rowe 2000	Bcal μ 1, Bcal μ 2, Bcal μ 3, Bcal μ 4, Bcal μ 5, Bcal μ 6, Bcal μ 7, Bcal μ 8

RESULTS

Population genetic diversity

The 8 microsatellite markers used were polymorphic in all populations and the number of alleles per *locus* varied from 14 for *Bcalμ7* at 36 for *Bcalμ4*. We identified a total of 174 different alleles; 32 were present in all populations (common alleles) and 72 were observed in unique populations (private alleles). H_E ranged from 0.44 at Cav2100 to 0.93 at Nal750 (Table 3), very close to H_o values. Deviation from Hardy-Weinberg equilibrium was observed for *Bcalμ3* in the Cav2100 population, caused by heterozygote deficiency that could be explained by non-amplifying alleles. After Bonferroni correction for multiple tests, a significant deviation from genotypic linkage equilibrium was found in 6 of the total 139 *locus*-pair tested (data not show).

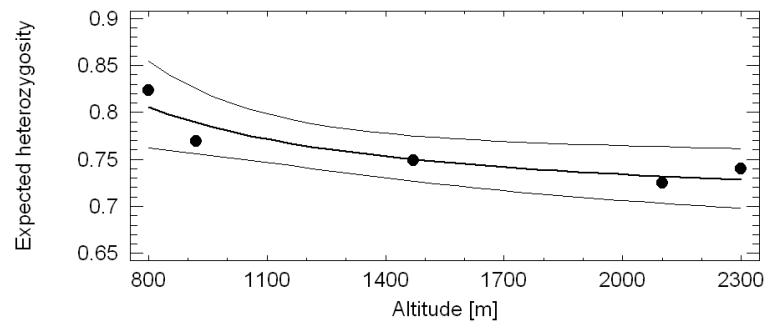
Table 3. Genetic diversity at sampling sites.

Locus	Nal750			Deh920			LaC1470			Cav2100			Nas2300		
	NA(#e/#)	H_E	H_o	NA(#e/#c)	H_E	H_o	NA(#e/#c)	H_E	H_o	NA(#e/#c)	H_E	H_o	NA(#e/#c)	H_E	H_o
<i>Bcalμ1</i>	17 (4/13)	0.93	0.85	13 (1/12)	0.84	0.65	10 (0/10)	0.75	0.58	11 (1/10)	0.80	0.56	12 (0/12)	0.89	0.69
<i>Bcalμ2</i>	12 (2/10)	0.82	0.74	12 (3/9)	0.80	0.49	13 (2/11)	0.72	0.76	6 (1/5)	0.45	0.28	2 (0/2)	0.51	0.29
<i>Bcalμ3</i>	12 (4/8)	0.70	0.72	7 (2/5)	0.66	0.78	9 (2/7)	0.76	0.75	7 (1/6)	0.64	0.78	5 (0/5)	0.58	0.60
<i>Bcalμ4</i>	18 (2/16)	0.91	0.87	21 (4/17)	0.89	0.98	16 (3/13)	0.86	0.92	15 (1/14)	0.91	0.90	16 (6/10)	0.90	0.74
<i>Bcalμ5</i>	13 (3/10)	0.82	0.85	12 (3/9)	0.75	0.85	11 (2/9)	0.79	0.93	10 (1/9)	0.76	0.52	10 (2/10)	0.79	0.78
<i>Bcalμ6</i>	18 (3/15)	0.94	0.83	12 (1/11)	0.86	0.66	12 (2/10)	0.76	0.68	17 (1/16)	0.92	0.87	15 (1/14)	0.93	0.84
<i>Bcalμ7</i>	8 (2/6)	0.67	0.77	10 (3/7)	0.57	0.67	7 (1/6)	0.61	0.88	4 (1/3)	0.55	0.71	3 (0/3)	0.62	0.89
<i>Bcalμ10</i>	11 (3/9)	0.81	0.92	8 (2/6)	0.79	0.98	6 (0/6)	0.73	0.90	8 (2/6)	0.77	0.72	4 (0/4)	0.70	0.69
Total	109			175			95			78			67		

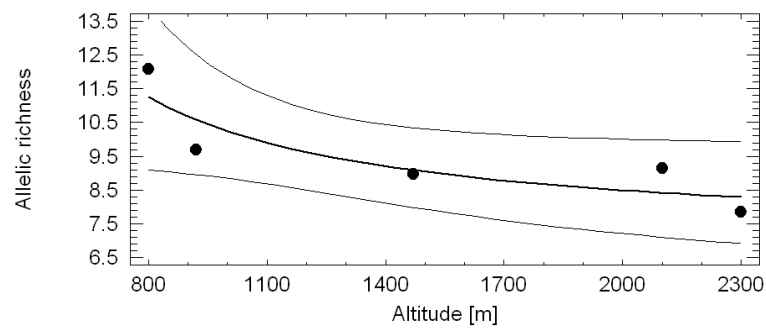
NA, number of alleles; #e, number of specific alleles; #c, number of common alleles; H_E , expected heterozygosity; H_o , observed heterozygosity.

Altitude correlated significantly and inversely with the parameters of genetic diversity, explaining the 85.02% of variance in percentage of H_E [$H_E = 1/(1.44276 + 161.275/\text{Altitude})$; $r = -0.922$, $P = 0.026$, Fig.1a], the 77.01% of variance in AR [$AR = \exp(1.95193 + 374.979/\text{Altitude})$; $r = 0.877$, $P = 0.05$; Fig.1b] and the 92.55% of variance in M_a [$M_a = 6.70675 + 5278.81/\text{Altitude}$; $r = 0.96$, $P = 0.008$; Fig.1c].

a)



b)



c)

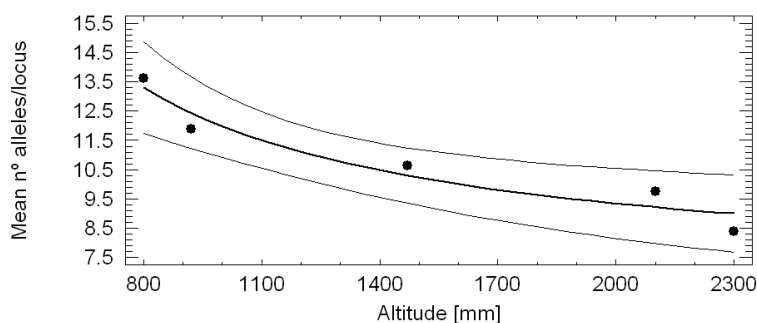


Figure 1. Genetic diversity and altitude relationship. Mean expected heterozygosity (a), Allelic richness (b) and Mean number of alleles per locus (c) for each population. Each point represents a population sample.

Results from AMOVA indicated a lack of population structure, with a 94.95% of overall variation within populations (Table 4). Differences between populations measured by F_{st} and R_{st} were low, ranging from 0.0115 to 0.1018 and 0.0062 to 0.1148, respectively. The measures obtained using D_{est} , ranging from 0.0124 to 0.2525, showed also low differences in allelic diversity between populations. The partial Mantel test used to evaluate the correlation of F_{st} matrices with geographic and altitudinal distance matrices, showed significant correlations of F_{st} ($r = 0.75$, $t=2.48$, $P_{two-tailed} = 0.013$) with geographic distance according to the hypothesis of isolation by distance. In addition, F_{st} ($r = -0.65$, $t=-2.22$, $P_{two-tailed} = 0.025$) correlated inversely with altitude.

Table 4. Molecular analysis of variance AMOVA for proportion of total variance accounted for by each source.

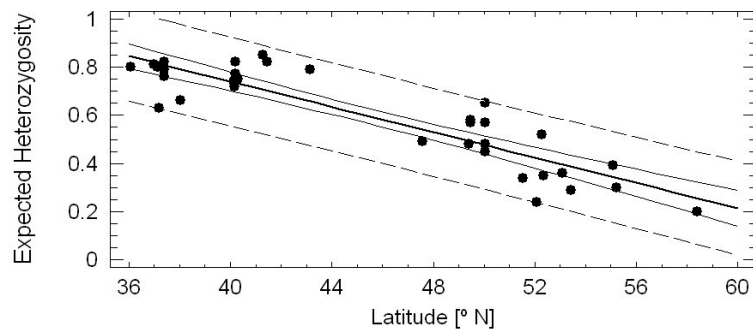
Source	df	SS	Varcomp	%Var
Among groups	2	23.66	0.053	2.69
Among populations within groups	2	10.58	0.046	2.35
Within populations	369	693.90	1.880	94.96
Total	373	728.15	1.980	100

df, degree freedom; SS, sum of squares; Varcomp, variance components and %Var, proportion of total variance accounted by each source.

Latitudinal genetic variation

In the 34 European populations distributed in a south-north latitudinal gradient, H_E correlated inversely with latitude, explaining 86.21% of the variance in percentage [regression model: $H_E = 1,79064 - 0,0262712*\text{latitude}$; $r = -0.902$, $P < 0.00001$; Fig. 2a]. M_a also showed an inversely significant relationship with latitude, explaining 81.35% of the variance [regression model: $M_a = \exp(5.78897 - 0.0883939*\text{latitude})$; $r = -0.928$, $P < 0.00001$; Fig. 2b]. The separately analysis considering the Iberian Peninsula as the glacial refuge showed that H_E and M_a not varied significantly with the latitude in the Iberian populations (H_E , lineal regression model: $r = 0.17$, $P = 0.51$, Fig 3a; M_a exponential regression model: $r = -0.16$, $P = 0.53$, Fig 3b) whereas these parameters correlated inversely across the latitudinal gradient since the barrier of the Pyrenees to the north [H_E regression model: $H_E = \exp(4,0582 - 0,0958323*\text{latitude})$, $r = -0.77$, $P = 0.002$, $R^2 = 60.37\%$, Fig. 3a; M_a regression model: $M_a = \exp(4,47651 - 0,0637442*\text{latitude})$, $r = -0.56$, $P = 0.017$, $R^2 = 32.24\%$, Fig. 3b]. Our results emphasise a decrease in genetic diversity at the glacial barrier to north latitudinal gradient in the European *B. calamita* distribution.

a)



b)

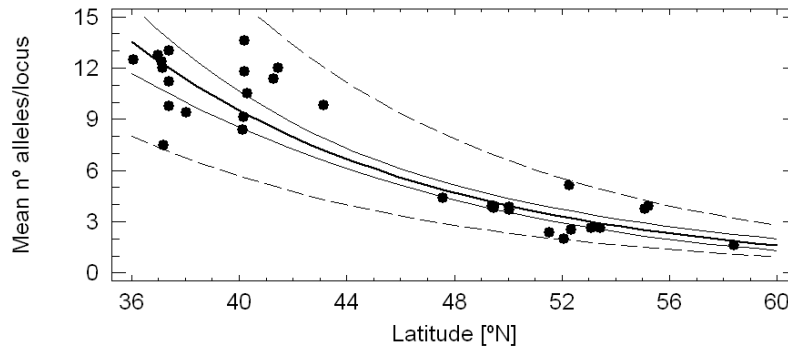
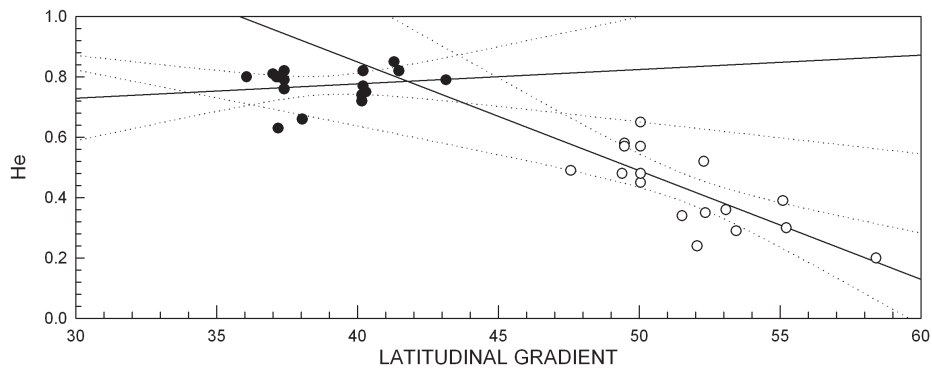


Figure 2. Genetic diversity and latitude relationship. Mean expected heterozygosity (a) and Mean number of alleles per locus (b) for each population. Each point represents a population of this study and compiled from literature (see Table 2 for details).

DISCUSSION

Amphibians are considered particularly vulnerable to environmental changes as a consequence of their low capacity for dispersal (Blaustein et al. 1994). Genetic studies in amphibians have been mainly focused in locally distributed populations with a high risk of isolation, especially those living in fragmented habitats, which can be at risk of extinction from demographic, environmental and genetic stochasticity (reviewed in Allentoft & O'Brien 2010). Nevertheless, the effects of environmental factors such as altitude and latitude on genetic differentiation can only be addressed by analysing data from species with a wide geographical range of distribution, as is the case of *Bufo calamita*. Our study is the first to analyse the genetic variation of *B. calamita* across broad altitudinal and latitudinal ranges. First we demonstrated how altitude constitutes a barrier decreasing genetic diversity from low altitudes to high altitudes, and second how the Pyrenees constituted a natural barrier across a European latitudinal gradient, demonstrating the relevance of mountain systems as barriers for amphibian populations.

a)



b)

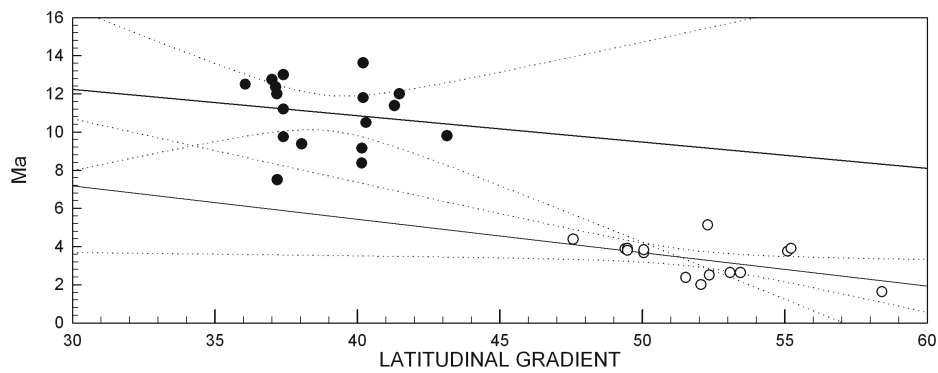


Figure 3. Genetic diversity and latitude relationship. The separately analysis considering the Iberian Peninsula as the glacial refuge. Mean expected heterozygosity (a) and Mean number of alleles per locus (b). Each point (black: Iberian populations, white: northern populations) represents a population.

Altitudinal variation and population genetic diversity

Our results show that altitude is inversely correlated with genetic diversity of *B. calamita* populations in the Sierra de Gredos. Geographic isolation at high altitudes and the low number of individuals per population that we observed could explain these results. Despite the decrease in genetic diversity found in highland populations of the Sierra de Gredos, H_E and M_a values are close to those

observed in lowland populations of the south Iberian Peninsula (Gomez-Mestre & Tejedo 2004; Marangoni 2006). In addition, the AMOVA results in our study indicate a lack of population structure. In the Sierra de Gredos, the landscape features seem to be adequate for gene flow between Gredos populations, influencing genetic variations within and between populations (Stéphanie et al. 2003). Therefore, the effects of altitude in the genetic population structure seem to be minor, as suggested by the high gene flow observed between populations which can induce an incomplete separation of populations over large geographical (Brede & Beebee 2004) and altitudinal (this study) distances.

Despite this lack of genetic structure, we have found significant correlations between genetic distance and altitudinal and geographical distance. We noted that, in addition to geographic distance, the altitude distance analysed in our study included some intrinsic variables such as temperature, slope and forest cover. Therefore, we suggest that the levels of genetic differentiation among populations of *B. calamita* in the altitudinal range of Sierra de Gredos are due to the combined effects of discontinuous habitat, produced mainly by the high slopes barriers, and geographical distance. As Marangoni (2006) did not find differences between populations in a geographical distance of more than 100 Km in a lowland area, we can consider the differences found in our study to be mainly contributed by the altitudinal gradient.

Latitudinal genetic differentiation

Genetic diversity of Sierra de Gredos populations differs from that of other European regions. Following the genetic characteristics found in the Iberian Peninsula (Gomez-Mestre & Tejedo 2004; Marangoni 2006), Gredos populations had higher allelic diversity than the same species in Europe (Rowe et al. 1998; Beebee & Rowe 2000). Whereas in Gredos populations the M_a ranged between 8.38 and 13.63, populations in northern Europe ranged between 1.63 and 5.13 (Beebee & Rowe 2000; Allentoft et al. 2009; Frantz et al. 2009). It was notable that genetic diversity in the extremes of species distribution is low, e.g. M_a in

Poland was 2, despite the fact that populations in these areas are numerous and large (Beebee & Rowe 2000). Populations are more isolated in the peripheral regions of the *B. calamita* distribution area (Beebee 1983) and the genetic variation decreased as a result of increased population differentiation (Petit et al. 2001). These results have been supported in studies about migratory ranges carried out in populations from Britain, which considered that these populations cannot maintain connectivity of neighbouring local populations (Sinsch et al. subm). On the contrary, metapopulation dynamics seem to be much greater in populations from the Iberian Peninsula (Sinsch et al. subm) with high genetic diversity and gene flow (Marangoni 2006).

As we noted, the Ma and H_E variables showed a decrease in genetic diversity in the latitudinal gradient of *B. calamita* distribution. This difference is especially exemplified with Ma , which shows a relatively high range of variation in the populations from Spain (range: 7.5-13.65) with no apparent latitudinal influence. On the contrary, in the populations from north of the Pyrenees to Sweden (at 49 to 58.5 °N) genetic variability decreases in the latitudinal gradient (range: 1.63-5.13). These results are consistent with the hypothesis that the Iberian Peninsula was a refuge for *B. calamita* during the Pleistocene, where all populations expanded from the south to the north during the postglacial period (Beebee & Rowe 2000).

In conclusion, the range of genetic variability of *B. calamita* showed throughout its geographic distribution area mainly reflects the colonization history of the species after the last glacial period. This was suggested by previous studies in the latitudinal gradient (Beebee & Rowe 2000) and by the present study at both latitudinal and altitudinal gradients. Despite this fact, the studies of genetic structure (this study) and home range (Sinsch et al. subm) evidenced that some populations can be genetically isolated by distance and prone to local extinction.

ACKNOWLEDGEMENTS

The permissions for field studies and handling of the toads were issued by the environmental agency responsible for the Sierra de Gredos (Consejería de Medio Ambiente, Junta de Castilla y León). The project was financed by Spain's Ministry of Science and Innovation and the regional administration (Diputació) of the province of Lleida.

REFERENCES

- Allentoft, M. E., H. R. Siegismund, L. Briggs, and L. W. Andersen. 2009. Microsatellite analysis of the natterjack toad (*Bufo calamita*) in Denmark: populations are islands in a fragmented landscape. *Conservation Genetics* **10**:15–28.
- Allentoft, M. E., and J. O'Brien. 2010. Global Amphibian Declines, Loss of Genetic Diversity and Fitness: a Review. *Diversity* 47–71.
- Arioli, M., C. Jakob, and H. Reyer. 2010. Genetic diversity in water frog hybrids (*Pelophylax esculentus*) varies with population structure and geographic location. *Molecular Ecology* **19**:1814–1828.
- Beebee, T. J. C. 1983. *The Natterjack Toad*. Oxford University Press, Oxford, UK:1–1.
- Beebee, T., and G. Rowe. 2000. Microsatellite analysis of natterjack toad *Bufo calamita* Laurenti populations: consequences of dispersal from a Pleistocene refugium. *Biological Journal of the Linnean Society* **69**:367–381.
- Blaustein, A., D. Wake, and W. Sousa. 1994. Amphibian Declines - Judging Stability, Persistence, and Susceptibility of Populations to Local and Global Extinctions. *Conservation biology: the journal of the Society for Conservation Biology* **8**:60–71.
- Brede, E., and T. Beebee. 2004. Contrasting population structures in two sympatric anurans: implications for species conservation. *Heredity* **92**:110–117.
- Crawford NG. 2010. SMOGD: software for the measurement of genetic diversity. *Molecular Ecology Resources*, 10, 556-557.

- Excoffier, L., G. Laval, and S. Schneider. 2006. Arlequin (version 3.1): an integrated software package for population genetics data analysis. Computational and Molecular Population Genetics Laboratory, University of Berne, Switzerland:1-4.
- Frantz, A., R. Proess, T. Burke, and L. Schley. 2009. A genetic assessment of the two remnant populations of the natterjack toad (*Bufo calamita*) in Luxembourg. Herpetological Journal **19**:53-59.
- Foulley, J. L., and L. Ollivier. 2006. Estimating allelic richness and its diversity. Livestock Science **101**: 150-158.
- Funk, W. C., M. S. Blouin, P. S. Corn, B. A. Maxell, D. S. Pilliod, S. Amish, and F. W. Allendorf. 2005. Population structure of Columbia spotted frogs (*Rana luteiventris*) is strongly affected by the landscape. Molecular Ecology **14**:483-496.
- Giordano, A. R., B. J. Ridenhour, and A. Storfer. 2007. The influence of altitude and topography on genetic structure in the long-toed salamander (*Ambystoma macrodactylum*). Molecular Ecology **16**:1625-1637.
- Gomez-Mestre, I., and M. Tejedo. 2004. Contrasting patterns of quantitative and neutral genetic variation in locally adapted populations of the natterjack toad, *Bufo calamita*. Evolution **58**:2343-2352.
- Goudet, J. 2001. FSTAT, a program to estimate ant test gene diversities and fixation indices (Version 2.9.3) Lausanne University. Lausanne, Switzerland.
- Hill, W.G., and J. Rasbash. 1986. Models of long term artificial selection in finite populations. Genet Res 48, 41-50.
- Jost, L. 2008. Gst and its relatives do not measure differentiation. Molecular Ecology **17**, 4015-4026.
- Marangoni, F. 2006. Variación clinal en el tamaño del cuerpo a escala microgeográfica en dos especies de anuros (*Pelobates cultriples* y *Bufo calamita*). PhD Tesis, University of Sevilla, Spain:1-299.

- Ninyerola, M., X. Pons, and J. Roure. 2005. Atlas climático digital de la Península Ibérica. Metodología y aplicaciones en bioclimatología y geobotánica Universidad Autónoma de Barcelona. Bellaterra.
- Palacios, D., R. Garcia, V. Rubio, and R. Vigil. 2003. Debris flows in a weathered granitic massif: Sierra de Gredos, Spain. *Catena* **51**:115–140.
- Palo, J., R. O'Hara, A. Laugen, A. Laurila, C. Primmer, and J. Merila. 2003. Latitudinal divergence of common frog (*Rana temporaria*) life history traits by natural selection: evidence from a comparison of molecular and quantitative genetic data. *Molecular Ecology* **12**:1963–1978.
- Palo, J. U., D. S. Schmeller, A. Laurila, C. R. Primmer, S. L. Kuzmin, and J. Merila. 2004. High degree of population subdivision in a widespread amphibian. *Molecular Ecology* **13**:2631–2644.
- Park, S. 2001. Trypanotolerance in West African Cattle and the Population Genetic Effects of Selection PhD Thesis, University of Dublin. Ireland.
- Petit R.J., El Mousadik A. and O., Pons. 1998. Identifying populations for conservation on the basis of genetic markers. *Conserv Biol* **12**, 844-855.
- Petit, E., F. Ballouxand, and J. Goudet. 2001. Sex-biased dispersal in a migratory bat: A characterization using sex-specific demographic parameters. *Evolution* **55**:635–640.
- Raymond, M. and F. Rousset. 1995. GENEPOP (Version 1.2)- Population-genetics software for exact tests and ecumenicism. *Journal of Heredity* **86**:248–249.
- Reed, D., and R. Frankham. 2003. Correlation between fitness and genetic diversity. *Conservation biology* **17**:230–237.
- Rosenberg, M.S. and C.D., Anderson. 2011. PASSaGE: Pattern Analysis, Spatial Statistics and Geographic Exegesis. Version 2. *Methods in Ecology and Evolution* **2**:229-232.
- Rowe, G., T. Beebee and T. Burke. 1997. PCR primers for polymorphic microsatellite loci in the anuran amphibian *Bufo calamita*. *Molecular Ecology* **6**:401–402.

- Rowe, G., T. Beebee and T. Burke. 1998. Phylogeography of the natterjack toad *Bufo calamita* in Britain: genetic differentiation of native and translocated populations. *Molecular Ecology* **7**:751–760.
- Rowe, G., and T. B. T. Burke. 2001. A further four polymorphic microsatellite loci in the natterjack toad *Bufo calamita*. *Conservation Genetics* **1**:371–372.
- Simianer H., 2005. Using expected allele number as objective function to design between and within breed conservation of farm animal biodiversity. *J Anim Breed Genet* **122**, 177-187.
- Sinsch, U. 2008. *Bufo calamita* Laurenti, 1768. (K. Grossenbacher, Ed.) Aula Verlag. Wiesbaden.
- Sinsch, U., N. Oromi, Miaud C., Denton J. and Sanuy, D. 2011 Connectivity of local *Bufo calamita* populations: modelling the migratory range of radio-tracked toads. *Animal Conservation*. Submitted manuscript.
- Slatkin, M. 1987. Gene Flow and the Geographic Structure of Natural Populations. *Science*:287–792.
- Smith, M., and D. Green. 2005. Dispersal and the metapopulation paradigm in amphibian ecology and conservation: are all amphibian populations metapopulations? *Ecography* **28**:110–128.
- Stéphanie, M., M. K. Schwartz, G. Luikart, and P. Taberlet. 2003. Landscape genetics: combining landscape ecology and population genetics. *Trends in Ecology & Evolution* **18**:189–197.
- Swindell, W.R., and J. L. Bouzat. 2006. Ancestral inbreeding reduces the magnitude of inbreeding depression in *Drosophila melanogaster*. *Evolution* **60**:762–767
- Walsh, P. S., D. A. Metzger, and R. Higuchi. 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *BioTechniques* **10**:506–513.

Zhan, A., C. Li, and J. Fu. 2009. Big mountains but small barriers: Population genetic structure of the Chinese wood frog (*Rana chensinensis*) in the Tsinling and Daba Mountain region of northern China. *BMC Genetics* **10**:17.

GENERAL DISCUSSION

Life history traits co-vary with altitude and latitude (Morrison & Hero, 2003). Variation can be due to multiple proximate causes ranging from genetic adaptation to phenotypic plasticity (Roff, 2001). This study about the variation of life history traits among different populations of *Bufo calamita* across its wide geographical distribution, has allowed to evaluate for the first time the mechanisms involved in this variation in a single species covering the complete latitudinal and altitudinal range.

The distribution of amphibians is constrained by their thermal requirements, which limits the extension towards lower and higher altitudes and southern and northern latitudes by constraining life history traits such as growth rate, fecundity and age (Morrison & Hero, 2003). In terrestrial toads, the strategies used to reduce ambient temperature variation in order to maintain body temperature in a viable range, are mainly based on the selection of microhabitats as in the case of *Bufo calamita* in Spain (Oromi et al., 2010; Chapter 1) and in Germany (Leskovar & Sinsch, 2001; Sinsch & Leskovar, 2011). In fact, the properties of local shelter microhabitats determine the thermal ecology of natterjack toads and their migratory ranges (Sinsch et al., subm., Chapter 2). Individuals of *B. calamita* inhabiting the semiarid zones of Mas de Melons have different thermoregulatory behaviour and migratory ranges depending on the water availability in the different season periods. The summers in semiarid zones are dry and the conditioning factor for toads during this period is the lack of moisture. Consequently, toads select moist shelters associated, in the Valley of Ebro River, with relatively low temperatures. In fact, toads do not thermoregulate during summer; the low variation in their body temperature reflexes the conditions of the choice shelter that is a simple consequence of hydroregulation. In contrast, winters are humid enough to avoid

dehydration stress. In this period, toads are thermal conformers with thermoregulatory behaviour restricted to the avoidance of lower lethal temperatures.

But, do thermoregulatory traits differ between natterjacks inhabiting moist sand habitats and those of semiarid areas? Some studies carried out in Germany in moist sand environment (Sinsch, 1998; Sinsch & Leskovar, 2011) show a superior level of summer body temperature (37.4 °C) than those in Mas de Melons (33.7 °C). This divergence is not due to significant differences in Critical Thermal Maxima, it is a consequence of shelter occupied. Whereas toads from German population burrow in moist sand, the dry and clay soil of semiarid zone of Spain prevented the toads from active burrowing. The only structures offering protection against temperature and dehydration stress are cavities in stone embankments bordering fields and farm tracks.

This fact also affects the migratory range of natterjack toads that varies depending to the surface substrate (Sinsch et al., *subm.*; Chapter 2). Individuals inhabiting the clay dominated localities from Spain, Mas de Melons and Balaguer, move more than those living in habitats that permit burrowing from sandy and moistly substrates of central and northern European populations. The type of summer shelter constrains the upper limit of core temperature because burrowed toads combine osmotic water uptake with absence of evaporative water loss, while toads within cavities are at least partially exposed to air flow and subsequent evaporative cooling (Hutchison & Dupré, 1992; Bartelt & Peterson, 2005; Sinsch & Lekovar, 2011). In addition, cavity shelters dry up faster than shelters in sandy soils. For this reason, contrarily of toads in clay soils, toads in moist shelter are not in the need of frequent movements to keep water economy balanced. These results suggest that the thermoregulatory behaviour of natterjack toads exhibits phenotypic plasticity in relation to shelter choice and corresponding maximum core temperatures during summer. During winter, temperature decreases, water availability increases and the dehydration

stress is a minor problem for toads. Whereas toads in Germany need to avoid low temperatures, toads in the semiarid zone of Mas de Melons, where winters are mild, are general conformers with air temperatures changing the shelter occupied in order to find a more comfortable warm shelter.

The thermal and hydroselctive behaviour influence strongly the migratory range of *B. calamita* that determines its ability to disperse and its metapopulations dynamics (Sinsch et al., subm., Chapter 2). In natterjack toads the movement capacity is not sex-biased and the dispersers are juveniles and also adults. The metapopulation systems are considerably larger in the south of the Pyrenees because the populations have greater migratory ranges than those in central and northern Europe as evidenced by the variation of distance movements between populations. For the management and conservation of local amphibian populations it is important to estimate the size of a buffer zone around breeding sites. These core habitat sizes differ considerably between populations. In Spain, dry agricultural areas with high mortality risks due to dehydration and predation do not impede long-distance movements neither in Germany populations that inhabiting urban and industrially areas. Our studies include populations from Britain which have to be considered isolated by distance and prone to local extinction, as a natural recolonisation by dispersers from neighbouring sites is highly improbable. These conclusions are congruent with the levels of genetic differentiation found in these populations. Whereas populations from Britain have been considered genetically isolated by distance (Beebee & Rowe, 2000), no evidence of genetic differentiation has been found in Spain between populations separated by more than 100 km in the same altitude (Marangoni, 2006) or in different altitudes separated by 1500 m (Oromi et al., in preparation; Chapter 5).

The radiotracking studies suggest that ambient air temperatures of less than 4 °C impede movement outside the shelter sites, but do not induce a continuous hibernation in natterjack toads. Short warm periods during winter are used for

foraging and subsequent growth periods, as evidenced in natterjack toads. These growth periods are marked in the periosteal bone section with the formation of more than one LAG per year found in all populations studied (Oromi et al., 2011; Chapter 4). However, these multiple LAGs are more pronounced in the population of lowlands specially living in semiarid zones with clayey substrate affecting the adult size of individuals. The incidence and frequency of multiple growth marks per year represent informative histological correlates of local environmental impacts on life history (Sinsch et al., 2007). Growth rate can be influenced by several factors as for example food availability or temperature limitations of annual growth periods (e.g. Jørgensen, 1992; Gramapurohit et al., 2004). The main source of size variation among natterjack populations seems to be the environmental conditions which juveniles experience during their first year of life, which is the period of major growth in anurans (Jørgensen, 1992). In fact, the geographical body size variation in *B. calamita* populations is probably the result of interacting among altitudinal and latitudinal temperature effects, the microhabitat of shelters choice (that depend on substrate type) and habitat constraints (Sinsch et al. 2010, Chapter 3; Oromi et al., 2011; Chapter 4)

Altitudinal variation of life history traits in natterjack toads is more notable than latitudinal variation despite the fact that the latitudinal and altitudinal effects cause similar responses as predicted the theory of Morrison & Hero (2003). Age-related altitudinal effects are distinct from latitudinal ones, affecting males and females in the same manner (delayed age at maturity, increased longevity, unchanged PRLS), whereas latitudinal effects are notable only in females (unchanged age at maturity, increased longevity, increased PRLS). Size, in contrast, decreases roughly towards north and in highland populations, but shows a wide variability in Iberian lowland populations. Thus, the size reduction towards north is indeed a reliable predictor of altitudinal size variation, whereas age-related traits are independently selected for and follow distinct latitudinal and altitudinal patterns. Variation of demographic life history traits seems to be

a generalised response of anurans to high-elevation conditions especially probably due to the temperature variation (Oromi et al., 2011; Chapter 4).

Proximate causes of the observed variation in age- and size related life-history traits may be genetic differentiation or phenotypic plasticity or a combination of both (Berven 1982a,b; Roff, 2001; Marquis & Miaud, 2008). Microsatellite analyses and migratory range denote a high gene flow and little support for a local genetic adaptation among populations of natterjack toads in an altitudinal gradient (Oromi et al., in preparation; Chapter 5). In addition, significant gene flow among seven natterjack populations has also been detected along a 100 km transect including an altitudinal range of 400 m (southern Spain; Marangoni 2006). Both studies agree in that there is little evidence for genetic isolation by distance or altitude to promote local modifications of the gene pool. However, in the absence of common garden experiments neither the microsatellite studies nor our data set presented here allow to distinguish, whether or not despite of gene flow local adaption contributes significantly to the variation of life-history traits in *B. calamita* (Richter-Boix et al., 2010). It remains currently open, whether or not observed variability of the life history traits studied is mainly due to the phenotypic plasticity of a “general purpose genotype”.

In conclusion, variation of life history traits of *B. calamita* seems to depend on the contraction of the annual activity period along latitudinal and altitudinal gradient. The substrate type determines the shelter used by toads for hibernation or aestivations that affects the size variation and the migratory ranges. Females respond more sensitively than males to environmental changes and PRLS of females are essential for the long-term viability of populations because with size determine the lifetime fecundity. Whereas the pattern of size variation suggests a covariance of females' size and PRLS to optimise the lifetime fecundity rather in a latitudinal cline, lifetime fecundity decreases at the upper altitudinal ranges because PRLS is similar to the lowland population but females

are smaller. This reduction in lifetime fecundity can explain the inability of natterjack toads to colonise elevations exceeding 2500 m and probably to expand geographical range further north. The high gene flow between populations suggests genetic cohesion of metapopulations systems as predicted the migratory capacity of dispersing natterjacks estimate in this study.

REFERENCES

- Bartelt, P.E., Peterson, C.R. (2005) Physically modeling operative temperatures and evaporation rates in amphibians. *Journal of Thermal Biology*, 30: 93-102.
- Beebee, T.J.C., Rowe G. (2000) Microsatellite analysis of natterjack toad *Bufo calamita* Laurenti populations: consequences of dispersal from a Pleistocene refugium. *Biological Journal of the Linnean Society*, 69: 367–381.
- Berven, K.A. (1982a) The genetic basis of altitudinal variation in the wood frog *Rana sylvatica*. I. An experimental analysis of life history traits. *Evolution*, 36: 962-983.
- Berven, K.A. (1982b) The genetic basis of altitudinal variation in the frog *Rana sylvatica* II. An experimental analysis of larval development. *Oecologia*, 52:360-369.
- Gramapurohit, N.P., Shanbhag, B.A., Saidapur, S.K. (2004) Growth, sexual maturation and body size dimorphism in the Indian bullfrog, *Hoplobatrachus tigerinus* (Daudin). *Herpetologica*, 60: 414–419.
- Hutchison, V. H, Dupré, R. K. (1992) *Thermoregulation*. In: Feder, M. E., Burggren, W. W. (Eds), *Environmental physiology of the amphibians*. The University of Chicago Press: 206-249.
- Jørgensen, C.B. (1992) Growth and reproduction. In *Environmental physiology of the amphibians*: 439–466. Feder, M.E. & Burggren, W.W. (Eds). Chicago: The University of Chicago Press.
- Leskovar, C., Sinsch, U. (2001) Hibernation behaviour of radiotracked natterjack toads *Bufo calamita* and green toads *Bufo viridis*. *Biota* (Suppl.), 2: 33-34.

- Leskovar, C., Oromi, N., Sanuy, D., Sinsch, U. (2006) Demographic life history traits of reproductive natterjack toads (*Bufo calamita*) vary between northern and southern latitudes. *Amphibia-Reptilia*, 27: 365-375.
- Marangoni, F (2006) Variación clinal en el tamaño del cuerpo a escala microgeográfica en dos especies de anuros (*Pelobates cultripes* y *Bufo calamita*). PhD Thesis, University of Seville, Spain.
- Marquis, O., Miaud, C. (2008) Variation in UV sensitivity among common frog *Rana temporaria* populations along an altitudinal gradient. *Zoology*, 111: 309-317.
- Morrison, C., Hero J. M. (2003) Geographic variation in life history characteristics of amphibian: a review. *Journal of Animal Ecology*, 72: 270–279.
- Oromi, N., Sinsch, U., Sanuy, D. (2010) Thermal ecology of natterjack toads (*Bufo calamita*) in a semiarid landscape. *Journal of Thermal Biology*, 25:34-40.
- Oromi, N., Sinsch, U., Sanuy, D. (2011a) Altitudinal variation of demographic life history traits in natterjack toads. *Zoology* (submitted a).
- Oromi, N., Richter-Boix, A., Sanuy, D., Fibla, J. (2011) Genetic variability of geographical populations of the natterjack toad (*Bufo calamita*). *Conservation Biology* (submitted b).
- Richter-Boix, A., Teplitsky, C., Rogell, B., Laurila, A. (2010) Local selection modifies phenotypic divergence among *Rana temporaria* populations in the presence of gene flow. *Molecular Ecology*, 19: 716-731.
- Roff, D.A. (2001). *Life History Evolution*. Sinauer Associates, Sunderland, MA.
- Sinsch, U. (1998) *Biologie und Ökologie der Kreuzkröte*. Laurenti Verlag.
- Sinsch, U., Oromi, N., Sanuy, D. (2007) Growth marks in natterjack toad (*Bufo calamita*) bones: histological correlates of hibernation and aestivation periods. *Herpetological Journal*, 17: 129-137.
- Sinsch, U., Marangoni, F., Oromi, N., Sanuy, D., Tejedo, M. (2010) Proximate mechanisms determining size variability in natterjack toads. *Journal of Zoology*, 281: 272-281.

Sinsch, U., Leskovar, C. (2011) Does thermoregulatory behaviour of green toads (*Bufo viridis*) constrain geographical range in the west? A comparison with the performance of syntopic natterjacks (*B. calamita*). *Journal of Thermal Biology* (accepted).

Sinsch, U., Oromi, N., Miaud, C., Denton, J., Sanuy, D. (2011) Connectivity of local *Bufo calamita* populations: modelling the migratory range of radio-tracked toads. *Animal Conservation* (submitted).

CONCLUSIONS

1. *Bufo calamita* is thermal conformer with thermoregulatory behaviour restricted to avoidance of upper and lower lethal temperatures. The variation of body temperatures of the toads reflects primarily the thermal proprieties of shelters sites, which permit the maintenance of water balance.
2. The differences in local migratory ranges depend on the predominant surface substrate (clay, sand). The dispersal models demonstrate that individuals living on clay substrates (suboptimal shelter availability) move about three times farther than those living on sand substrate.
3. The buffer zones and the distance to maintain connectivity, predict that metapopulations dynamics is much greater in Iberian populations than in those located in the north of the Pyrenees. Some populations in the limit of the distribution species have to be considered isolated by distance and prone to local extinction.
4. The geographical variation in body size of *B. calamita* populations is probably the result of interaction between latitudinal temperature effects, microhabitat choice and habitat constrains to growth.
5. Latitudinal variation in body size of *B. calamita* populations is the evolutionary by-product of optimized lifetime fecundity.
6. Age-and size-related life history traits in *B. calamita* show a significant co-variation with either altitude or latitude, as predicted by life history

theory. However, altitudinal effects are distinct from latitudinal ones, affecting males and females in the same way (delayed age at maturity, increased longevity, unchanged PRLS), whereas latitudinal effects are notable only in females (unchanged age at maturity, increased longevity, increased PRLS).

7. Size depends on substrate type, and decreases roughly towards north and in highland populations, but shows a wide variability in Iberian lowland populations. Age and size related life history traits of *B. calamita* seem to respond independently and directly to the contraction of the annual activity period along a south-north axis or an altitudinal gradient.
8. Reduced lifetime fecundity in highland populations can be the last reason for the natterjacks' inability to colonize elevations exceeding 2500 m, and probably to expand geographical range further north.
9. The altitude constitutes a barrier decreasing genetic diversity from low altitudes to high altitudes.
10. The range of genetic variability of *B. calamita* showed among its geographic distribution area mainly reflects the colonization history of the species after the last glacial period.

The general conclusion of this study is that the variation of life history traits in natterjack toads (*Bufo calamita*) seems to depend on the contraction of the annual activity period along latitudinal and altitudinal gradient. This variation is influenced by substrate type. It remains currently open, whether or not observed variability of the life history traits studied is mainly due to the phenotypic plasticity of a "general purpose genotype".

CONCLUSIONS (Català)

1. El *Bufo calamita* és una espècie tèrmicament conforme amb la temperatura ambiental que presenta un comportament termoregulador limitat per tal d'evitar les temperatures letals -inferiors i superiors-. La variació de la temperatura corporal dels gripaus reflecteix primordialment les característiques tèrmiques dels refugis que permeten el manteniment del balanç hídric corporal.
2. Les diferències trobades en els rangs de migració locals entre les poblacions estudiades depenen del tipus de substrat predominant en el sòl (argila, arena). Els models de dispersió mostren que els individus que viuen en sòls argilosos (amb disponibilitat de refugis subòptims) es mouen considerablement més que els gripaus que viuen en sòls arenosos.
3. Les “buffer zones” i la distància necessària per tal de mantenir la connectivitat estimada entre poblacions, prediuen que la dinàmica metapoblacional és més gran en les poblacions ibèriques que en les poblacions del nord dels Pirineus. Algunes poblacions en el límit de la distribució de l'espècie, aïllades geogràficament, s'han de considerar subjectes a l'extinció.
4. La variació de la mida corporal del individu de les poblacions de *B. calamita* es probablement deguda al resultat de la interacció entre la variació latitudinal de la temperatura, el microhàbitat escollit i les limitacions per al creixement.

5. La variació latitudinal de la mida de les poblacions de *B. calamita* és el conseqüència de la seva evolució per tal d'optimitzar la fecunditat.
6. Els caràcters del cicle vital, edat i mida corporal del *B. calamita*, covarien significativament en el gradient altitudinal i latitudinal tal i com prediu la teoria dels caràcters del cicle vital en els amfibis. Tot i això, els efectes altitudinals són diferents dels latitudinals. En el gradient altitudinal, els mascles i les femelles presenten una variació similar (retard a l'arribada a la maduresa sexual, increment de la longevitat i semblant potencial reproductiu) mentre que els efectes latitudinals només són notoris en les femelles (no es troben efectes en l'arribada a la maduresa sexual, s'incrementa la longevitat i el potencial reproductiu).
7. La mida corporal dels gripaus depèn del tipus de sòl on viuen i presenta una tendència a disminuir en les poblacions del nord i en les d'altitud. Tanmateix, mostra una ampla variabilitat en les poblacions ibèriques de zones baixes. La variació en l'estructura d'edats del *B. calamita* respon de manera independent a la variació en la mida corporal. Malgrat això, ambdós caràcters responen de manera similar i directa a la reducció del període d'activitat anual produïda a través del gradient latitudinal -sud-nord-, i altitudinal -de zones baixes a zones altes-.
8. La reducció de la fecunditat en les poblacions en altitud, podria ser una de les raons per les quals el gripau corredor es incapaç de colonitzar altituds superiors als 2500 m i probablement per aquest motiu, no podria expandir-se més al nord de la seva distribució.
9. L'altitud constitueix una barrera que causa una reducció de la diversitat genètica des de baixes a elevades altituds.

10. L'ampli rang de variabilitat genètica trobat al llarg de l'àrea de distribució geogràfica de l'espècie reflexa, majoritàriament, la història de la colonització del *B. calamita* després de l'últim període de glaciació.

El present estudi conclou que la variació dels caràcters del cicle vital del gripau corredor (*Bufo calamita*) sembla dependre de la reducció del període d'activitat anual en el decurs del gradient altitudinal i latitudinal. Aquesta variació es troba influenciada pel tipus de sòl en el que viuen. Els anàlisis genètics efectuats no permeten afirmar amb certesa si la variació fenotípica observada és majoritàriament conseqüència de la plasticitat fenotípica d'un genotip general ("general purpose genotype").

