Universidad de Oviedo
Universidá d'Uviéu
University of Oviedo

Departamento de Biología de Organismos y Sistemas
Programa de Doctorado en Biogeociencias

Diversity and modes of reproduction in *Salamandra salamandra*: morphological and evolutionary implications in a polymorphic species

Diversidad y modos de reproducción en *Salamandra salamandra*: implicaciones morfológicas y evolutivas en una especie polimórfica

PhD Thesis by

Lucía Alarcón Ríos

under the supervision of

Dr. Alfredo González Nicieza and Dr. Guillermo Velo Antón

TESIS DOCTORAL

Oviedo 2019

RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
Español/Otro Idioma: Diversidad y modos de reproducción en *Salamandra salamandra:* implicaciones morfológicas y evolutivas en una especie polimórfica	Inglés: Diversity and modes of reproduction in *Salamandra salamandra:* morphological and evolutionary implications in a polymorphic species

2.- Autor	
Nombre: LUCÍA ALARCÓN RÍOS	DNI/Pasaporte/NIE:
Programa de Doctorado: BIOGEOCIENCIAS	
Órgano responsable: CENTRO INTERNACIONAL DE POSTGRADO	

RESUMEN (en español)

El viviparismo, entendido como la retención de huevos y embriones en el tracto reproductivo de la madre a lo largo de un periodo de tiempo durante el cual se proporcionan nutrientes adicionales a los contenidos en el huevo, y que concluye con la liberación de individuos juveniles en estados avanzados de desarrollo, ha evolucionado independientemente en todos los taxones de vertebrados, excepto aves y ciclóstomos, y ha sido objeto de múltiples estudios y disciplinas. La evolución del viviparismo lleva asociados una serie de cambios a diversos niveles, de comportamiento, de desarrollo, fisiología y morfología para permitir el correcto desarrollo de los embriones dentro del oviducto, así como para facilitar la provisión de nutrientes por diversas vías. La mayor protección de las fases iniciales del desarrollo reduce las limitaciones impuestas por el ambiente para la reproducción y desarrollo inicial, lo que puede favorecer la colonización de nuevos hábitats, el aislamiento entre grupos y, en última instancia, la diversificación o especiación.

La especie *Salamandra salamandra* presenta un polimorfismo reproductivo: a lo largo de la mayor parte de su distribución, esta especie es larvípara, liberando larvas acuáticas de vida libre. Sin embargo, se han identificados dos núcleos en los que el viviparismo (o pueriparismo), donde las hembras liberan juveniles terrestres completamente desarrollados, ha evolucionado de manera independiente. Uno al norte de la Península Ibérica que incluye todas las poblaciones de la subespecie *S. s. bernardezi*. El segundo aparece en dos poblaciones insulares en el noroeste de la Península Ibérica pertenecientes a la subespecie *S. s. gallaica*, larvípara en el resto de su distribución. La adquisición del viviparismo en *S. salamandra* ha tenido lugar mediante una serie de modificaciones heterocrónicas en el desarrollo embrionario respecto a las poblaciones larvíparas, que involucran la tasa de desarrollo, el momento de eclosión y las estructuras implicadas en la alimentación temprana intrauterina.

Por la gran diversidad ecológica, morfológica y reproductiva que presenta, constituye un sistema de estudio ideal para llevar a cabo estudios comparativos y explorar las consecuencias morfológicas, reproductivas y evolutivas de la adquisición de un nuevo modo reproductivo a diferentes niveles de la organización biológica, así como profundizar en la comprensión acerca del origen y evolución de la complejidad biológica que podemos observar en la naturaleza.

Para ello, a lo largo de esta tesis se ha descrito un método no invasivo de morfometría geométrica para la exploración de la variación morfológica en la vista dorsal de la cabeza de urodelos. Utilizando dos poblaciones de la subsespecie *S. s. gallaica* se ha testado su precisión y su utilidad para detectar cambios a nivel intra-poblacional (CAPÍTULO 1). Mediante la aplicación de éste método en un estudio comparativo que incluía poblaciones de las dos subespecies que presentan viviparismo, así como de ambos modos reproductivos, hemos encontrado que la morfología cefálica adulta constituye un rasgo subespecífico, y que no deriva de las modificaciones en el desarrollo asociadas con la adquisición del viviparismo (CAPÍTULO 2). Asimismo, los diversos morfos dentro de la subespecie puerípara *S. s. bernardezi* difieren en el tamaño corporal y en la forma de la cabeza, y presentan una estructuración geográfica de la diversidad fenotípica, con un alto grado de concordancia con la estructura de la diversidad genética neutra, que sigue un patrón de aislamiento por distancia (CAPÍTULO 3). Finalmente, el viviparismo presenta una serie de consecuencias reproductivas, ya que está relacionado con una mayor incidencia de múltiple paternidad y número de padres, a pesar la considerable reducción en el tamaño de puesta (CAPÍTULO 4).

En conclusión, los resultados muestran que la adquisición del viviparismo en *S. salamandra* y las modificaciones en el desarrollo asociadas no tienen consecuencias sobre la morfología adulta de la cabeza, que constituye un rasgo diferencial y altamente variable sujeto a un elevado número de fuerzas evolutivas tanto entre como dentro de las subespecies. Por otro lado, la evolución ha favorecido mecanismos para garantizar el éxito reproductor de las hembras vivíparas, minimizando el riesgo de incompatibilidades así como compensando la reducción de los tamaños de puesta aumentando el número de padres implicados en las mismas. Finalmente, nuestros resultados consolidan a la salamandra común como un sistema de estudio ideal para abordar diversas cuestiones acerca del origen, evolución y consecuencias de la adquisición de un modo reproductor vivíparo.

RESUMEN (en Inglés)

Viviparity is understood as eggs and embryos retention within the mother's genital tract throughout development until fully developed juveniles are delivered. During oviductal retention, developing offspring is provided with further nutrients after yolk is resorbed. It has independently evolved in all vertebrate taxa excepting birds and cyclostomes and has received lot of attention from different disciplines of biology. The evolution of viviparity is associated to a number of implications at different levels: behaviour, development, physiology and morphology, in order to allow embryos' development and nutrients provision. Higher protected early developmental stages reduce environmental constraints on reproduction, oviposition site and early development, which might favour colonization of new habitats, isolation and, ultimately, diversification and speciation.

The species *Salamandra salamandra* is a reproductive polymorphic species. Across most part of its range of distribution it presents a larviparous mode of reproduction (i.e. females lying free-living aquatic larvae). But two nuclei have independently evolved pueriparity or viviparity (i.e. females deliver fully metamorphosed terrestrial juveniles), one on the north of the Iberian Peninsula, within the subspecies *S. s. bernardezi*. The second one is located in two insular populations in the northwest of the Iberian Península, which belongs to the subspecies *S. s. gallaica*, which is larviparous across the rest of its distribution. The evolution of viviparity took place through a number of heterochronic modifications in development regarding larviparous populations. Most of those changes are focused on developmental rates acceleration, hatching pre-displacement and precocious formation of structures involved in early intrauterine feeding behavior.

The general aim of the present thesis is to explore the morphological and reproductive consequences of the evolution of a new reproductive mode in the polymorphic species *Salamandra salamandra*. For that, this thesis takes advantage of this particular natural system, in which viviparity (pueriparity) have independently evolve twice across its range of distribution, allowing for comparisons between different origins as well as different reproductive strategies. The singularity of this system allows gaining further insights into the mechanisms and consequences of evolutionary life-strategies transitions. To this end, we developed and described a non-invasive geometric morphometrics method for exploring dorsal head shape variation in urodeles, and checked its accuracy in detecting fine scale patterns using *S. salamandra* as model system (Chapter 1). Through the implementation of described methodology in a comparative approach among populations from both lineages displaying viviparity and from both modes of reproduction, we found that adult head morphology constitutes a subspecific trait, unaffected by changes in early development associated with the acquisition of viviparity (Chapter 2). Indeed, within the polymorphic pueriparous subspecies *S. s. bernardezi*, morphs differ in body size and head shape, and display geographic structuration of phenotypic diversity, highly concordant with neutral genetic diversity structure, which follow an isolation-by-distance pattern (Chapter 3). Finally, viviparity in the fire salamander demonstrated to entail several reproductive consequences, as it is related to a higher incidence of multiple paternity (in frequency and in number of mates) despite considerable smaller broods in this reproductive strategy (Chapter 4).

Summarizing, the results of this thesis show that the acquisition of viviparity in the urodele *S. salamandra* and the associated developmental modifications do not have an impact on adults head morphological diversity, which constitutes a differential and highly variable trait subjected to a number of evolutionary forces among and within subspecies. On the other hand, evolution has favored mechanisms to ensure reproductive success of viviparous females, balancing the reduction in brood size though increasing multiple mating behavior. Finally, results herein adds to the bunch of evidences consolidating the fire salamander system as an exceptional model to address different questions about the origin, evolution and consequences of the acquisition of viviparity.

La investigación presentada en esta Tesis Doctoral se ha desarrollado en el Departamento de Biología de Organismos y Sistemas de la Universidad de Oviedo, la Unidad Mixta de Investigación en Biodiversidad (UMIB-UO-CSIC-PA) y en el Centro de Investigación en Biodiversidad y Recursos Genéticos de la Universidad de Porto (CIBIO-InBIO).

FINANCIACIÓN

La realización de esta tesis está financiada por una beca predoctoral para la Formación de Profesorado Universitario (FPU14/03015) del Ministerio de Educación, Cultura y Deporte (MECD) y los proyectos de investigación CGL2012-40246-C02-02 y CGL2017-86924-P del Ministerio de Economía y Competitividad del Gobierno de España (MINECO). Además la realización de los trabajos de la presente tesis fue financiada por los proyectos de investigación FCOMP-01-0124-FEDER-028325 y POCI-01-0145-FEDER-006821 a través de fondos FEDER del Programa Operativo de Factores de Competitividad (COMPETE) y los proyectos EVOVIV: PTDC/BIA-EVF/3036/2012 y SALOMICS: PTDC/BIA-EVL/28475/2017 de la Fundación para la Ciencia y Tecnología del Gobierno de Portugal (FCT). También se concedió una ayuda complementaria de movilidad para estancias breves en otros centros dentro del subprograma de formación del profesorado universitario por el MECD (EST16/00992).

A papá y mamá,

“Nothing in life is to be feared, it is only to be understood”

“I am among those who think that science has great beauty.
A scientist in his laboratory is not only a technician: he is also a child
placed before natural phenomena which impress him like a fairy tale”

Marie Skłodowska-Curie

Agradecimientos

> *“Tuve así, en el curso de mi vida, muchísimas relaciones con muchísima gente seria. [...]*
>
> *Cuando encontré alguna que me pareció un poco lúcida, hice la experiencia de mi dibujo número 1, que siempre he conservado. Quería saber si era verdaderamente comprensiva. Pero siempre me respondía: ‘Es un sombrero’. Entonces no le hablaba ni de serpientes boas, ni de bosques vírgenes, ni de estrellas. Me ponía a su altura. Le hablaba de bridge, de golf, de política y de corbatas. Y la persona grande se quedaba muy satisfecha de haber conocido a un hombre tan razonable”.*
>
> ***Antoine de Saint-Exupéry***
> *Le Petit Prince*

Supongo que cada uno de nosotros tenemos nuestros dibujos, que solo nosotros creemos entender, pero que nos empujan a buscar a aquellas personas con los que poder hablar de nuestras propias serpientes boas, selvas vírgenes y estrellas. Así, esta tesis se ha convertido uno de esos dibujos, pero uno muy especial, ya que no hubiera sido posible sin todas aquellas personas ‘verdaderamente comprensivas’, que de una forma u otra, han contribuido a hacerla posible.

En primer lugar, quiero agradecer a mis directores, el Dr. Alfredo G. Nicieza y el Dr. Guillermo Velo-Antón, todo lo aprendido a lo largo de estos años. Alfredo, gracias por tu paciencia explicándome las cosas una y mil veces, por confiar en mí y por ‘consentirme’ llevar a cabo cualquier idea que se me ocurriera, por descabellada que pudiera ser. Por compartir conmigo tu experiencia y conocimientos, guiarme cuando me perdía o bloqueaba, soportar mis agobios (sobre todo al final), exigirme que (¡por favor!) sintetice más cuando escribo y todas las anécdotas durante las jornadas de campo. En definitiva, por todo lo que me has enseñado tanto profesional como personalmente. Guillermo, darte las gracias por tu confianza y enseñarme tantas cosas del mundo ‘salamandrero’, por ser una fuente inagotable de nuevas ideas y proyectos, pero sobre todo, por haberme guiado y enseñado tantas cosas, que de otra forma hubiera tardado mucho en aprender, y también por saber meterme caña cuando me dormía en los laureles. Eso sí, sigue pendiente esa visita a la Isla de Ons, que tantas alegrías nos da!. Gracias a los dos, de verdad.

Además de mis directores, hay un buen número de investigadores e investigadoras sin cuya ayuda esta tesis no hubiera salido adelante. En primer lugar, quiero dar las gracias a la Dra. Antigoni Kaliontzopoulou, ya que sin su participación, la realización de esta tesis no hubiera sido posible. Lo cierto es que prácticamente todo lo que se de morfometría geométrica y sus entresijos te lo debo a ti, pero no solo eso. Gracias por estar siempre disponible a mis llamadas (mails) de socorro, por enseñarme cómo mejorar la forma de trabajar, pero también cómo abordar y solucionar los múltiples problemas que pueden surgir.

I also want to express my gratitude to André Lourenço (although by the time this thesis is printed he may be Dr. Lourenço) for his help in the lab during my stay in Portugal, for sharing you knowledge on genetic secrets of salamanders, and your patience explaining things and even when I messed up (remember those samples in the heater…). Despite the long and stressful days, I really learned and enjoyed a lot. O brigada.

Agradecer también al Dr. David Buckley por su amabilidad, disponibilidad y generosidad a la hora de compartir su extenso conocimiento sobre los modos de reproducción en salamandra, el viviparismo y el desarrollo. Desde luego esta tesis bebe en gran medida de tu trabajo, y te agradezco realmente tus contribuciones y la ayuda que siempre que has podido me has brindado.

Siempre he creído que la colaboración solo puede hacer que las cosas sean mejores, y en ciencia esto se convierte en un pilar fundamental. Por ello, gracias a todas aquellos y aquellas profesionales a los que en un momento u otro recurrí en busca de ayuda y consejo sobre diversos aspectos y que la ofrecieron desinteresadamente. Aunque no coincidimos, gracias a todxs los que participaron en el campo del capítulo tres y al Dr. Fernando Sequeira por su trabajo para obtener los datos moleculares de dicho capítulo. Un agradecimiento especial para la Dra. Eugenia Sanchez y el Dr. Alexander Kupfer, por sus informes respaldando mi solicitud de doctorado con mención internacional.

Por otro lado, y porque siempre están ahí y sin su labor una gran parte de nuestro trabajo no saldría adelante, quiero dar las gracias a todo el personal técnico con el que he trabajado, tanto en campo como en el laboratorio y que siempre han mostrado una disponibilidad y capacidad de trabajo increíbles. Andrés, gracias por aguantar mis noches de muestreo intensivas queriendo ir a 10 puntos diferentes en una sola jornada; Leti, por tu diligencia y profesionalidad siempre que acudía a ti desesperada, y aun así recibirme siempre con una sonrisa; Jorge, por tu forma de ser, tu profesionalidad y por solucionarme tantos y tantos problemas con los ordenadores; Miriam, por tu dulzura y disponibilidad siempre que lo necesitaba; Luis, por estar siempre dispuesto a ayudar en lo que fuera…y otrxs muchxs que pasaron por el BOS o la UMIB y a quienes 'arrastraba' a mis noches de dragones. ¡Gracias!.

Hablando de dragones no puedo dejar de mencionar al Dr. David Álvarez, que desde el momento uno estuvo ahí, ofreciendo todo lo que tenía y pendiente de cómo me iba todo. Gracias por tu interés, opiniones y por todas las fotos que me has proporcionado para la tesis y que me ofreciste sin dudar. También por tu trabajo de divulgación con el que disfruto, aprendo y me río a partes iguales. Espero que podamos seguir compartiendo inquietudes sobre salamandras durante mucho tiempo ahora que las has convertido en el animal más famoso de la ciudad!

Gracias al Dr. Florentino Braña por toda la ayuda, consejos y recursos (esos artículos 'clásicos' imposibles de encontrar online). Un gran profesional, pero también una gran persona, con quien en cada conversación que hemos mantenido a lo largo de estos años he aprendido algo nuevo, de temas extremadamente diversos.

Quiero dar las gracias de forma muy especial a Félix González, no solo por darme mi primera oportunidad laboral, sino por todo lo que desinteresadamente me ha brindado y enseñado. Gracias por tus consejos y truquillos con los que siempre logras sorprenderme, por recomendarme herramientas de campo que me han sido tan útiles, por identificarme todas las especies que te envío y por su apoyo y confianza en mi trabajo.

En cuanto a la gente de UniOvi, me gustaría dar las gracias a los profesores, profesoras, investigadores e investigadoras del área de Ecología y Zoología, por vuestra acogida y confianza para las tareas docentes, así como vuestra ayuda y muchos consejos para poder sobrevivir a las mismas. También a las directoras y a los miembros de la Comisión Académica de Doctorado, por solucionarme tantas y tantas dudas y problemas.

Cuando hablo de gente 'verdaderamente comprensiva', no puedo pasar por alto a todos mis compañeros y compañeras del BOS. Aunque cada locx con su tema, tenemos demasiado en común como para no congeniar, debatir, aportar, reírnos y ayudarnos mutuamente. Ha sido mucha gente la que ha pasado por nuestro querido 'antro', pero cada cual siempre aporta algo. Quisiera agradecer a Gemma, mi 'hermana mayor' en ciencia, muy presente en lo profesional, pero siempre ahí en lo personal, gracias por todos los momentos vividos (menos el del ascensor, que me asusté un poco…). A Isa, mi compi de despacho forever and ever (y yoguini favorita, ya lo sabes), por toda esa energía positiva que desprendes, me alegro infinidad de que nuestras respectivas tesis nos cruzaran. A Deivid, que aunque me ha abandonado en Oviedo, solo él consigue que salga de mi agujero hobbit para ir a tomar una cerveza aunque caiga el diluvio universal. A Ro, por tu vitalidad, por tu compañía y apoyo en la recta final, pero por supuesto, gracias también por todos los buenos momentos. A Irene, gracias por tu entusiasmo y positividad, y por embarcarte conmigo en cualquier aventura que te propusiera (o me propusieras), hacemos un gran equipo. A Roberta, por compartir el estrés pre-tesis, documentos, deadlines y demás, pero también tu calidez y por ser tan comprensiva siempre. A Anais, porque siempre presta hablar contigo de cualquier cosa. A Sara Aguado, miembro destacado del team salamander de muestreo, por echarme un cable siempre que te lo pedí y retroalimentarnos creando ideas locas. A Carlos, que aunque sé que preferías estar en la sección de 'reproches', tienes un sitio irremplazable en agradecimientos. A Giulia, por tu amabilidad y tener siempre una sonrisa. A Axa, porque sigo echando de menos tu sonido de trompeta marcando la hora del café y de la comida. A Sonia, por compartir tus secretos culinarios y dejarnos disfrutarlos en las comidas de Navidad. A Sara Gómez, por ir a clases de step a ser descoordinadas juntas. A Mauro, por su buen rollo, y por sumarse siempre al carro de la locura. A Carlinos, por procuparte por mis cervicales y conseguirme una pantalla de ordenador en condiciones cuando estaba empezando. A Richi, por su alegría y por estar siempre localizándome salamandras por las inmediaciones. A María, que sin apenas conocernos me ofreció su ayuda y consejo muy valiosos para avanzar con la tesis. A Manuel, por suministrarnos en la sala dulces exóticos. A Katja, por tu interés por todas las cosas y por siempre ofrecer tus 'excedentes' marinos. A Urtzi, por su ayuda y buenos consejos desde el principio, y a Albert, que siempre intentó que sacáramos adelante proyectos en grupo. Soy consciente de que me dejo a mucha gente en el tintero (perdonadme, estoy quemando los últimos cartuchos neuronales…), pero aunque no se os nombre y estemos ahora desperdigadxs por medio mundo, tened en cuenta que el camino hasta aquí hubiera sido más duro, pero sobre todo mucho menos divertido, sin todos vostrxs. Gracias BOS people.

Quiero dar las gracias también al personal de secretaría del departamento, por hacerse cargo de mis grillos ruidosos y que hicieron que cualquier papeleo fuera siempre menos pesado. Gracias también a Feli y Pepa, por cuidarnos y alegrarnos con sus buenos días y energía matutina.

Un agradecimiento especial también a la maravillosa gente del CIBIO por su acogida y que desde luego ayudaron a que cualquier estrés fuera menos duro de sobrellevar: Lucía, Antonio, Gemma, Mike, Pili, Jesús, Angelica…, ¡gracias por tantas risas y cafés!. Y si hablo de mi estancia en Portugal, no puedo olvidarme de mi familia portuguesa: Zinda, David, Carolina y Gabriela (y todos los miembros cuadrúpedos!). Gracias de verdad por hacer que me sintiera como en casa, por compartir tantas cosas conmigo y por vuestra forma de ver la vida. Muito o brigada.

Quiero dar las gracias a mis amigas, amigos y familia, aunque no sé muchas veces dónde se dibuja el límite entre unos y otros. Este agradecimiento va mucho más allá de esta tesis, porque estaban antes, sabiendo lo que esto significaba para mí, y durante, con todas las sesiones intensivas que a veces puedo protagonizar. Todos y todas han sabido entender mis ausencias, mi falta de comunicación con el mundo exterior y mis momentos de estrés. Gracias a Selma, porque pese a la distancia siempre ha estado ahí, celebrando mis victorias como si fueran suyas. Gracias al Wildside y a la Jumera, por toda la vida. A Iria, que aunque seamos un caos comunicativo, cuando nos juntamos siempre encontramos el orden. Gracias a toda mi familia, a mis queridos abuelos Paca y Juan, siempre pendientes de mis 'bichitos', a Lala, por tu orgullo de abuela. A mis tíos y tías, por vuestro cariño y apoyo infinitos. A mis peques (aunque cada vez menos), Pablo, Gonzalo, Sergio, y Carmen, por alegrarme la vida. A María Jesús y Ramón, por entenderme y preocuparos tanto por mí, a Samu, por tu apoyo y tu ayuda para conseguir el mejor musgo para mis terrarios. Y no olvido a mi Quinto, que odia esta tesis, porque me quitaba tiempo de estar con él.

Finalmente. Gracias mamá, gracias papá. Sin vuestros esfuerzos, confianza y apoyo no hubiera sido capaz de llegar hasta aquí. Gracias por todo. Por último, tengo que dar las gracias a mi compañero en la vida, que desde el primer momento supo ver más que un sombrero en mis dibujos, y con quien he creado mil dibujos más. Gracias Rubén, por acompañarme en este trecho del camino y por creer siempre en mí.

Esta tesis también es vuestra.

Table of Contents

General Introduction

General Introduction

Reproduction is a crucial life-history trait of organisms that have attracted the attention of naturalists, philosophers and scientists for centuries, even since the time of Aristotle and his work "*Historia Animalium*" and "*De generatione animalium*". Different combinations of reproductive traits, such as oviposition or development site, number and size of ova, stage of hatchling/birth, or the amount and type of parental care result in a wide diversity of reproductive modes in nature (Salthe 1969; Angelini and Ghiara 1984; Lombardi 1998; Blackburn 1999; Wake 2018). Reproductive strategies range from species in which a high number of gametes are released in the aquatic environment, where fertilization and embryos development take place without any parental care or further nutrient provisioning beyond the egg yolk, to modes of reproduction in which fertilization and development of, normally, a small number of offspring occur within the body of one of the parents (usually the mother), where embryos and parent establish a close relationship by means of highly specialized structures (e.g. placenta). Between the two extremes, we can found a wide variety of life histories for which different adaptations and specializations have evolved, with important consequences at different biological levels (Shine 1983; Blackburn 1992; Lombardi 1998; Bruce 2003; Hamlett 2005; Wells 2007). After all, "Modification of reproductive modes is among the most complex of the 'Great Transitions' that vertebrates have achieved in their morphology, function, and ecology" (M. Wake in *Great Transformation in Vertebrates Evolution* 2015a p. 375).

I. VIVIPARITY IN VERTEBRATES

Maybe because of mammalian reproduction, but also because the important evolutionary leap and its ubiquity in nature, the evolution of viviparity has received great attention from different disciplines of biology (e.g. Amoroso et al. 1979; Shine 1985; Wake 1992; Griffith et al. 2016). Viviparity has independently evolved in all vertebrate taxa excepting birds and cyclostomes (Blackburn and Evans 1986; Blackburn 1999, 2015). It is a homoplastic trait (Wake 1992), and are among the vertebrate's innovations that have evolved more times in the tree of life (Blackburn 2015). Herein viviparity is understood as the retention and development of eggs and embryos within the mother's genital tract until fully developed juveniles are delivered. During oviductal retention, developing offspring is provided with nutrients through egg yolk (lecitotrophy) but also by a number of means of maternal provisioning (matrotrophy) after yolk is resorbed. Embryos nutrition can be accomplish through vascular provision by placenta, absorption of nutrients through the skin or gills, or ingestion of oviductal skin (epitheliophagy), eggs (oophagy) or their own siblings (adelphophagy) (Wake 2015b).

Note on terminology. As terminology can be quite confusing across cited literature, it is worth devoting some lines in order to clarify terms and make important specifications. Throughout this text, we will consider *life-bearing* all those species laying individuals that actively interact with their environment, either aquatic larvae or juveniles, not considering the source of nutrition during development. *Viviparity* will be considered a form of life-bearing that occur when any kind of matrotrophic nutrition is provided (oophagy, adelphophagy, histophagy, epitheliophagy, placentophagy, or dermatophagy). In addition, to specify the developmental stages of birth we will apply the term *larviparity* for those life-bearing species that deposit free aquatic larvae, and *pueriparity* (in this case, as a synonym of viviparity) restricted to those in which females deposit terrestrial metamorphosed juveniles (*sensu* Greven 2002, 2003) and any sort of matrotrophy exist (Wake 2015b; but see Blackburn 2000).

***I.a.* Benefits and costs of a viviparous mode of reproduction.** Transition from an oviparous to a viviparous mode of reproduction would entail several putative benefits. Long-term retention of developing embryos within a parent provides higher protection of eggs and early-developing progeny against different biotic and abiotic pressures, like predation or adverse environmental conditions (Wourms and Lombardi 1992; Shine 2002). In addition, increased investment in offspring by nutrient provisioning results in larger and more developed offspring with increased fitness (Wourms and Lombardi 1992; Andrews 2000; Shine 2002; While et al. 2009). On the other hand, viviparity entails several costs, such as increased energetic costs during pregnancy derived of oviductal retention, nutrient transfer and development of specialized structures (Hopkins et al. 1995; Schultz et al. 2008). In addition, carrying offspring during development constitutes a physical burden that could affect females locomotion, reducing their performance in hunting or predation avoidance (Shine 1980; Ghalambor et al. 2007). Finally, viviparity present important trade-offs with other life-history traits such as offspring size, fecundity (i.e. egg number per clutch and lifetime reproductive events), parental reproductive effort, degree of parental investment, and maternal size (Thibault and Schultz 1978; Goodwin et al. 2002; Wells 2007; Roitberg et al. 2013), which could constraint the evolutionary potential of this reproductive mode. Only when benefits overcome costs, the evolution of viviparity would be favoured (Shine 2015).

***I.b.* The evolution of viviparity entails changes at multiple levels.** Concomitant with intraoviductal period of developing offspring, both parents (pregnant sex mostly) and progeny display several changes at the developmental, physiological, morphological, and behavioural level to face this new scenario. Generally, internal fertilization is a necessary, although not a sufficient, condition for viviparity, and it requires the transfer of sperm to the oviduct (Blackburn 1999). This would promote the acquisition of behavioural (e.g. modified amplexus position in some anurans for cloacal apposition) or structural specializations (e.g. copulatory organs) (Kühnel et al. 2010). In addition, oviductal retention or gestation requires the appropriate structural and functional mechanisms to meet the respiratory, physiological, and nutritional needs of developing embryos in this somewhat “new environment”. Therefore, viviparous females should adapt their physiology, endocrinology, immunological system, as well as the anatomy and features of their reproductive tract to facilitate gestation ensuring gas exchange, osmoregulation, excretion of embryos waste products, and immunological function (Guillette 1987; Greven and Guex 1994; Guex and Greven 1994; Greven 1998, 2011; Wake and Dickie 1998; Bainbridge 2014; Sandberger-Loua et al. 2017). Moreover, as embryo development and growth require energy, mechanisms for nutrient provision and uptake are crucial in the evolution of viviparity. Both pregnant parent and developing offspring have developed a variety of alternative means to accomplish intraoviductal nutrition once the lecitotrophic phase ends (Blackburn 2015). In some cases, specialized structures have evolved, as for instance the modification of a part of the mother's oviduct to produce nutritive substances and the development of a specialized fetal dentition to scrape on this modified epithelium (Wake 2015b). In other species, matrotrophy is fulfilled by the precocious formation of feeding structures through heterochronic shifts in developmental pathways, allowing the exploitation of maternal products (e.g. precocious formation of jaws and digestive tract) (Buckley et al. 2007; Skov et al. 2010; Blackburn 2015). Nonetheless is important to take into account that means of acquisition of viviparity are different among different groups and even among closely related species, and thus, the mechanisms through which viviparity is accomplished vary broadly.

***I.c.* Viviparity implies important ecological and evolutionary consequences.** Viviparous offspring are more protected from the environmental variability during development than oviparous species, thus, viviparity is often considered an adaptation in response to highly variable environments and stresses on early development stages (e.g. free larval stage) (Shine 1989; Wourms and Lombardi 1992; Wake 2018; Vági et al. 2019). Furthermore, it can be seen as a novel phenotypic trait that increase the potential of colonization of new ecological niches or geographic regions, as it reduces the dependence from suitable oviposition sites and environmental conditions for progeny survival and development (Wourms and Lombardi 1992). For instance, the viviparous strategy in amphibians, with the parturition of terrestrial metamorphosed juveniles and the avoidance of the aquatic larval stage, confers greater independence from water to accomplish successful reproduction and adult survival. Thus, the reduction of the constraints imposed by the dependence on water allow the colonization of new environments and the exploitation of new resources (Salthe and Duellman 1973; Salthe and Mecham 1974; Lourenço et al. 2017). In addition, viviparity within amphibians might arise as an adaptive response to different abiotic conditions like the steepness of the terrain in montane habitats (Liedtke et al. 2017) or the dryness of environments such as offshore islands and karstic limestones substrates ('dry-climate' hypothesis: Velo-Antón et al. 2012). In the case of reptiles, where not only oviposition site, but also different environmental factors that affect eggs development, such as temperature or oxygen levels, constraints the successful colonization of new habitats, viviparity have allowed for mother control of eggs developmental environment, making possible the colonization of diverse climatic regions and environments ('cold-climate' and 'maternal manipulation' hypotheses: Shine 1985, 1995; Andrews 2000; Webb et al. 2006; Braña and Ji 2007; Rodríguez-Díaz and Braña 2012; Pincheira-Donoso et al. 2013; and 'hypoxia' hypothesis: Pincheira-Donoso et al. 2017).

At the evolutionary level, the acquisition of viviparity has also been hypothesized to promote lineages diversification (Wourms and Lombardi 1992; Lynch 2009). Colonization of new habitats or geographic regions favoured by the greater independence from breeding sites, and followed by geographic isolation, can ultimately result in speciation events (e.g. Helmstetter et al. 2016). In addition, the development of embryos within the mother allows for an antagonistic coevolution resulting from genomic conflicts between mother and embryos, developing siblings, and parental genomes within individuals, accelerating the evolution of interpopulation post-zygotic isolation, and promoting speciation in viviparous lineages (Zeh and Zeh 2000, 2008). This pattern is, however, variable. Bursts of diversification has been observed in viviparous fishes (Helmstetter et al. 2016), while in some reptiles diversification within viviparous taxa are similar to oviparous groups (Pyron and Burbrink 2014).

II. AMPHIBIANS REPRODUCTIVE DIVERSITY

Among vertebrates, amphibians stand out due to the high number of life histories they have evolved. The ancestral, or ‘standard’, biphasic life-cycle consisting on the external fertilization of aquatic eggs, from which aquatic larvae hatch and freely grow and develop until they metamorphose into terrestrial juveniles has been modified multiple times across the three extant orders of amphibians: Anura, Caudata and Gymniophiona (Duellman and Trueb 1986; Duellman 1989). Modifications occur in a broad range of reproductive traits, such as mating behaviour, patterns of fertilization and egg deposition, as well as sites and pathways of development, and are usually associated with differences in egg and clutch size and reproductive effort (Wells 2007). Attempts to classify different modes of amphibians reproduction have been done, mostly in anurans species which present the highest diversity of reproductive modes (Duellman and Trueb 1986; Haddad and Prado 2005; Iskandar et al. 2014), followed by urodeles, and, lastly, caecilians, although the reproductive biology of most of their species remains unknown (Duellman 1989; Wells 2007; Wake 2015b). In general, although with different frequency of occurrence, the three orders present species that have evolved aquatic, partially aquatic, terrestrial and live-bearing reproduction (Wells 2007). Many of those life-histories are linked to aquatic environments either during a specific stage of development (e.g. aquatic eggs or larvae), or throughout all their lives, as paedomorphic species, which are fully-aquatic and retain larval phenotype during all their lifetime (Hanken 1989; but see Denoël et al. 2005). Conversely, species from the three living orders have evolved strategies that allowed a complete independence from water for reproduction, such as the direct developers, in which the larval stage is absent (Hanken et al. 1997; Wake and Hanken 2004), or viviparous species, in which birth products are completely metamorphosed terrestrial juveniles (Wake 1992, 1993, 2015b).

***II.a*. Viviparism in Salamandridae (Caudata).** Although the diversity of reproductive strategies found across urodeles species is smaller than within anurans (Wells 2007), diversity within this group is not trivial (Buckley 2012). However, viviparity is restricted to the family Salamandridae (Greven and Guex 1994; Weisrock et al. 2006; Buckley 2012; Wake 2015b). At the same time, within family Salamandridae, only species within the two sister genus *Salamandra* and *Lyciasalamandra* have independently evolved a viviparous mode of reproduction (Buckley 2012). The genus *Lyciasalamandra* include seven viviparous species (Özeti 1979; Polymeni 1994; Veith et al. 2016), while genus *Salamandra* includes six either larviparous and/or pueriparous species (Buckley 2012).

Viviparity independently evolved in four of those species: *S. atra*, *S. lanzai*, *S. algira* and *S. salamandra*. The first two, the so-called Alpine salamanders, *S. atra* and *S. lanzai*, are strictly viviparous (Häfeli 1971; Guex and Greven 1994; Miaud et al. 2001). However, *S. algira* and *S. salamandra* populations present both larviparity as well as pueriparity throughout its distribution (Joly 1986; Bass and Gasser 1994; Dopazo and Alberch 1994; Greven and Guex 1994; Joly et al. 1994; Donaire-Barroso and Bogaerts 2003; Velo-Antón et al. 2014, 2015; Dinis and Velo-Antón 2017). In both species larviparity is the predominant and ancestral reproductive mode, but some geographically restricted populations have evolved a pueriparous strategy. In the case of *S. algira*, which appears in north Africa, from north-western Morrocco to Algeria, pueriparity is restricted to some populations of a single subspecies from its north-western distribution (Beukema et al. 2010; Dinis and Velo-Antón 2017). Similarly, throughout most part of *S. salamandra* distribution, which extends from the Iberian Peninsula towards the Balkans (Steinfartz et al. 2000; Velo-Antón and Buckley 2015) it presents a larviparous mode of reproduction. Nonetheless, two subspecies from the Iberian Peninsula (*S. s. bernardezi* and *S. s. gallaica*) have independently evolve a pueriparous mode of reproduction, in which females deliver completely metamorphosed terrestrial juveniles (Fachbach 1969; Greven 1976; Gasser 1978; Joly 1986; Dopazo and Alberch 1994; Alcobendas et al. 1996; Dopazo et al. 1998; Dopazo and Korenblum 2000; Buckely et al. 2007; Velo-Antón et al. 2007; 2015). Populations of the subspecies *S. s. bernardezi* are exclusively pueriparous and appear in the western Cantabrian Range, in the north-centre of the Iberian Peninsula. Pueriparity in those populations evolved in allopatry during the Pleistocene, and they present contact zones with adjacent larviparous subspecies where hybridization events occur. In fact, the presence of pueriparity in the neighbouring subspecies *S. s. fastuosa*, which actually display both modes of reproduction, is hypothesized to be the result of introgression events from *S. s. bernardezi* distribution as they present continuous distribution with no apparent barriers between them (García-París et al. 2003). The second pueriparous origin within *S. salamandra* occur in two insular populations of the subspecies *S. s. gallaica* in the north-west Iberian Peninsula during the Holocene (Velo-Antón et al. 2007, 2012), meanwhile throughout the rest of its mainland distribution it present a larviparous mode of reproduction (Velo-Antón et al. 2015).

All pueriparous salamanders present similar patterns, such as the presence of high number of arrested or unfertilized eggs, reduced clutch size, independence from water for reproduction and the evolution of specialized adaptations to facilitate matrotrophic nutrition after yolk is resorbed (Buckley 2012). On the other hand, despite all those species deliver terrestrial juveniles after a period of gestation within the body of the mother, there are substantial differences in the mechanisms driving the evolution of the pueriparous mode of reproduction (Greven 1998). For instance, *Lyciasalamandra ssp*., *S. atra* and *S. lanzai* females present long gestation periods and reduced broods of 1 or 2 descendants. In the case of *S. atra* females ovulate over 100 eggs from which only one per oviduct receive all the jelly coats and thus, can be successfully fertilized and start development (Häfeli 1971). At first stages, developing embryos go through a lecitotrophic stage, but once they run out of yolk, they start a matrotrophic stage with two sort of nutrients: oophagy and epitheliophagy, as the mother secretes a nourishment substance in an specialized portion of the uteri, the zona trophica, where embryos scrape with a specialized dentition (Guex and Chen 1986; Guex and Greven 1994). Intraoviductal development extend over a period of 2 to 3 years depending on environmental conditions, after which one or two well developed, metamorphosed juveniles are born (Guex and Greven 1994). Less is known about the processes that led to viviparity in the other alpine salamander, *S. lanzai*, but similar mechanisms to *S. atra* has been suggested (Miaud et al. 2001). In addition, reproductive cycle of the pueriparous genus *Lyciasalamandra* is also similar to *S. atra*, although it has been studied in just one species of the seven assigned to the genus (Özeti 1979). Main differences involve courtship behaviour, the existence of a specialized structure, the 'dorsal tail tubercle', which is inserted in the female's cloaca before the deposition of a spermatophore on the ground by the male (Sever et al. 1997), and the length of gestation, which is shorter than in *S. atra* (Özeti 1979; Polymeni 1994; Buckley 2012).

***II.b.* Diversity and modes of reproduction in *Salamandra salamandra* (Linnaeus, 1758).** Conversely, sister species *S. algira* and *S. salamandra* present reproductive polymorphism with two differentiated modes of reproduction across their distribution. Meanwhile little is known about the mechanisms driving the evolution of viviparity in pueriparous populations of *S. algira* (Donarire-Barroso et al. 2001; Beukema et al. 2010; Reinhard et al. 2015; Dinis and Velo-Antón 2017), the pueriparous mode of reproduction within *S. salamandra* has evolved through different mechanisms regarding the alpine species (Greven 1977; Dopazo and Korenblum 2000; Buckley et al. 2007). Larviparous *S. salamandra* females ovulate and fertilize ca. 20 and 80 eggs (Joly et al. 1994). During development, embryos are exclusively lecitotrophic, as they depend on the nutrients supplied by the egg yolk. After a variable time of intrauterine development dependent on environment, female deliver aquatic larvae in ponds or stream. Hatching typically take place just before being delivered or at birth, when free aquatic stage of development starts. During this period, larvae feed and grow until metamorphosis into terrestrial juveniles, which also depends on environmental conditions (from three months to up one year in mountain populations). The evolution of a pueriparous mode of reproduction results from heterochronic modifications in the ontogenetic sequence regarding the larviparous mode of reproduction (Figure I-1). Those heterochronic changes include: i) a general acceleration of development and the lecitotrophic phase; ii) a precocious development of feeding structures (jaws, jaw musculature, digestive tract and pharyngeal structures); iii) less developed larval tail fins; iv) earlier intrauterine hatching; v) intrauterine active feeding through oophagy or adelphophagy (matrotrophy), and vi) faster completion of metamorphosis regarding the larviparous strategy (90 against 120 days respectively) (Buckley et al. 2007). Despite ovulating almost the same number of eggs as larviparous salamanders, not all ovulated eggs are fertilized, and part of them arrest their development (Dopazo and Korenblum 2000; Buckley et al. 2007), which, together with cannibalistic events, result in considerably reduced brood sizes (1-35 terrestrial juveniles against 20-80 larvae). Briefly, ontogenetic modifications lead to an accelerated development, which allow the exploitation of new resources that allowed a faster growth and earlier metamorphosis.

Above mentioned processes and mechanisms underlying the acquisition of pueriparity were described from *S. s. bernardezi* females (Buckley et al 2007), one of the two suggested origins of pueriparism in *S. salamandra*. However, a number of evidences point to similar mechanisms driving the evolution of pueriparity in insular populations of *S. s. gallaica*: i) intrauterine active feeding; ii) females deliver a reduced number of heavier terrestrial juveniles; and iii) accelerated development (Velo-Antón et al. 2015). Therefore, *S. salamandra* is one of the few cases among vertebrates displaying intraspecific reproductive polymorphism. More strikingly, viviparity has independently evolved in two subspecies, with *S. s. gallaica* presenting both modes of reproduction, allowing for further comparisons between both evolutionary nuclei as well as, between strategies at the intraspecific level.

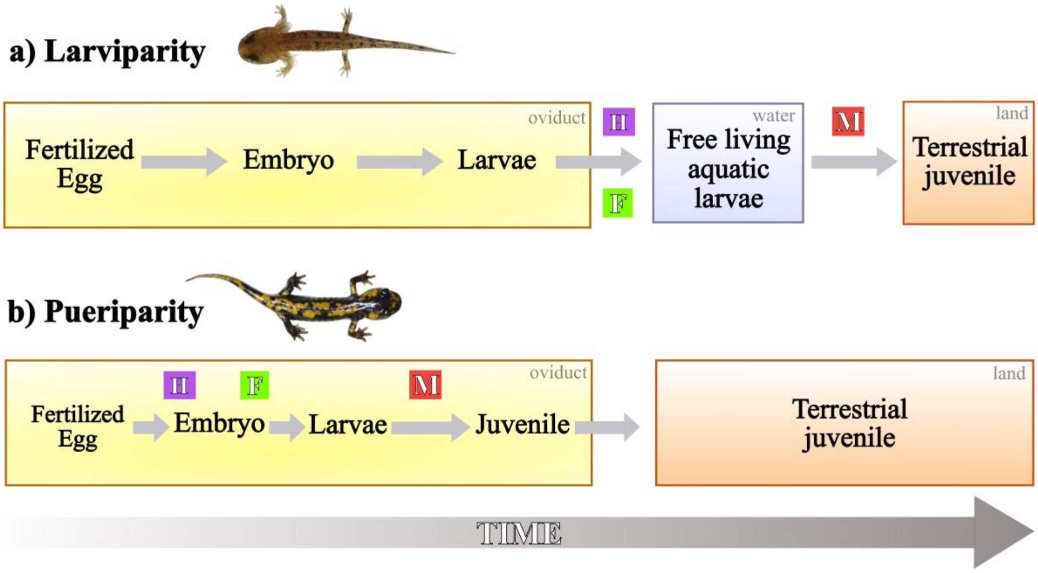


Figure I-1. Comparative scheme of the main differences in developmental sequence of both reproductive strategies within *Salamandra salamandra*, larviparity (a) and pueriparity or viviparity (b). In both sequences, each box represents discrete environments (the mother's oviduct, aquatic environment, and land). Small color boxes highlight main heterochronic events related to the acquisition of pueriparity: hatching (H; purple), onset of exogenous active feeding (F; green) and metamorphosis (M; red). Adapted from Buckley et al. 2007.

III. *SALAMANDRA SALAMANDRA* AS A STUDY SYSTEM

The striking intraspecific diversity observed in *S. salamandra* is not only represented at the reproductive level but also at the evolutionary (Steinfartz et al. 2000; García-París et al. 2003; Velo-Antón et al. 2007; Pereira et al. 2016; Bisconti et al. 2018), behavioural (Manenti and Ficetola 2013; Velo-Antón and Cordero-Rivera 2017), ecological and morphological levels (Gasser 1978; Bass and Gasser 1994; Joly et al. 1994; Rebelo and Leclair 2003; Weitere et al. 2004; Cordero-Rivera et al. 2007; Velo-Antón et al. 2015; Beukema et al. 2016). This diversity have led to the description of up to 13 subspecies, 9 within the Iberian Peninsula, although recent studies suggest the need of a taxonomic revision within this species integrating molecular, environmental and morphological information (Beukema et al. 2016).

Overall, the intraspecific diversity and polymorphisms displayed by the fire salamander makes it an exceptionally system to address several evolutionary, ecological and biological questions about inherent consequences of the evolution of a new reproductive strategy. Integrative studies, including multiple perspectives and techniques from several areas of biology provide a more complete insight into the origin and evolution of the biological complexity observed in nature, as well as to understand the evolutionary implications of the appearance of new traits. For instance, the comparative studies of life-bearing using different evolutionary lineages can be of interest for the study of similarities (homoplasies) and differences among different origins (see Wake 2015). Nonetheless, comparative studies between recently diverged lineages or within a polymorphic lineage can shed light on the processes that lead to patterns observed at higher biological scales. Therefore, the conclusions that can be reached would be highly determined by the study systems and the availability of natural diversity. In that sense, the occurrence of different life-histories within the species *S. salamandra* makes of it an exceptional natural system for exploring different hypothesis about the origin, the evolution, and the consequences of the appearance of new reproductive strategies at different levels of biological organization.

Objectives and thesis outline

The general aim of the present thesis is to explore the morphological and reproductive consequences of a new reproductive mode in the polymorphic species *Salamandra salamandra*. This thesis takes advantage of this particular natural system, in which viviparity (pueriparity) have independently evolve twice across its range of distribution, allowing for comparisons between different origins as well as different reproductive strategies. Therefore, by using comparative analyses at different levels, the potential consequences of the acquisition of a viviparous mode of reproduction on head morphology differentiation and diversity are addressed, as well as the relationship between viviparity and other reproductive trait such as multiple paternity. In that sense, the singularity of this system allows gaining further insights into the mechanisms and consequences of evolutionary life-strategies transitions.

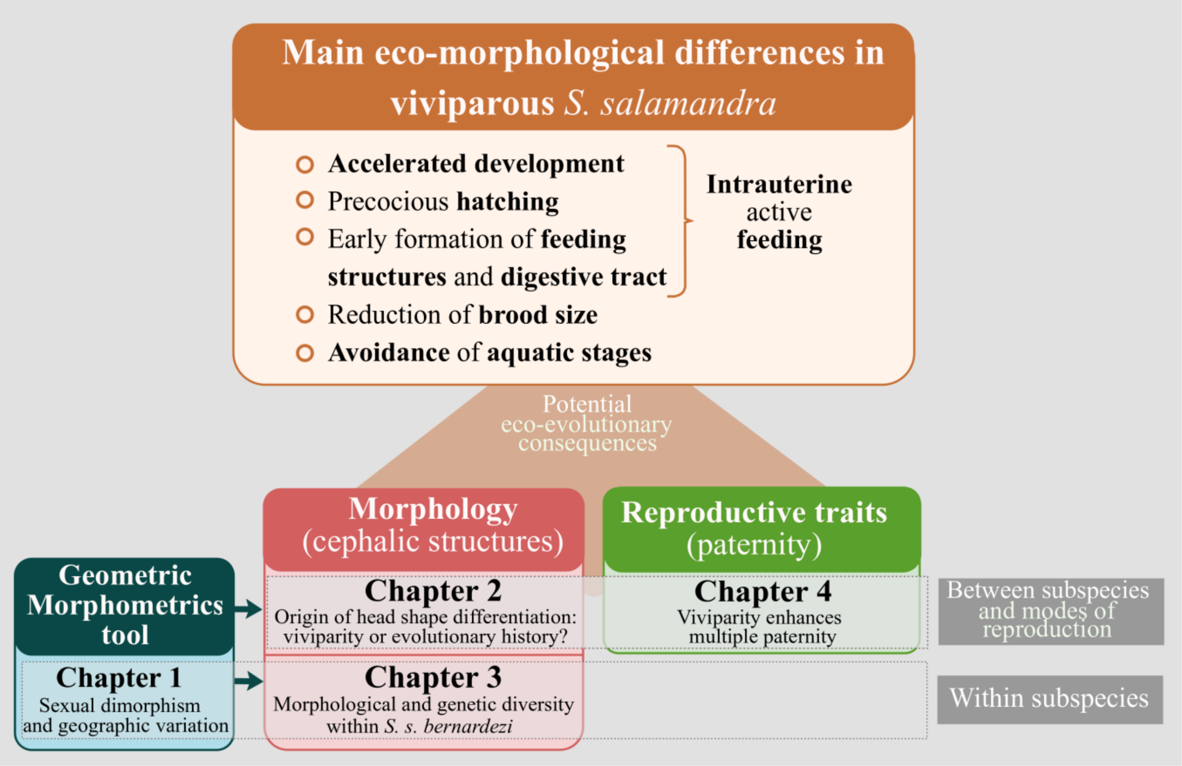


Figure I-2. Schematic diagram highlighting key developmental events and life-history traits within pueriparous salamanders (orange box) and the links with each chapter. In red, green and blue boxes are summarized the aspects related to the acquisition of viviparity explored in each chapter and how they are interrelated. In grey and dashed-line boxes, chapters are grouped according to the level at which studies were performed (inter or intraspecific).

First, we developed a geometric morphometrics non-invasive method for studying head morphology (**Chapter 1**). It allowed us to further test several morphological hypotheses regarding the potential evolutionary or developmental origin of head shape differences within both subspecies displaying pueriparism (**Chapter 2**); as well as morphological diversity patterns and drivers within a highly diverse pueriparous lineage (**Chapter 3**). Finally, we studied the reproductive consequences of the evolution of a new reproductive mode (**Chapter 4**) (see Figure I-2).

The specific objectives addressed are detailed below:

1) Describe a non-invasive, landmark-based, geometric morphometrics method for the exploration of dorsal head shape of urodeles using *Salamandra salamandra* as a model system, evaluate its accuracy, and explore its applicability for testing different hypothesis regarding biological differentiation using natural populations (Chapter 1).

2) Characterize head morphological diversity within and between both *Salamandra salamandra* subspecies displaying viviparity.

- **1.1.** Assess the validity and usefulness of head shape as a subspecific discriminant trait within *Salamandra salamandra* (Chapter 2).
- **1.2.** Explore head shape diversity patterns within a polymorphic viviparous subspecies and its correspondence with other phenotypic (coloration) and genetic traits (neutral genetic structure) (Chapter 3).

3) Determine whether the acquisition of a viviparous mode of reproduction entail any consequence at different levels through comparative analyses between subspecies and modes of reproduction.

- **1.1.** Determine whether differences in developmental sequences between reproductive modes result in different head morphologies (Chapter 2).
- **1.2.** Identify paternity patterns within both viviparous *Salamandra salamandra* independent origins (Chapter 4).
- **1.3.** Compare paternity patterns between viviparous subspecies and between reproductive modes, and evaluate the potential benefits of multiple paternity for viviparous systems (Chapter 4).

REFERENCES

Alcobendas, M., H. Dopazo, and P. Alberch. 1996. Geographic variation in allozymes of populations of *Salamandra salamandra* (Amphibia: Urodela) exhibiting distinct reproductive modes. Journal of Evolutionary Biology 9:83–102.

Amoroso, E. C., R. B. Heap, and M. B. Renfree. 1979. Hormones and the evolution of viviparity. Pages 925-989 *in* E. J. W. Barrington, ed. Hormones and Evolution. Volumen 2. Academic Press, New York.

Andrews, R. M. 2000. Evolution of viviparity in squamate reptiles (*Sceloporus spp*.): a variant of the cold-climate model. Journal of Zoology 250:243–253.

Angelini, F., and G. Ghiara. 1984. Reproductive modes and strategies in vertebrate evolution. Italian Journal of Zoology 51:121–203.

Bainbridge, D. R. J. 2014. The evolution of pregnancy. Early human development 90:741–745.

Bass, S., and F. Gasser. 1994. Polytipism of *Salamandra salamandra* (L.) in north-western Iberia. Mertensiella 4:41–74.

Beukema, W., P. De Pous, D. Donarire-Barroso, D. Escoriza, S. Bogaerts, A. G. Toxopeus, C. A. J. M. De Bie, et al. 2010. Biogeography and contemporary climatic differentiation among Moroccan *Salamandra algira*. Biological Journal of the Linnean Society 101:626–641.

Beukema, W., A. G. Nicieza, A. Lourenço, and G. Velo-Antón. 2016. Colour polymorphism in *Salamandra salamandra* (Amphibia: Urodela), revealed by a lack of genetic and environmental differentiation between distinct phenotypes. Journal of Zoological Systematics and Evolutionary Research 54:127–136.

Blackburn, D. G. 1992. Convergent evolution of viviparity, matrotrophy, and specializations for fetal nutrition in reptiles and other vertebrates. Integrative and Comparative Biology 32:313–321.

———. 1999. Viviparity and oviparity: evolution and reproductive strategies. Pages 994–1003 *in* T. Knobil and J. Neill, eds. Encyclopedia of Reproduction. Academic Press. New York, USA.

———. 2000. Classification of the reproductive patterns of amniotes. Herpetological Monographs 371–377.

———. 2015. Evolution of vertebrate viviparity and specializations for fetal nutrition: a quantitative and qualitative analysis. Journal of Morphology 276:961–990.

Blackburn, D. G., and H. E. Evans. 1986. Why are there no viviparous birds? The American Naturalist 128:165–190.

Braña, F., and X. Ji. 2007. The selective basis for increased egg retention: early incubation temperature determines hatchling phenotype in wall lizards (*Podarcis muralis*). Biological Journal of the Linnean Society 92:441–447.

Bruce, R. C. 2003. Life histories. Pages 477–525 *in* B. Jamieson, ed. Reproductive biology and phylogeny of Urodela (Volume 1). Science Publishers.

Buckley, D. 2012. Evolution of Viviparity in Salamanders (Amphibia, Caudata) *in* eLS, ed. doi:10.1002/9780470015902.a0022851

Buckley, D., M. García-París, and M. H. Wake. 2007. Heterochrony, canibalism, and the evolution of viviparity in *Salamandra salamandra*. Evolution and Development 9:105–115.

Denoël, M., P. Joly, and H. H. Whiteman. 2005. Evolutionary ecology of facultative paedomorphosis in newts and salamanders. Biological reviews of the Cambridge Philosophical Society 80:663–671.

Dinis, M., and G. Velo-Antón. 2017. How little do we know about the reproductive mode in the north African salamander, *Salamandra algira*? Pueriparity in divergent mitochondrial lineages of *S. a. tingitana*. Amphibia-Reptilia 38:540–546.

Donarire-Barroso, D., and S. Bogaerts. 2003. A new subspecies of *Salamandra algira*, Bedriaga, 1883 from northern Morocco. Podarcis 4:84–100.

Donarire-Barroso, D., S. Bogaerts, and D. Herbert. 2001. Confirmación de desarrollo larvario completo intrauterino en *Salamandra algira* (Bedriaga, 1883) del Noroeste de Marruecos. Butlletí de la Societat Catalana d’Herpetologia 5:107–110.

Dopazo, H., and P. Alberch. 1994. Preliminary results on optional viviparity and intrauterine siblicide in *Salamandra salamandra* populations from Northern Spain. Mertensiella 4:125–137.

Dopazo, H., L. Boto, and P. Alberch. 1998. Mitochondrial DNA variability in viviparous and ovoviviparous populations of the urodele *Salamandra salamandra*. Journal of Evolutionary Biology 2:365–378.

Dopazo, H., and M. Korenblum. 2000. Viviparity in *Salamandra salamandra* (Amphibia: Salamandridae): adaptation or exaptation? Herpetologica 144–152.

Duellman, W. E. 1989. Alternative life-history styles in anuran amphibians: evolutionary and ecological implications. Pages 101–126 *in* M. N. Bruton, ed. Alternative life-history styles of animals. Springer. Dordrecht.

Duellman, W. E., and L. Trueb. 1986. Biology of amphibians. New York. JHU Press.

Fachbach, G. 1969. Zur Evolution der Embryonal-bzw. Larvalentwicklung bei *Salamandra* L. Journal of Zoological Systematics and Evolutionary Research 7:128–145.

García-París, M., M. Alcobendas, D. Buckley, and D. B. Wake. 2003. Dispersal of viviparity across contact zones in Iberian populations of fire salamanders (*Salamandra*) inferred from discordance of genetic and morphological traits. Evolution 57:129–43.

Gasser, F. 1978. Le polytypisme de l’espece paléarctique *Salamandra salamandra* (L.) (Amphibien, Urodele). II. Systématique, relations génétiques et tendences évolutives dans l’aire de répartition. Archives de Zoologie Expérimentale et Générale 119:635–668.

Ghalambor, C. K., J. K. McKay, S. P. Carroll, and D. N. Reznick. 2007. Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. Functional Ecology 21:394–407.

Goodwin, N. B., N. K. Dulvy, and J. D. Reynolds. 2002. Life-history correlates of the evolution of live bearing in fishes. Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences 357:259–267.

Greven, H. 1976. Notizen zum Geburtsvorgang beim Feuersalamander, *Salamandra salamandra* (L.). Salamandra 12:87–93.

———. 1977. Comparative ultrastructural investigations of the uterine epithelium in the viviparous *Salamandra atra* Laur. and the ovoviviparous *Salamandra salamandra* (L.) (Amphibia, Urodela). Cell and tissue research 181:215–237.

———. 1998. Survey of the oviduct of salamandrids with special reference to the viviparous species. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology 282:507–525.

———. 2002. The urodele oviduct and its secretions in and after G. von Wahlert's doctoral thesis" Eileiter, Laich und Kloake der Salamandriden". Bonner Zoologische Monographien 50:25-61.

———. 2003. Larviparity and Pueriparity. Pages 447–475 *in* D. M. Sever, ed. Reproductive Biology and Phylogeny (Volume 1). Enfield, New Hampshire.

———. 2011. Maternal adaptations to reproductive modes in amphibians. Pages 117–141 *in* D. O. Norris, and K. H. Lopez, eds. Hormones and reproduction of vertebrates. Volume 2: Amphibians. Elsevier.

Greven, H., and G. D. Guex. 1994. Structural and physiological aspects of viviparity in *Salamandra salamandra*. Mertensiella 4:139–160.

Griffith, O. W., M. C. Brandley, K. Belov, and M. B. Thompson. 2016. Reptile pregnancy is underpinned by complex changes in uterine gene expression: A comparative analysis of the uterine transcriptome in viviparous and oviparous lizards. Genome biology and evolution 8:3226–3239.

Guex, G. D., and P. S. Chen. 1986. Epitheliophagy: Intrauterine cell nourishment in the viviparous alpine salamander, *Salamandra atra* (Laur.). Experientia 42:1205–1218.

Guex, G. D., and H. Greven. 1994. Structural and physiological aspects of viviparity in *Salamandra atra*. Mertensiella 4:161–208.

Guillette, L. J. 1987. The evolution of viviparity in fishes, amphibians and reptiles: An endocrine approach. Pages 523–562 *in* D. O. Norris and R. E. Jones, eds. Hormones and reproduction in fishes, amphibians, and reptiles. Springer.

Haddad, C. F. B., and C. P. A. Prado. 2005. Reproductive modes in frogs and their unexpected diversity in the Atlantic Forest of Brazil. BioScience 55:207–217.

Häfeli, H. P. 1971. Reproductive biology of the alpine salamander (*Salamandra atra* Laur). Revue suisse de zoologie 78:235–293.

Hamlett, W. C. 2005. Reproductive biology and phylogeny of Chondrichthyes. Sharks, Batoids, and Chimaeras. Volume 3. Science publishers, Inc. Enfield, NH, USA.

Hanken, J. 1989. Development and evolution in amphibians. American Scientist 77:336–343.

Hanken, J., D. H. Jennings, and L. Olsson. 1997. Mechanistic basis of life-history evolution in anuran amphibians: direct development. American Zoologist 37:160–171.

Helmstetter, A. J., A. S. Papadopulos, J. Igea, T. J. Van Dooren, A. M. Leroi, and V. Savolainen. 2016. Viviparity stimulates diversification in an order of fish. Nature Communications 7:11271.

Hopkins, T. E., M. B. Eldridge, and J. J. Cech. 1995. Metabolic costs of viviparity in yellowtail rockfish, *Sebastes flavidus*. Environmental Biology of Fishes 43:77–84.

Iskandar, D. T., B. J. Evans, and J. A. McGuire. 2014. A novel reproductive mode in frogs: a new species of fanged frog with internal fertilization and birth of tadpoles. PLoS One 9:e115884.

Joly, J. 1986. La reproduction de la salamandre terrestre (*Salamandra salamandra* L.). Traite de Zoologie. Amphibiens 14:471–495.

Joly, J., F. Chesnel, and D. Boujard. 1994. Biological adaptations and reproductive strategies in the genus *Salamandra*. Mertensiella 4:255–269.

Kühnel, S., S. Reinhard, and A. Kupfer. 2010. Evolutionary reproductive morphology of amphibians: an overview. Bonn Zoological Bulletin 57:119–126.

Liedtke, H. C., H. Müller, J. Hafner, J. Penner, D. J. Gower, T. Mazuch, M.-O. Rödel, et al. 2017. Terrestrial reproduction as an adaptation to steep terrain in African toads. Proceedings of the Royal Society B: Biological Sciences 284:20162598.

Lombardi, J. 1998. Comparative vertebrate reproduction. Kluwer Academic Publisher. USA.

Lourenço, A., D. Álvarez, I. J. Wang, and G. Velo-Antón. 2017. Trapped within the city: Integrating demography, time since isolation and population-specific traits to assess the genetic effects of urbanization. Molecular Ecology 26:1498–1514.

Lynch, V. J. 2009. Live-birth in vipers (Viperidae) is a key innovation and adaptation to global cooling during the Cenozoic. Evolution 63:2457–2465.

Manenti, R., and G. F. Ficetola. 2013. Salamanders breeding in subterranean habitats: local adaptations or behavioural plasticity? Journal of Zoology 289:182–188.

Miaud, C., F. Andreone, A. Ribéron, S. De Michelis, V. Clima, J. Castanet, H. Francillon-Vieillot, et al. 2001. Variations in age, size at maturity and gestation duration among two neighbouring populations of the alpine salamander (*Salamandra lanzai*). Journal of Zoology 254:251–260.

Özeti, N. 1979. Reproductive biology of the salamander *Mertensiella luschani antalyana*. Herpetologica 193–197.

Pincheira-Donoso, D., M. Jara, A. Reaney, R. García-Roa, M. Saldarriaga-Córdoba, and D. J. Hodgson. 2017. Hypoxia and hypothermia as rival agents of selection driving the evolution of viviparity in lizards. Global ecology and biogeography 26:1238–1246.

Pincheira-Donoso, D., T. Tregenza, M. J. Witt, and D. J. Hodgson. 2013. The evolution of viviparity opens opportunities for lizard radiation but drives it into a climatic cul-de-sac. Global Ecology and Biogeography 22:857–867.

Polymeni, R. 1994. On the biology of *Mertensiella luschani* (Steindachner, 1891): a review. Mertensiella 4:301–314.

Pyron, R. A., and F. T. Burbrink. 2014. Early origin of viviparity and multiple reversions to oviparity in squamate reptiles. Ecology letters 17:13–21.

Rebelo, R., and M. H. Leclair. 2003. Site tenacity in the terrestrial salamandrid *Salamandra salamandra*. Journal of Herpetology 37:440–446.

Reinhard, S., S. Renner, and A. Kupfer. 2015. Age and fecundity in *Salamandra algira* (Caudata: Salamandridae). Salamandra 51:19–24.

Rodríguez-Díaz, T., and F. Braña. 2012. Altitudinal variation in egg retention and rates of embryonic development in oviparous *Zootoca vivipara* fits predictions from the cold-climate model on the evolution of viviparity. Journal of Evolutionary Biology 25:1877–1887.

Roitberg, E. S., V. N. Kuranova, N. A. Bulakhova, V. F. Orlova, G. V Eplanova, O. I. Zinenko, R. R. Shamgunova, et al. 2013. Variation of reproductive traits and female body size in the most widely-ranging terrestrial reptile: testing the effects of reproductive mode, lineage, and climate. Evolutionary biology 40:420–438.

Salthe, S. N. 1969. Reproductive modes and the number and sizes of ova in the urodeles. American Midland Naturalist 467–490.

Salthe, S. N., and W. E. Duellman. 1973. Quantitative constraints associated with reproductive mode in anurans. Evolutionary biology of the anurans 229–249.

Salthe, S. N., and J. S. Mecham. 1974. Reproductive and courtship patterns. Physiology of the Amphibia 2:309–521.

Sandberger-Loua, L., H. Müller, and M. O. Rödel. 2017. A review of the reproductive biology of the only known matrotrophic viviparous anuran, the West African Nimba toad, *Nimbaphrynoides occidentalis*. Zoosystematics and Evolution 93.

Schultz, T. J., J. K. Webb, and K. A. Christian. 2008. The physiological cost of pregnancy in a tropical viviparous snake. Copeia 2008:637–642.

Sever, D. M., M. Sparreboom, and G. Schultschik. 1997. The dorsal tail tubercle of *Mertensiella caucasica* and *M. luschani* (Amphibia: Salamandridae). Journal of Morphology 232:93–105.

Shine, R. 1980. “Costs” of reproduction in reptiles. Oecologia 46:92–100.

———. 1983. Reptilian reproductive modes: the oviparity-viviparity continuum. Herpetologica 1–8.

———. 1985. The evolution of viviparity in reptiles: an ecological analysis. Biology of the Reptilia 15:605–694.

———. 1989. Ecological influences on the evolution of vertebrate viviparity. Pages 263–278 *in* D. B. Wake, and G. Roth, eds. Complex organismal functions: integration and evolution in vertebrates. Wiley, Chichester.

———. 1995. A new hypothesis for the evolution of viviparity in reptiles. The American Naturalist 145:809–823.

———. 2002. Reconstructing an adaptationist scenario: what selective forces favor the evolution of viviparity in montane reptiles? The American Naturalist 160:582–593.

Shine, R. 2015. The evolution of oviparity in squamate reptiles: an adaptationist perspective. Journal of Experimental Zoology Part B: Molecular and Developmental Evolution 324:487–492.

Skov, P. V., J. F. Steffensen, T. F. Sørensen, and K. Qvortrup. 2010. Embryonic suckling and maternal specializations in the live-bearing teleost *Zoarces viviparus*. Journal of Experimental Marine Biology and Ecology 395:120–127.

Steinfartz, S., M. Veith, and D. Tautz. 2000. Mitochondrial sequence analysis of *Salamandra* taxa suggests old splits of major lineages and postglacial recolonizations of central Europe from distinct source populations of *Salamandra salamandra*. Molecular ecology 9:397–410.

Thibault, R. E., and R. J. Schultz. 1978. Reproductive adaptations among viviparous fishes (Cyprinodontiformes: Poeciliidae). Evolution 32:320–333.

Vági, B., Z. Végvári, A. Liker, R. P. Freckleton, and T. Székely. 2019. Parental care and the evolution of terrestriality in frogs. Proceedings of the Royal Society B 286:20182737.

Veith, M., B. Göçmen, K. Sotiropoulos, S. Kieren, O. Godmann, and S. Steinfartz. 2016. Seven at one blow: the origin of major lineages of the viviparous Lycian salamanders (*Lyciasalamandra* Veith and Steinfartz, 2004) was triggered by a single paleo-historic event. Amphibia-Reptilia 37:373–387.

Velo-Antón, G., and D. Buckley. 2015. Salamandra común – *Salamandra salamandra* (Linnaeus, 1758) Departamento de Biodiversidad y Biología Evolutiva . Museo Nacional de Ciencias Dirección actual : 1 Bailey Mews , Auckland Road . Cambridge CB5 8DR. Enciclopedia Virtual de los Vertebrados Españoles 2–12.

Velo-Antón, G., and A. Cordero-Rivera. 2017. Ethological and phenotypic divergence in insular fire salamanders: diurnal activity mediated by predation? Acta Ethologica 20:243–253.

Velo-Antón, G., L. García-Cardenete, F. J. Cazalla, and F. Martínez-Freiría. 2014. New record of *Salamandra algira* isolated on the north-western Tingitana peninsula, with some notes on the reproductive modes within the species. Boletín Asociación Herpetológica Española 25:46–50.

Velo-Antón, G., M. García-París, P. Galán, and A. Cordero-Rivera. 2007. The evolution of viviparity in holocene islands: ecological adaptation versus phylogenetic descent along the transition from aquatic to terrestrial environments. Journal of Zoological Systematics and Evolutionary Research 45:345–352.

Velo-Antón, G., X. Santos, I. Sanmartín-Villar, A. Cordero-Rivera, and D. Buckley. 2015. Intraspecific variation in clutch size and maternal investment in pueriparous and larviparous *Salamandra salamandra* females. Evolutionary Ecology 29:185–204.

Velo-Antón, G., K. R. Zamudio, and A. Cordero-Rivera. 2012. Genetic drift and rapid evolution of viviparity in insular fire salamanders (*Salamandra salamandra*). Heredity 108:410.

Wake, D. B., and J. Hanken. 2004. Direct development in the lungless salamanders: what are the consequences for developmental biology, evolution and phylogenesis? International Journal of Developmental Biology 40:859–869.

Wake, M. H. 1992. Evolutionary scenarios, homology and convergence of structural specializations for vertebrate viviparity. American Zoologist 32:256–263.

———. 1993. Evolution of oviductal gestation in amphibians. Journal of Experimental Zoology 266:394–413.

———. 2015a. How do homoplasies arise? Origin and maintenance of reproductive modes in amphibians. Pages 375-394 *in* K. P. Dial, N. Shubin, and E. L. Brainerd, eds. Great Transformations in Vertebrate Evolution. University of Chicago Press.

———. 2015b. Fetal adaptations for viviparity in amphibians. Journal of Morphology 276:941–960.

———. 2018. Modes of Reproduction Verts: Hermaphroditism, Viviparity, Oviparity, Ovoviviparity: General Definition With Examples. Reference Module in Life Sciences

Wake, M. H., and R. Dickie. 1998. Oviduct structure and function and reproductive modes in amphibians. The Journal of experimental zoology 282:477–506.

Webb, J. K., R. Shine, and K. A. Christian. 2006. The adaptive signigicance of reptilian viviparity in the tropics: testing the maternal manipulation hypothesis. Evolution 60:115–122.

Weisrock, D. W., T. J. Papenfuss, J. R. Macey, S. N. Litvinchuk, R. Polymeni, I. H. Ugurtas, E. Zhao, et al. 2006. A molecular assessment of phylogenetic relationships and lineage accumulation rates within the family Salamandridae (Amphibia, Caudata). Molecular Phylogenetics and Evolution 41:368–383.

Weitere, M., D. Tautz, D. Neumann, and S. Steinfartz. 2004. Adaptive divergence vs. environmental plasticity: tracing local genetic adaptation of metamorphosis traits in salamanders. Molecular ecology 13:1665–1677.

Wells, K. D. 2007. The ecology and behavior of amphibians. University of Chicago Press.

While, G. M., T. Uller, and E. Wapstra. 2009. Offspring performance and the adaptive benefits of prolonged pregnancy: experimental tests in a viviparous lizard. Functional ecology 23:818–825.

Wourms, J. P., and J. Lombardi. 1992. Reflections on the evolution of piscine viviparity. American Zoologist 32:276–293.

Zeh, D. W., and J. A. Zeh. 2000. Reproductive mode and speciation: The viviparity-driven conflict hypothesis. BioEssays 22:938–946.

Zeh, J. A., and D. W. Zeh. 2008. Viviparity-driven conflict. Annals of the New York Academy of Sciences 1133:126–148.

Chapter 1

A NON-INVASIVE GEOMETRIC MORPHOMETRICS METHOD FOR EXPLORING VARIATION IN DORSAL HEAD SHAPE IN URODELES: SEXUAL DIMORPHISM AND GEOGRAPHIC VARIATION IN *SALAMANDRA SALAMANDRA*[*]

ABSTRACT

The study of morphological variation among and within taxa can shed light on the evolution of phenotypic diversification. In the case of urodeles, the dorso-ventral view of the head captures most of the ontogenetic and evolutionary variation of the entire head, which is a structure with a high potential for being a target of selection due to its relevance in ecological and social functions. Here, we describe a non-invasive procedure of geometric morphometrics for exploring morphological variation in the external dorso-ventral view of urodeles' head. To explore the accuracy of the method and its potential for describing morphological patterns we applied it to two populations of *Salamandra salamandra gallaica* from NW Iberia. Using landmark-based geometric morphometrics, we detected differences in head shape between populations and sexes, and an allometric relationship between shape and size. We also determined that not all differences in head shape are due to size variation, suggesting intrinsic shape differences across sexes and populations. These morphological patterns had not been previously explored in *S. salamandra*, despite the high levels of intraspecific diversity within this species. The methodological procedure presented here allows to detect shape variation at a very fine scale, and solves the drawbacks of using cranial samples, thus increasing the possibilities of using collection specimens and alive animals for exploring dorsal head shape variation and its evolutionary and ecological implications in urodeles.

---

[*] This chapter is based on the article:

Alarcón-Ríos, L., Velo-Antón, G., & Kaliontzopoulou, A. (2017). A non-invasive geometric morphometrics method for exploring variation in dorsal head shape in urodeles: sexual dimorphism and geographic variation in *Salamandra salamandra.* **Journal of morphology, 278(4), 475-485**

INTRODUCTION

Geometric morphometrics (GM) is a powerful tool for studying shape and its covariation with other variables, testing hypotheses about its causes, exploring morphological variation among and within species, and uncovering unsuspected morphological variation with biological significance (Zelditch et al. 2004). Indeed, since their introduction more than 20 years ago (Corti 1993; Rohlf and Marcus 1993) several fields of biology have benefited from its broad range of applications (e.g., Rohlf 2002; Adams et al. 2004, 2013; Lawing and Polly 2010; Slice 2005; Klingenberg 2010).

The high potential of landmark-based GM is based on (see review in Adams et al. 2013) its ability of preserving the geometric properties of the studied structures, enabling an accurate description of all aspects of shape without the bias introduced by employed measures, as occurs in linear morphometrics (Zelditch et al. 2004). In addition, due to the high number of variables evaluated by geometric morphometrics, the detection of even small differences in shape is possible (Collyer et al. 2015). This is particularly useful when investigating fine-scale patterns of variation, as is the case in biological systems with reduced morphological variability or when working on the populational level. The detection and description of these shape patterns is key to understand the mechanisms and processes underlying observed morphological diversification among and within species. Another advantage of GM when exploring shape variation is its potential to describe allometric relationships. Because size and shape are tightly linked (e.g., Gould 1966; Klingenberg 2016), observed variation in shape may be, at least partially, based on allometric relationships. Through the Generalized Procrustes Analysis (GPA), GM allows to clearly define and mathematically separate shape and size, and thus elucidating the level of correlation among both traits (Rohlf and Slice 1990; Swiderski 2003; Mitteroecker et al. 2013). Finally, evolutionary biology, ecology and developmental biology frequently involve studies of shape change between groups or along evolutionary, ecological or developmental gradients. Such studies of shape change can be efficiently carried out in the framework of GM by studying the vectors of phenotypic change, which allow detecting such variation, but also describing the reasons why this variation is meaningful (Collyer and Adams 2007; Adams and Collyer 2009; Collyer and Adams 2013).

The study of shape using GM has been extensively applied in a wide variety of taxa, including mammals (Marcus et al. 2000), birds (Marugán-Lobón et al. 2016), fishes (Bichuette et al. 2014) and insects (Dujardin 2008), to mention just a few examples. Nonetheless, reptilian sauropsids and amphibians have long constituted a group of great interest in the study of morphology due to the high diversity of forms and lifestyles found across these taxa. Particularly, GM tools have been intensively applied to both groups to obtain a better understanding of phenotypic diversification and its causes (reviewed in Kaliontzopoulou 2011), but also to provide theoretical advances and extend GM applications (Magwene 2001; Collyer and Adams 2013; Adams 2014; Adams and Felice 2014; Adams et al. 2016). In amphibians, GM is helpful for exploring morphological patterns and investigating the ecological and evolutionary causes of diversification given the morphological conservatism observed in this group (Cherty et al. 1978; Moen et al. 2013) that results in many cryptic species (Hanken 1999; Stuart et al. 2006), and potentially an underestimation of diversity. For instance, the use of GM-tools to study phenotypic variation in amphibians has advanced our knowledge of the shape changes occurring throughout development (Ivanović et al. 2007; Jaekel and Wake 2007; Adams and Nistri 2010; Ivanović et al. 2011; Ponssa and Candioti 2012; Cvijanović et al. 2014), and enhanced our understanding of the contribution of phenotypic plasticity in the diversity observed in some taxa (Johnson et al. 2008; Van Buskirk 2009). Similarly, the development and application of GM tools for addressing evolutionary questions has shed light on the mechanisms underlying character displacement and parallel evolution due to interspecific competition and environmental adaptation (Adams and Rohlf 2000; Adams 2004, 2010; Adams and Nistri 2010; Deitloff et al. 2013).

The aforementioned studies have mostly focused on exploring variation in head shape, due to its relevance for a number of biological functions, including feeding (Adams and Rohlf 2000), defence against predators (Brodie 1986), and agonistic behaviour related to territoriality and mate acquisition (Kästle 1986; Adams 2004, 2010; Arif et al. 2007). In most studies, the lateral view of the head has been targeted (Adams and Rohlf 2000; Adams 2004, 2011), because of its relevance for understanding the biomechanical properties of the jaw. However, the architecture of the head of urodeles, which is wide and flattened, results in large part of the ontogenetic and evolutionary variation occurring in the dorso-ventral direction (Trueb 1993). Several studies have applied GM on both the dorsal and ventral view of the skull to investigate sexual dimorphism or phylogenetic relatedness (Ivanović et al. 2007, 2009, 2012; Ivanović and Kalezić 2012). While the study of the cranium of both anurans and urodeles has been very fruitful for understanding sources of head shape variation in the dorso-ventral direction, the procedures required for obtaining cranial elements are prohibitive when working with museum specimens and above all with living individuals from natural populations. To the best of our knowledge, only one study has applied GM to the analysis of external ventral head shape in urodeles (Alcorn et al. 2013), giving clues of the potential of the exploration of external head shape in these organisms.

Here, we use the fire salamander (*Salamandra salamandra*, Linnaeus 1758) as a model system to develop a non-invasive methodological framework for applying two-dimensional landmark-based GM to the study of dorsal head shape variation in urodeles. By the application of this method on males and females from two populations of the Iberian subspecies *S. s. gallaica* we aim to determine the utility of the methodological procedure in quantifying dorsal head shape, evaluate its precision, and explore its potential for identifying morphological patterns.

MATERIAL AND METHODS

STUDY SYSTEM

Salamandra salamandra, Linnaeus 1758, is widespread throughout much of southern, central and eastern Europe, with most of the morphological and genetic variation occurring in the Iberian Peninsula, where nine subspecies have been described (see Velo-Antón and Buckley 2015). Moreover, it shows a remarkable intraspecific variation in reproductive modes (Velo-Antón et al. 2015), morphological polytypism (Bas and Gasser 1994; Alcobendas et al. 1996; García-París et al. 2003) and polymorphism (Cordero-Rivera et al. 2007; Beukema et al. 2016). In spite of the high morphological diversity observed at the intraspecific level, there is a limited number of morphological studies on this species, most of which are focused on coloration (Bosch and López-Bueis 1994; Pasmans and Keller 2000; Balogová and Uhrin 2015; Beukema et al. 2016). Some studies have also explored sexual dimorphism in size (Degani 1986; Cordero-Rivera et al. 2007; Balogová et al. 2015) and shape (Labus et al. 2013), showing that, as in many other urodeles, females are the larger sex, with relatively larger heads, interlimb distances and parotid glands while males have longer limbs and feet (Labus et al. 2013; Reinhard et al. 2015). However, although snout shape has been considered as a key trait to identify sexual dimorphism (Labus et al. 2013) and to distinguish between subspecies in *S. salamandra* (Bas and Gasser 1994; Velo-Antón and Buckley 2015) head shape variation has been poorly studied.

SAMPLING AND IMAGE ACQUISITION

For this study we examined a total of 120 adult individuals of *S. s. gallaica*, Seoane 1884, from two localities in the Atlantic coast of the Iberian Peninsula: Mindelo (41.32N, 8.72W; 38 males and 42 females) and Coiro (42.30N, 8.75W; 25 males and 15 females). We sampled individuals in rainy or high-moisture, no-windy nights in March, April and October 2015, and transported them to CIBIO´s laboratory facilities, where we anesthetized them placing the animals in a container with 10 ml of benzocaine 10% dissolved in 0.5L of water for two minutes. Then, we sexed them by checking the swollen gland near the cloaca (more pronounced in males). We took high-resolution photographs of the dorsal view of the head using an OLYMPUS TOUGH TG1 camera, and ensuring that the objective was always parallel to the head surface, which we dried out using soft paper to reduce reflections that could mask important shape features of the salamanders' heads. We placed graph paper under the salamanders to record scale.

Animals were captured and measured with the corresponding permits from regional administration (Galicia, Ref. 410/2015; Portugal Ref. 36490/2016/DRNCN/DGEFF). Once measured, all animals were rinsed with dechlorinated water and placed in a container with wet soft paper until they were completely awake. Finally, they were released in the same place where they were captured. Images used in the study are available in: http://morphobank.org/permalink/?P2569.

GEOMETRIC MORPHOMETRICS

To record dorsal head shape and size variation across our sample, we used tpsDig2 (Rohlf 2015) to digitize the position of 13 fixed landmarks and 32 sliding semilandmarks. Landmarks should provide a comprehensive sampling of morphology, capturing the features of relevance for testing the biological hypotheses at hand (Zelditch et al. 2004). Taking this into account, we selected landmarks that capture snout and jaw shape, and delimit the contour of the parotid glands and the eyes (Figure 1.1). It is important to note that all subsequent statistical analyses were performed using Randomized residual permutation procedure (RRPP; Collyer et al. 2015). This permutational procedure allows using a higher number of variables (landmarks and semilandmarks), leading to a better description of shape and increasing statistical power while avoiding problems related to the number of variables and sample size when working on high-dimensional data (Collyer et al. 2015).

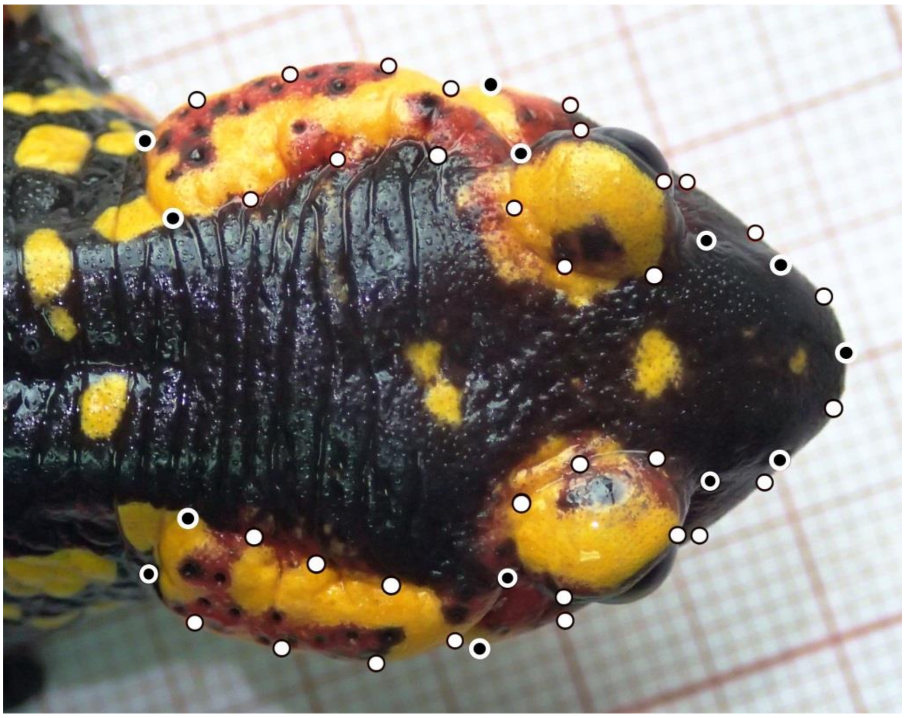


Figure 1.1. Landmarks (black-filled points) and semilandmarks (white-filled points) recorded on the dorsal view of salamanders' head.

Because this is the first time that landmark-based GM are used to quantify dorsal head shape externally in urodeles, we were interested in validating the accuracy of the proposed method. Landmark digitizing for data collection adds a source of variation that should be quantified and evaluated to assess its impact on the shape variation (Cramon-Taubadel et al. 2007). In order to quantify the amount of variation due to observer-induced error, one of us (LAR) digitized the landmarks of individuals from the population of Mindelo twice.

Because the position of some landmarks could not be accurately determined due to light reflections or tissue irregularities, we recorded them as missing data and estimated their location using a multivariate regression (Gunz et al. 2009; Arbour and Brown 2014). Then, in order to obtain shape variables for subsequent multivariate analyses and remove all information unrelated with shape, we standardized in size, translated and rotated landmark configurations from all individuals through a generalized least-squares Procrustes superimposition (GPA; Rohlf and Slice 1990; Rohlf 1999). During the GPA, we optimized the position of semilandmarks by sliding them along their tangent directions using the Procrustes distance criterion that minimizes the Procrustes Distance across specimens and the average shape in the sample (Bookstein 1997). In this approach the sliding of each semilandmark is not influenced by the position of the rest of landmarks and semilandmarks because each of them slides separately (Gunz and Mitteroecker 2013). Because we were not interested in head asymmetry, we eliminated asymmetry effects by averaging the position of corresponding bilateral landmarks across the midline.

STATISTICAL ANALYSES

To assess measurement error and put it in the context of variation among individuals we tested for the effects of repetition and individual on head shape using a Procrustes ANOVA. In this analysis, the effect of repetition captures variation due to measurement error, while the effect of individual captures the total variation across the subsample of individuals from Mindelo. Finally, their interaction expresses variation in the amount and type of multivariate measurement error across individuals. A non-significant interaction term would indicate that measurement error is consistent across individuals, making it systematic. Instead, a significant interaction suggests that measurement error is random across individuals. To visualize the magnitude of error-induced vs. biological variation, we quantified mean measurement error as the mean Procrustes distance between repeats of each individual, and variation among specimens as the mean distance to the centroid of all landmark configurations after GPA superimposition. When calculating total variation, we removed digitizing effects by using the mean configuration of the two digitizations of each individual.

To investigate biological sources of variation in salamander head shape and size we performed distance-based ANOVAs as implemented in the function *procD.lm* of the R package *geomorph* (Adams et al. 2016), with population, sex and their interaction as factors. Statistical significance was always assessed based on 10000 random permutations, using Residual Randomization (Collyer et al. 2015). As a measure of head size we used the logarithm of Centroid Size (logCS). CS is calculated as the square root of the summed squared distances of each landmark from the centroid of the landmark configuration (Bookstein 1991; Zelditch et al. 2004), and it is uncorrelated with shape in the absence of allometry. Then, in order to analyze the allometric relationship between shape and size, we repeated the Procrustes ANOVA, including size as a covariate and its interaction with other variables. This allowed us to investigate the covariation between shape and size, test for common allometric slopes among groups, and evaluate population differentiation and sexual dimorphism in shape while accounting for size effects. To visualize and compare allometric trends between groups, we calculated predicted values from a linear model that included all significant terms from the previous Procrustes ANOVA (three main effects and sex by population interaction, see Results), and plotted the first principal component of those predicted values versus logCS (Adams and Nistri 2010).

To further explore shape variation across populations and sexes, we performed a phenotypic trajectory analysis (PTA: Adams and Collyer 2009; Collyer and Adams 2013). This sort of analysis is grounded in the study of geometric attributes of phenotypic change vectors: size (d) and direction (θ). Size of shape change vector describe how much shape change occurs per unit change in other independent variable while the direction of shape change vector describes the relative covariations of shape variables per unit change in other independent variable. Through the statistical comparison of their geometric attributes it is possible to describe in what aspect shape change occurs. In the present study, the conducted PTAs compared sexual dimorphism shape change vectors in each population and were based on a) the Procrustes residuals, and b) size-corrected Procrustes residuals, obtained as the residuals of the regression of shape on log(CS) under a common slope for all groups (see also Results). Shape variation across groups, both between sexes of the same population and between individuals of the same sex from different populations, was visualized using vector plots. We implemented all GM-operations and statistical analyses using package *geomorph* (Adams and Otárola-Castillo 2013; Adams et al. 2016) in the R-language for statistical programming (R Development Core Team, 2016).

RESULTS

The permutational ANOVA performed to test the accuracy of the method showed that the interaction term was significant suggesting that the amount and type of measurement error varied across individuals (Table 1.1). However, total variation across individuals of the same population was much higher than the measurement error, with up to twice as much variation occurring across individuals as that observed between digitizations of the same individual (Figure 1.2).

Table 1.1. Results of the Procrustes ANOVA used to assess the accuracy of the method by testing the variation due to measurement error (Replicate variation), to total variation in the sample (Individual variation) and to its interaction. df: degrees of freedom, SS: Sum of Squares, Z: Standarized Z-scores, *P*: p-value based on 10000 permutations.

	df	**SS**	**Z**	***P***
Replicate	1	0.002	1.020	0.267
Individual	79	0.197	1.836	<0.0001
Replicate x Individual	79	0.018	2.011	<0.0001
Residuals	0	0		

Table 1.2. Results of permutational ANOVAs used to test: a) the effect of population, sex, and their interaction on dorsal head shape; b) the effect of population, sex, and their interaction on dorsal head size as represented by the centroid size of the landmark configuration (CS); c) the shape-size covariation, population differentiation, sexual dimorphism and common allometric slopes among groups. df: Degrees of freedom, SS: Sums of Squares, F: ANOVA F-value, Z: Standardized effect Z-score, *P*: p-value based on 10000 residual permutations.

	df	SS	F	Z	*P*
a) Shape					
Population	1	0.008	6.375	4.9552	<0.0001
Sex	1	0.010	7.678	6.2395	<0.0001
Population x Sex	1	0.003	2.621	2.3202	0.015
Residuals	116	0.144	0.001		
b) Size					
Sex	1	0.263	12.036	6.405	0.001
Population	1	0.006	0.275	0.164	0.600
Sex x Population	1	0.016	0.728	0.429	0.390
Residuals	116	2.532			
c) Shape vs. Size					
Size	1	0.009	7.743	5.826	<0.0001
Population	1	0.007	6.286	4.989	<0.0001
Sex	1	0.007	6.191	5.205	<0.0001
Size x Population	1	0.002	1.778	1.571	0.082
Size x Sex	1	0.001	1.257	1.132	0.225
Population x Sex	1	0.002	2.030	1.827	0.049
Size x Pop. x Sex	1	0.002	1.368	1.264	0.166
Residuals	112	0.133			

Procrustes ANOVA revealed that population, sex, as well as their interaction had significant effects on dorsal head shape variation (Table 1.2a). As the degree of sexual shape dimorphism varied among populations, this significant interaction was further investigated by PTA, which showed significant differences in both the size ($\Delta d = 0.012$, $P = 0.04$) and the direction ($\theta = 0.362$, $P = 0.015$) of the vectors describing phenotypic change between males and females within each population (Figure 1.3a). Generally, males of both populations exhibited rounder and relatively larger eyes than females (Figure 1.4 a,b), but head shape sexual dimorphism was more marked in Mindelo (Figure 1.3a). In addition, Coiro males had slightly shorter heads than females, with relatively smaller parotids (Figure 1.4a). On the other hand, Mindelo males had relatively narrow and long heads and larger parotids as compared to respective females (Figure 1.4b), similarly to the pattern of shape change found between females (Figure 1.4c). Those differences between females are also more pronounced than differences between males (Figure 1.4d), where males from Coiro had slightly shorter and broader heads than those from Mindelo.

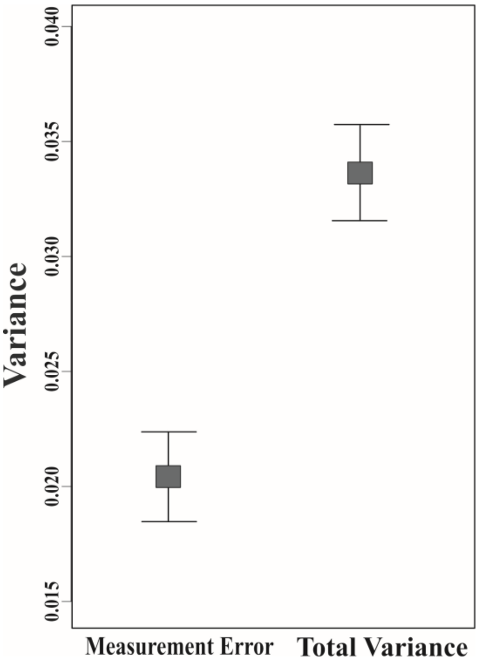


Figure 1.2. Measurement error vs. total variance among individuals in the subsample from Mindelo used to test digitizing accuracy. Vertical bars denote 95% confidence intervals obtained through bootstrapping.

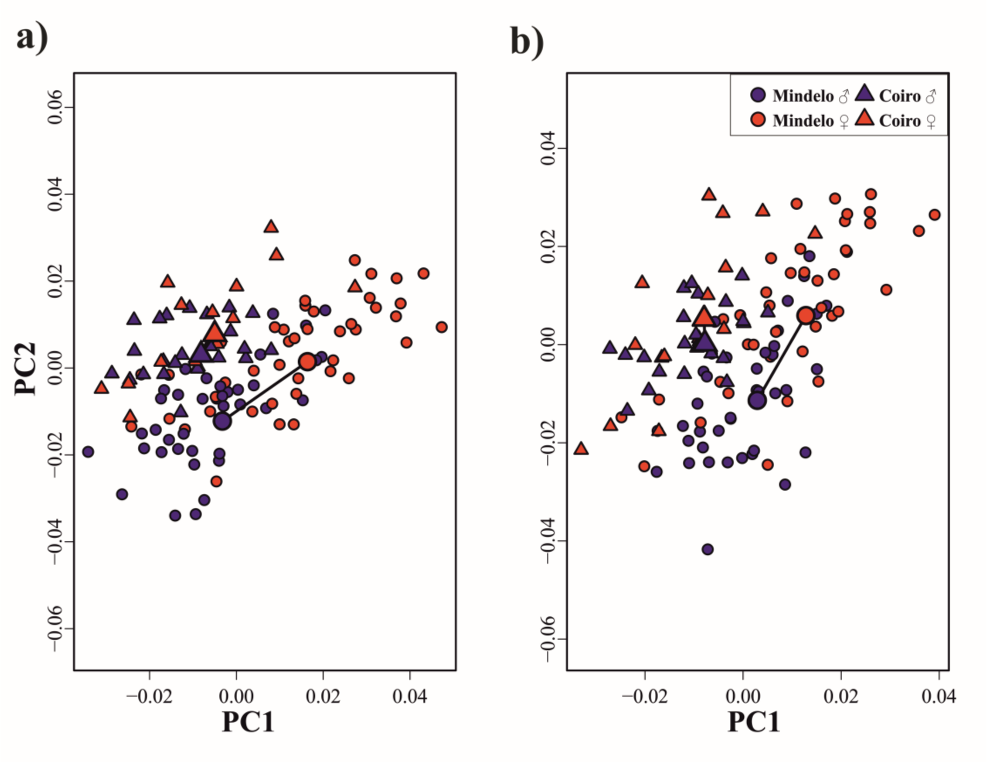


Figure 1.3. Shape trajectories and shape variation across populations and sexes, visualized as the two first principal components of the morphospace to maximize differences among group trajectories. (a) Raw shape variation (PC1= 33.23% and PC2= 16.98% of total variance); (b) shape variation after size correction (PC1= 32.27% and PC2= 17.01% of total variance).

The permutational ANOVA conducted showed that sexual size dimorphism existed in both populations of *S. salamandra*, where females had larger heads than males (Figure 1.5a). By contrast, head size did not differ between populations, nor did the degree of sexual size dimorphism between populations (Table 1.2b). Procrustes ANOVA including size as a covariate (Table 1.2c) revealed that a significant allometric relationship existed between head shape and head size, and that it followed a common slope across all groups. However, differences among populations, sexes and their interaction continued to exist after taking size variation into account. Inspection of shape-size allometry across groups revealed that this pattern was due to a marked differentiation in shape, independently of size variation, of females from Mindelo as compared to the rest of the groups (Figure 1.5b). Finally, the PTA on size-corrected data revealed that phenotypic change vectors for each population were not significantly different in size ($\Delta d = 0.008$; $P = 0.145$), but differences in vector direction were ($\theta = 1.728$; $P = 0.018$) (Figure 1.3b). Inspection of vector plots after size correction revealed similar patterns as those observed for Procrustes residuals, therefore, only the vector plots from size-corrected data are shown for visualization of shape change (Figure 1.4). This consistency in shape change among groups indicated that shape differences between the sexes in each population and between individuals of the same sex across populations were generally not influenced by size variation.

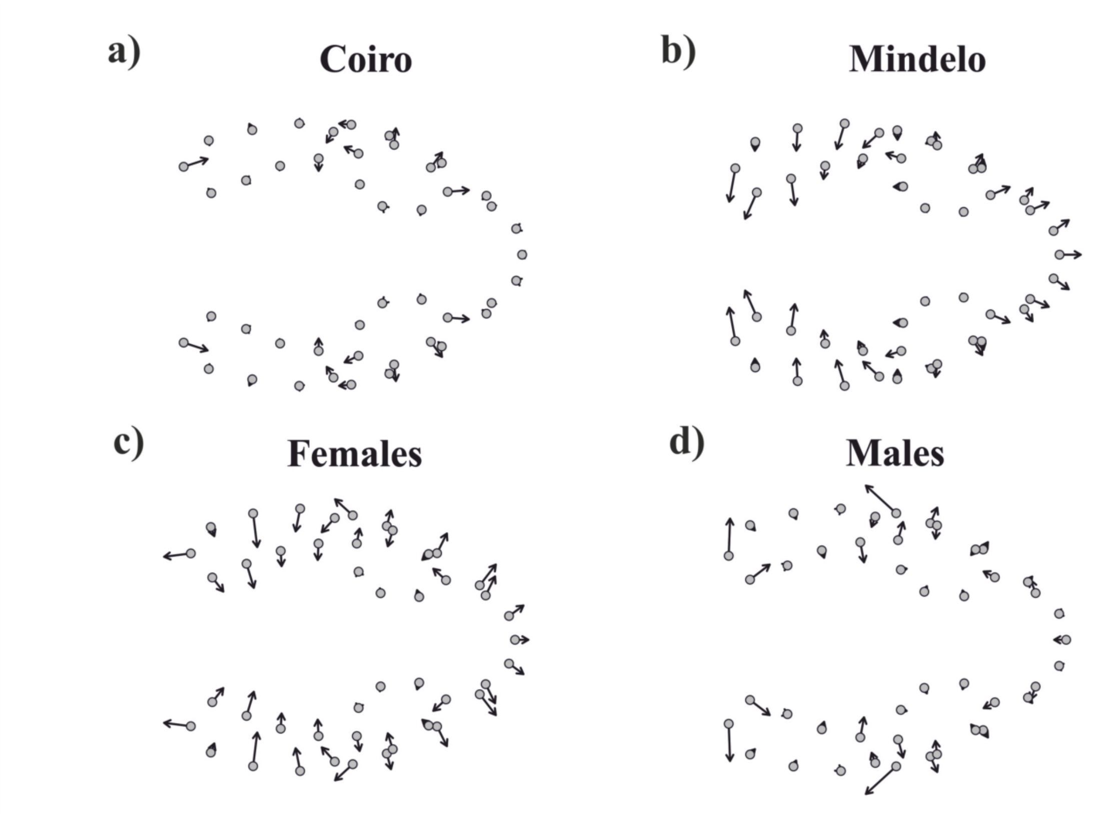


Figure 1.4. Vector plots showing shape change for the dorsal head view between the sexes (reference: females, target: males) from Coiro and Mindelo (a, b); and vector plots of shape change between the same sex from each population (reference: Mindelo, target: Coiro) (c, d). Shape change has been magnified by a factor of seven to facilitate visualization. All showed vectors were performed using shape data after size correction.

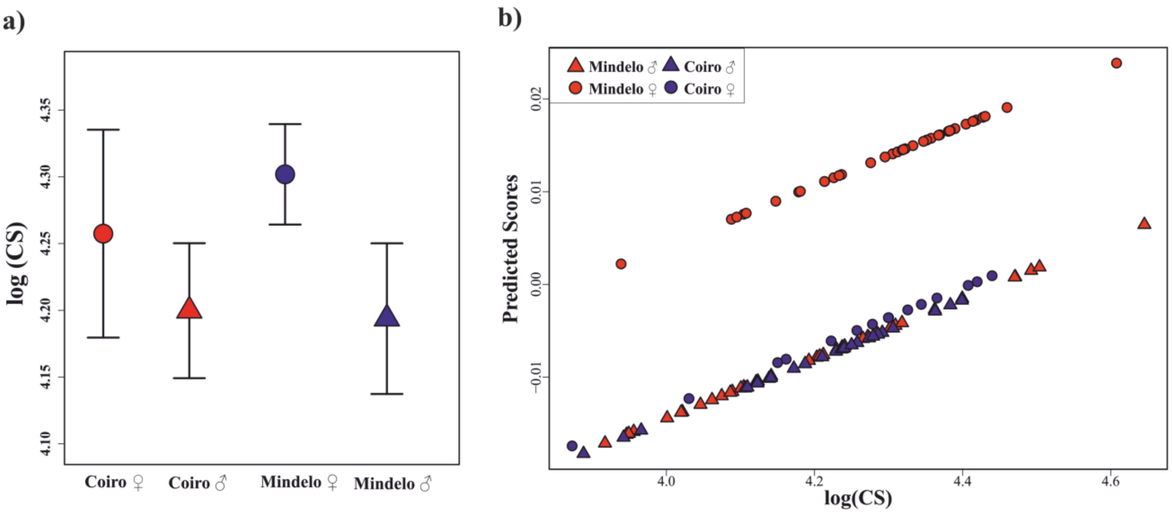


Figure 1.5. (a) Variation in mean head size (CS) across groups. Vertical bars denote 95% confidence intervals. (b) Shape-size allometry visualized as the relationship between predicted values of dorsal head shape and head size (CS) in each group.

DISCUSSION

Geometric morphometrics is a powerful tool for exploring the morphological diversification found in nature and for unmasking patterns of variation. However, the applicability of landmark-based GM could be constrained by a variety of sampling issues, such as the accessibility of different biological structures. Therefore, the development of methods allowing an accurate description, quantification and analysis of shape variation in a wider range of situations is of great interest. The non-invasive procedure described here for the exploration of dorsal head shape variation in urodeles using photographs of the external surface of this structure adds to the toolkit available for detecting fine-scale shape variation in amphibians (e.g., between and within populations of the same lineage), while exhibiting low error rate and high accuracy.

ACCURACY OF THE METHOD

Accurate landmark identification and digitization is the basis of GM methods, but the process of acquisition of landmark coordinates may involve several potential sources of error that can compromise repeatability and thus, the validity of the results obtained (Cramon-Taubadel et al. 2007). The use of skeletal elements in GM-studies facilitates landmark localization, because the structures used are more clearly defined (e.g. Ivanović et al. 2007; Clemente-Carvalho et al. 2008; Cjivanović et al. 2014). However, this approach also implies some drawbacks that restrict its range of applicability. That is the case of studies using museum specimens, where it is difficult to directly access bone structures without damaging the samples except through the use of resource-demanding solutions such as CT-scanning or other image-acquisition techniques. This limitation becomes even more relevant when working with natural populations, where one optimally tries to avoid sacrificing individuals.

The non-invasive GM-procedure described here potentially solves the aforementioned shortcomings, although its generality of application should be further investigated, for example by testing its efficiency when using museum specimens rather than living animals. Here, it has been proven to exhibit high accuracy and that intra-observer measurement error is much smaller in magnitude than mean variation across individuals (Figure 1.2). Most importantly, measurement error was non-systematic across individuals as the significant interaction between replicate and individual indicates (Table 1.1). When going through the photographs used in an a posteriori attempt to elucidate potential sources of measurement error, we found that individuals exhibiting the highest shape difference between replicate digitizations were those in which the parotids were not as well defined, being small and less conspicuous. In these cases, it was more difficult to empirically establish the limit of this structure, which calls for caution when locating landmarks in soft tissues of this type.

Although it has not been addressed in the present study, another potential and important source of error could come from the positioning of individuals when taking photographs. In the future, it could be interesting to test how it affects the accuracy of detected shape differences particularly when working on living, non-immobilized animals where the position of the head would depend on the animal movements. In our case, animals were completely anesthetized and head position was easily handled and positioned. Note, however, that differences across photographs of the same individual have been shown to constitute a minor source of shape variation, as compared to other sources, when a strict positioning protocol is followed (e.g., San Millán et al. 2015).

BIOLOGICAL SHAPE VARIATION

The method presented was remarkably efficient for describing shape patterns in our case study. Thus, the example dataset explored here illustrates how this landmark-based GM-procedure allows describing shape variation in the dorsal head shape of urodeles at a very fine biological scale (i.e., at the intra-lineage level).

First, the methodological procedure introduced allowed us to identify shape differences between the sexes, although these are known to be very subtle in *Salamandra salamandra* and had been scarcely studied in the past. The same occurs in most urodeles, in which sexual dimorphism in size has been broadly addressed whereas sexual dimorphism in shape remains much less studied (e.g., Romano et al. 2009; Ivanović and Kalezić 2012; Labus et al. 2012, 2013; Alcorn et al. 2013; Reinhard et al. 2015). The study of proportions has highlighted the presence of sexual shape dimorphism in several urodeles with weak differentiation between the sexes (Romano et al. 2009; Amat et al. 2015), even though its effectiveness in detecting shape variation could be too dependent of the structure or traits under study (Fabre et al. 2014). In the case of the fire salamander the only available study indicated that females have wider heads and longer parotid glands than males (Labus et al. 2013). In agreement with this observation, females from both populations exhibited relatively larger parotids than males, although only in one of the studied populations females had wider heads than males (Figure 1.4 a,b).

Furthermore, by applying the novel procedure described herein we could identify differences in shape between populations, as well as variation in the degree of sexual dimorphism between them, despite the fact that the populations belong to the same lineage (Velo-Antón et al. 2007; Mulder et al. 2016). Several morphological studies in *S. salamandra* have addressed differences at the intraspecific level to aid the description of subspecies (Bosch and López-Bueis 1994; Alcobendas et al. 1996; García-París et al. 2003), but diversity within subspecies has not been as widely explored (but see Cordero-Rivera et al. 2007; Beukema et al. 2016). Patterns observed from vector plots and PTA also reflect the existence of differences in sexual shape dimorphism in each population, with bigger differences in shape between sexes in the Mindelo population. While the reasons underlying this variation cannot be assessed with the data at hand, the observed patterns might reflect different levels of sexual selection, ecological selection, or both, acting differentially on each population, and within them, on each sex. Future studies should focus on the adaptive nature of detected sexual dimorphism and the causes that generated the observed patterns in these populations.

The proposed GM-techniques also allowed identifying variation in size. In agreement with previous studies that investigated head size variation in this species (Labus et al. 2013; Balogová and Uhrin 2015), females from both populations had larger heads than males, which might reflects variation in body size, where fecundity selection may be favouring larger body size in females (Kupfer 2007).

In addition, the study of the covariation between size and shape variation provided interesting insights into the proximate causes of head shape differentiation in *S. salamandra*. This phenotypic variation detected in the dorsal head shape as well as differences in the degree of sexual shape dimorphism result partially from differences in size. However, another part of variation is unrelated to size, indicating that other factors act exclusively on head shape both within and among populations. Differences in the direction of shape change vector between the sexes from each population after correcting for size effects could be reflecting different processes acting on each location. With respect to size effects on shape, however, the analysis of allometric relationships reveals common patterns of size-related shape change for both sexes and in both populations, suggesting that dorsal head shape is controlled by relatively rigid developmental mechanisms in *S. salamandra* (Larson 2004; Kaliontzopoulou et al. 2008; Lazić et al. 2015). Furthermore, the comparison of allometric trajectories allowed us to identify that size-independent differences among groups are mainly linked to modifications of head morphology occurring in females from Mindelo, while the morphology of males from this population are closer to Coiro individuals than to females of the same location (Figure 1.5b). This pattern was also detectable in vector plots, where shape change between females and males from Mindelo was similar to the shape change between females from both populations (Figure 1.4b,c) while differences between males from both populations, although present, were smaller (Figure 1.4d). Despite the data at hand do not allow inferring the biological causes of observed differences, they could have an adaptive significance, related for instance to variation across populations in the strength of sexual selection, sex-specific natural selection (e.g., related to diet), or if they are showing sex-specific phenotypic plasticity. Shape variation in Mindelo females could also reflect non-adaptive processes, such as genetic drift or sex-biased dispersal events, occurring at different rates on each population. Further studies considering more aspects of the biology of the species are needed to reach a robust biological conclusion. In the case of *S. salamandra*, it would be of interest to explore if and how differences in dorsal head morphology are related to the diversity that this species exhibits in reproductive styles, ontogeny, and ecology (García-París et al. 2003; Buckley et al. 2007; Velo-Antón and Buckley 2015; Velo-Antón et al. 2015).

In conclusion, the described patterns of morphological variation in *S. salamandra* exemplify how the presented GM procedure enhances the detection of fine-scale biological variation in the dorso-ventral view of the head of urodeles. This augments the tools available for the implementation of landmark-based GM-techniques to explore morphological variation in this group, and it could help to elucidate the biological processes responsible for the high levels of diversity observed within and across urodele taxa, as well as the implications of shape variation for species performance and adaptability.

REFERENCES

Adams, D. C. 2004. Character displacement via aggressive interference in Appalachian salamanders. Ecology 85:2664–2670.

———. 2010. Parallel evolution of character displacement driven by competitive selection in terrestrial salamanders. BMC Evolutionary Biology 10:72.

———. 2011. Quantitative genetics and evolution of head shape in *Plethodon* salamanders. Evolutionary Biology 38:278.

———. 2014. A method for assessing phylogenetic least squares models for shape and other high-dimensional multivariate data. Evolution 68:2675–2688.

Adams, D. C., and M. L. Collyer. 2009. A general framework for the analysis of phenotypic trajectories in evolutionary studies. Evolution 63:1143–1154.

Adams, D. C., M. L. Collyer, A. Kaliontzopoulou, and E. Sherratt. 2016. geomorph: Software for geometric morphometric analyses.

Adams, D. C., and R. N. Felice. 2014. Assessing trait covariation and morphological integration on phylogenies using evolutionary covariance matrices. PloS one 9:e94335.

Adams, D. C., and A. Nistri. 2010. Ontogenetic convergence and evolution of foot morphology in European cave salamanders (Family: Plethodontidae). BMC Evolutionary Biology 10:216.

Adams, D. C., and E. Otárola-Castillo. 2013. geomorph: An R package for the collection and analysis of geometric morphometric shape data. Methods in Ecology and Evolution 4:393–399.

Adams, D. C., and F. J. Rohlf. 2000. Ecological character displacement in *Plethodon*: biomechanical differences found from a geometric morphometric study. Proceedings of the National Academy of Sciences 97:4106–4111.

Adams, D. C., F. J. Rohlf, and D. E. Slice. 2004. Geometric morphometrics: ten years of progress following the ‘revolution.’ Italian Journal of Zoology 71:5–16.

———. 2013. A field comes of age: geometric morphometrics in the 21st century. Hystrix 24:7.

Alcobendas, M., H. Dopazo, and P. Alberch. 1996. Geographic variation in allozymes of populations of *Salamandra salamandra*. Journal of Evolutionary Biology 9:83–102.

Alcorn, M. A., J. Deitloff, S. P. Graham, and E. K. Timpe. 2013. Sexual dimorphism in head shape, relative head width, and body size of *Eurycea aquatica* and *Eurycea cirrigera*. Journal of Herpetology 47:321–327.

Amat, F., N. Oromí, D. Sanuy, and S. Carranza. 2015. Sexual dimorphism and age structure of the Montseny newt (*Calotriton arnoldi*). Amphibia-Reptilia 36:245–252.

Arbour, J., and C. Brown. 2014. Incomplete specimens in geometric morphometric analyses. Methods in Ecology and Evolution 5:16–26.

Arif, S., D. C. Adams, and J. A. Wicknick. 2007. Bioclimatic modelling, morphology, and behaviour reveal alternative mechanisms regulating the distributions of two parapatric salamander species. Evolutionary Ecology Research 9:843.

Balogová, M., E. Nelson, M. Uhrin, M. Figurová, V. Ledecký, and B. Zyśk. 2015. No sexual dimorphism detected in digit ratios of the fire salamander (*Salamandra salamandra*). The Anatomical Record 298:1786–1795.

Balogová, M., and M. Uhrin. 2015. Sex-biased dorsal spotted patterns in the fire salamander (*Salamandra salamandra*). Salamandra 51:12–18.

Bas, S., and F. Gasser. 1994. Polytypism of *Salamandra salamandra* (L.) in north-western Iberia. Mertensiella 4:41–74.

Beukema, W., A. G. Nicieza, A. Lourenço, and G. Velo-Antón. 2016. Colour polymorphism in *Salamandra salamandra* (Amphibia: Urodela), revealed by a lack of genetic and environmental differentiation between distinct phenotypes. Journal of Zoological Systematics and Evolutionary Research 54:127–136.

Bichuette, M. E., B. Rantin, E. Hingst-Zaher, and E. Trajano. 2015. Geometric morphometrics throws light on evolution of the subterranean catfish *Rhamdiopsis krugi* (Teleostei: Siluriformes: Heptapteridae) in eastern Brazil. Biological journal of the Linnean Society 114:136–151.

Bookstein, F. 1991. Morphometric Tools for Landmark Data: Geometry and Biology. Cambridge: Cambridge University Press.

Bookstein, F. L. 1997. Landmark methods for forms without landmarks: morphometrics of group differences in outline shape. Medical image analysis 1:225–243.

Bosch, J., and I. López-Bueis. 1994. *Salamandra bejarae* (wolterstorff, 1934) and (Müller & Hellmich, 1935). Herpetological Journal 4:46–48.

Brodie, E. D. 1983. Antipredator adaptations of salamanders: evolution and convergence among terrestrial species. Pages 109–133 *in* N. S. Margaris, M. Arianoutsou-Faraggitaki, and R. J. Reiter, eds. Adaptations to terrestrial environments. Springer, Boston.

Buckley, D., M. García-París, and M. H. Wake. 2007. Heterochrony, canibalism, and the evolution of viviparity in *Salamandra salamandra*. Evolution and Development 9:105–115.

Cherty, L. M., S. M. Case, and A. C. Wilson. 1978. Frog perspective on the morphological difference between humans and chimpanzees. Science 200:209–211.

Clemente-Carvalho, R. B. G., L. R. Monteiro, V. Bonato, H. S. Rocha, G. R. Pereira, D. F. Oliveira, R. T. Lopes, et al. 2008. Geographic variation in cranial shape in the pumpkin toadlet (*Brachycephalus ephippium*): a geometric analysis. Journal of Herpetology 42:176–186.

Collyer, M. L., and D. C. Adams. 2007. Analysis of two-state multivariate phenotypic change in ecological studies. Ecology 88:683–692.

———. 2013. Phenotypic trajectory analysis : comparison of shape change patterns in evolution and ecology. Hystrix, Italian Journal of Mammalogy. 24:75–83.

Collyer, M. L., D. Sekora, and D. C. Adams. 2015. A method for analysis of phenotypic change for phenotypes described by high- dimensional data. Heredity 115:357–365.

Cordero Rivera, A., G. Velo-Antón, and P. Galán. 2007. Ecology of amphibians in small coastal Holocene islands: local adaptations and the effect of exotic tree plantations. Munibe 25:94–103.

Corti, M. 1993. Geometric morphometrics: An extension of the revolution. Trends in Ecology and Evolution 8:302–303.

Cramon-Taubadel, N., B. C. Frazier, and M. M. Lahr. 2007. The problem of assessing landmark error in geometric morphometrics: theory, methods, and modifications. American Journal of Physical Anthropology: The Official Publication of the American Association of Physical Anthropologists 134:24–35.

Cvijanović, M., A. Ivanović, M. L. Kalezić, and M. L. Zelditch. 2014. The ontogenetic origins of skull shape disparity in the *Triturus cristatus* group. Evolution & development 16:306–317.

Degani, G. 1986. Plasma proteins and morphology of *Salamandm salamandra* in Israel. Amphibia-Reptilia 7:105-114

Deitloff, J., J. D. Petersen, and D. C. Adams. 2013. Complex species interactions lead to unpredictable outcomes in *Plethodon*. Herpetologica 69:1–10.

Dujardin, J.-P. 2008. Morphometrics applied to medical entomology. Infection, Genetics and Evolution 8:875–890.

Fabre, A., R. Cornette, K. Huyghe, D. V Andrade, and A. Herrel. 2014. Linear versus geometric morphometric approaches for the analysis of head shape dimorphism in lizards. Journal of Morphology 275:1016–1026.

García-París, M., M. Alcobendas, D. Buckley, and D. B. Wake. 2003. Dispersal of viviparity across contact zones in Iberian populations of fire salamanders (*Salamandra*) inferred from discordance of genetic and morphological traits. Evolution 57:129–43.

Gould, S. J. 1966. Allometry and size in ontogeny and phylogeny. Biological Reviews 41:587–638.

Gunz, P., and P. Mitteroecker. 2013. Semilandmarks: a method for quantifying curves and surfaces. Hystrix, the Italian Journal of Mammalogy 24:103–109.

Gunz, P., P. Mitteroecker, S. Neubauer, G. W. Weber, and F. L. Bookstein. 2009. Principles for the virtual reconstruction of hominin crania. Journal of Human Evolution 57:48–62.

Hanken, J. 1999. Larvae in amphibian development and evolution. Pages 61–108 *in* B. K. Hall, and M. H. Wake, eds. The origin and evolution of larval forms. Elsevier.

Ivanović, A., M. Cvijanović, and M. L. Kalezić. 2011. Ontogeny of body form and metamorphosis: Insights from the crested newts. Journal of Zoology 283:153–161.

Ivanović, A., and M. L. Kalezić. 2012. Sexual dimorphism in the skull geometry of newt species of *Ichthyosaura*, *Triturus* and *Lissotriton* (Salamandridae, Caudata, Amphibia). Zoomorphology 131:69–78.

Ivanović, A., K. Sotiropoulos, G. Džukić, and M. L. Kalezić. 2009. Skull size and shape variation versus molecular phylogeny: a case study of alpine newts (*Mesotriton alpestris*, Salamandridae) from the Balkan Peninsula. Zoomorphology 128:157–167.

Ivanović, A., K. Sotiropoulos, N. Üzüm, G. Džukić, K. Olgun, D. Cogălniceanu, and M. L. Kalezić. 2012. A phylogenetic view on skull size and shape variation in the smooth newt (*Lissotriton vulgaris*, Caudata, Salamandridae). Journal of Zoological Systematics and Evolutionary Research 50:116–124.

Ivanović, A., T. D. Vukov, G. Džukić, N. Tomašević, and M. L. Kalezić. 2007. Ontogeny of skull size and shape changes within a framework of biphasic lifestyle: a case study in six *Triturus* species (Amphibia, Salamandridae). Zoomorphology 126:173–183.

Jaekel, M., and D. B. Wake. 2007. Developmental processes underlying the evolution of a derived foot morphology in salamanders. Proceedings of the National Academy of Sciences 104:20437–20442.

Johnson, J. B., D. B. Burt, and T. J. DeWitt. 2008. Form, function, and fitness: pathways to survival. Evolution 62:1243–1251.

Kaliontzopoulou, A. 2011. Geometric morphometrics in herpetology: modern tools for enhancing the study of morphological variation in amphibians and reptiles. Basic and Applied Herpetology 25:5–32.

Kaliontzopoulou, A., M. Carretero, and G. Llorente. 2008. Head shape allometry and proximate causes of head sexual dimorphism in *Podarcis* lizards: Joining linear and geometric morphometrics. Biological Journal of the Linnean Society 93:111–124.

Kästle, W. 1986. Rival combats in *Salamandra salamandra*. Studies in herpetology 525–528.

Klingenberg, C. P. 2010. Evolution and development of shape: Integrating quantitative approaches. Nature Reviews Genetics 11:623–635.

———. 2016. Size, shape, and form: concepts of allometry in geometric morphometrics. Development genes and evolution 226:113–137.

Kupfer, A. 2007. Sexual size dimorphism in amphibians: an overview. Pages 50-59 *in* D. J. Fairbrain, W. U. Blanckenhorn, and T. Szèkely, eds. Sex, size and gender roles: evolutionary studies of sexual size dimorphism. Oxford University Press. Oxford.

Labus, N., M. Cvijanović, and T. D. Vukov. 2013. Sexual size and shape dimorphism in *Salamandra salamandra* (Amphibia, Caudata, Salamandridae) from the central Balkans. Archives of Biological Science 65:969–975.

Labus, N., T. D. Vukov, K. Ljubisavljević, and G. Džukić. 2012. Morphological variability without geographical structuring in the fire salamander (*Salamandra salamandra,* Salamandridae) from the Central Balkans. North-Western Journal of Zoology 8.

Larson, P. M. 2004. Chondrocranial morphology and ontogenetic allometry in larval *Bufo americanus* (Anura, Bufonidae). Zoomorphology 123:95–106.

Lawing, A. M., and P. D. Polly. 2010. Geometric morphometrics: Recent applications to the study of evolution and development. Journal of Zoology 280:1–7.

Lazić, M. M., M. A. Carretero, J. Crnobrnja-Isailović, and A. Kaliontzopoulou. 2015. Effects of environmental disturbance on phenotypic variation: an integrated assessment of canalization, developmental stability, modularity, and allometry in lizard head shape. The American Naturalist 185:44–58.

Magwene, P. M. 2001. Comparing ontogenetic trajectories using growth process data. Systematic Biology 50:640–656.

Marcus, L., E. Hingst-Zaher, and H. Zaher. 2000. Application of landmark morphometrics to skulls representing the orders of living mammals. Hystrix, the Italian Journal of Mammalogy 11.

Marugán-Lobón, J., A. Watanabe, and S. Kawabe. 2016. Studying avian encephalization with geometric morphometrics. Journal of anatomy 229:191–203.

Mitteroecker, P., P. Gunz, S. Windhager, and K. Schaefer. 2013. A brief review of shape, form, and allometry in geometric morphometrics, with applications to human facial morphology. Hystrix, the Italian Journal of Mammalogy 24:59–66.

Moen, D. S., D. J. Irschick, and J. J. Wiens. 2013. Evolutionary conservatism and convergence both lead to striking similarity in ecology, morphology and performance across continents in frogs. Proceedings of the Royal Society B: Biological Sciences 280:20132156.

Mulder, K. P., A. Lourenço, M. Carneiro, and G. Velo-Antón. 2016. The complete mitochondrial genome of *Salamandra salamandra* (Amphibia: Urodela: Salamandridae). Mitochondrial DNA Part B 1:880–882.

O'Leary, M. A., and S. G. Kaufman. 2012. MorphoBank 3.0: Web application for morphological phylogenetics and taxonomy. Available at website http://www. morphobank. org.

Pasmans, F., and H. Keller. 2000. Morphological variation in neighbouring populations of *Salamandra salamandra bernardezi* in northern Spain. Zeitschr Feldherp 7:77–84.

Ponssa, M. L., and M. F. V. Candioti. 2012. Patterns of skull development in anurans: size and shape relationship during postmetamorphic cranial ontogeny in five species of *the Leptodactylus fuscus* Group (Anura: Leptodactylidae). Zoomorphology 131:349–362.

R Development Core Team. 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Reinhard, S., S. Renner, and A. Kupfer. 2015. Sexual dimorphism and age of Mediterranean salamanders. Zoology 118:19–26.

Rohlf, F. J. 1999. Shape statistics: Procrustes superimpositions and tangent spaces. J Classif 16:197–223.

———. 2002. Geometric morphometrics and phylogeny. Systematics Association Special Volume 64:175–193.

———. 2015. TpsDig2, digitize landmarks and outlines. State University of New York.

Rohlf, F. J., and L. Marcus. 1993. A revolution in morphometrics. Trends in Ecology and Evolution 8:129–132.

Rohlf, F. J., and D. E. Slice. 1990. Extensions of the Procrustes method for the optimal superimposition of landmarks. Systematic Biology 39:40–59.

Romano, A., G. Bruni, and C. Paoletti. 2009. Sexual dimorphism in the Italian endemic species *Salamandrina perspicillata* (Savi, 1821) and testing of a field method for sexing salamanders. Amphibia-Reptilia 30:425.

San Millán, M., A. Kaliontzopoulou, C. Rissech, and D. Turbón. 2015. A geometric morphometric analysis of acetabular shape of the primate hip joint in relation to locomotor behaviour. Journal of human evolution 83:15–27.

Slice, D. E. 2005. Modern morphometrics. Pages 1–45 *in* D. E. Slice, ed. Modern morphometrics in physical anthropology. Springer. USA.

Stuart, B. L., R. F. Inger, and H. K. Voris. 2006. High level of cryptic species diversity revealed by sympatric lineages of Southeast Asian forest frogs. Biology letters 2:470–474.

Swiderski, D. L. 2003. Separating size from allometry: analysis of lower jaw morphology in the fox squirrel, *Sciurus niger*. Journal of Mammalogy 84:861–876.

Trueb, L. 1993. Patterns of cranial diversity among the Lissamphibia. Pages 255–343 *in* J. Hanken, and B. K. Hall. The skull. Volume 2. Uiversity of Chicago Press.

Van Buskirk, J. 2009. Natural variation in morphology of larval amphibians: phenotypic plasticity in nature? Ecological monographs 79:681–705.

Velo-Antón, G., and D. Buckley. 2015. Salamandra común – *Salamandra salamandra* (Linnaeus, 1758). Enciclopedia Virtual de los Vertebrados Españoles 2–12.

Velo-Antón, G., M. García-París, P. Galán, and A. Cordero-Rivera. 2007. The evolution of viviparity in holocene islands: ecological adaptation versus phylogenetic descent along the transition from aquatic to terrestrial environments. Journal of Zoological Systematics and Evolutionary Research 45:345–352.

Velo-Antón, G., X. Santos, I. Sanmartín-Villar, A. Cordero-Rivera, and D. Buckley. 2015. Intraspecific variation in clutch size and maternal investment in pueriparous and larviparous *Salamandra salamandra* females. Evolutionary Ecology 29:185–204.

Zelditch, M. L., D. L. Swiderski, D. H. Sheets, and W. L. Fink. 2004. Geometric morphometrics for biologists: A primer. Elsevier Academic Press. Waltham, MA.

Chapter 2

EVOLUTIONARY HISTORY AND NOT HETEROCHRONIC MODIFICATIONS ASSOCIATED WITH VIVIPARITY DRIVE HEAD SHAPE DIFFERENTIATION IN A REPRODUCTIVE POLYMORPHIC SPECIES, *SALAMANDRA SALAMANDRA*[†]

ABSTRACT

Identifying the evolutionary processes that underlie morphological variation at the intraspecific level is cornerstone for understanding the drivers of phenotypic diversity at higher macro-evolutionary scales. The fire salamander, *Salamandra salamandra*, exhibits exceptional intraspecific variation in multiple phenotypic traits (i.e. body size, head shape, coloration, reproductive modes). Pueriparity (females lying fully metamorphosed juveniles) in *S. salamandra* entails modifications during embryonic development in comparison to larviparity (females laying aquatic larvae), which is the ancestral reproductive mode. These heterochronic modifications involve a general acceleration of development, and intrauterine active feeding. In the present study, we i) describe the main features of head shape variation in adults of the two distinct subspecies of *Salamandra salamandra* that independently evolved to pueriparity, and ii) explore the morphological consequences of developmental and functional changes related to this major evolutionary shift. Our results show that evolutionary history, and not reproductive mode, is the main driver of head shape variation in *S. salamandra*. These results suggest different evolutionary processes acting differentially on each subspecies, at least at the adult stage. The present study highlights the importance of comparative studies integrating evolutionary histories and ontogenetic trajectories to explore the different sources of observed morphological diversification.

[†] This chapter in based on:

Alarcón-Ríos, L., Nicieza A. G., Kaliontzopoulou A., Buckley D. & G. Velo-Antón. (2019). Evolutionary history and not heterochronic modifications associated with viviparity drive head shape differentiation in a reproductive polymorphic species, *Salamandra salamandra.* **Evolutionary Biology.** ***Accepted.***

INTRODUCTION

Phenotypic variation is the raw material that might be later filtered by selection to shape phenotypic diversity among populations or species, driving evolutionary change. It results from the combined action of internal (genetic, developmental and physical) and external (environmental) factors, and their interactions (Alberch 1980; Carroll and Carrol 2001; Gould 1977; McNamara and McKinney 2005; West-Eberhard 2003). Therefore, the characterization of patterns of phenotypic diversity and the identification of their underlying mechanisms are crucial to understand how phenotypic variation is generated and maintained (Wake et al. 2011). This is especially true for species with complex life cycles, since the developmental processes and selective forces acting at specific developmental stages may be constraining, biasing, or modifying the patterns of phenotypic diversity observed at other stages of the life cycle (Moran 1994).

The evolution of reproductive strategies is tightly linked to phenotypic diversity resulting from modifications during development (e.g. McKinney, 1988; Sears 2014; Smith 2003). Amphibians constitute a clear example of how ontogenetic modifications drive changes in life-history traits and morphological diversity (Bruce 2003; Hanken 1989, 1999; Wake and Hanken, 1996; Wake, 2003). From the ancestral biphasic reproductive mode comprising aquatic eggs and larvae that undergo metamorphosis into terrestrial phenotypes, amphibian life histories have changed multiple times in the three living orders resulting in a wide variety of modes of reproduction and ontogenies (Gomez-Mestre et al. 2012; Wells 2007). Those innovations have major phenotypic implications, comprising changes in clutch and egg sizes, fertilization modes, behavior, physiology, and morphology (e.g. Duellman and Trueb 1986; Hanken 1999; Wells 2007). For instance, in urodele species with direct development, the formation of adult structures directly from the embryo averts constrains imposed by larval structures, either suppressed or simplified, and allows a higher morphological diversification (Hanken 1989; Wake and Hanken 1996; Wake 2003). Likewise, live-bearing, viviparity or pueriparity (sensu Greven 2003) (i.e. the retention of developing eggs within the females followed by the delivery of fully developed juveniles), is another example of how reproductive shifts result from developmental modifications of the ancestral amphibian life cycle (Wake 2003). In viviparous amphibian species, placental structures used to transfer nutrients from mother to progeny are substituted by elaborated maternal and fetal adaptations for matrotrophy, which imply the evolution of structural and functional modifications to facilitate pueriparity (e.g. the secretion of uterine nutrients and the evolution of special embryonic dentition for feeding) (Wells 2007; Blackburn 2015; Wake 2015 and references therein).

The fire salamander, *Salamandra salamandra* (Linnaeus 1758), is a widespread European amphibian renowned for its reproductive (Velo-Antón et al. 2015) and coloration polymorphism (see Beukema et al. 2016a; Donaire-Barroso and Rivera 2016), among other traits. It also exhibits high levels of morphological variation which, together with the observed intra-specific genetic structure, led to the description of 13 subspecies throughout its range (but see Beukema et al. 2016a), most occurring within the Iberian Peninsula (Velo-Antón and Buckley 2015). This species constitutes an exceptional case of reproductive polymorphism, with two reproductive modes co-occurring along its distribution range: an ancestral larviparous mode (i.e. females giving birth to free aquatic pre-metamorphic larvae), which occurs along most of its distribution and subspecies (Velo-Antón et al. 2015; Figure 2.1); a derived pueriparous mode (i.e. where females give birth to fully metamorphosed terrestrial juveniles), which is highly restricted, both geographically and phylogenetically. Pueriparity in *S. salamandra* evolved in *S. s. bernardezi* and expanded across northern Iberian Peninsula into the neighboring subspecies *S. s. fastuosa* range during the Pleistocene climatic oscillations (García-París et al. 2003; Uotila et al. 2013). At least one independent transition to pueriparity also occurred in two Holocene insular populations of *S. s. gallaica* from north-western Iberia Velo-Antón et al. 2007, 2012), while mainland populations of *S. s. gallaica* (excluding a hybrid zone with *S. s. bernardezi*) retain the ancestral larviparity mode (Velo-Antón et al. 2015).

The evolution of pueriparity in *S. s. bernardezi* is associated to ontogenetic changes along the embryonic development (Buckley et al. 2007; Dopazo and Alberch 1994). In particular, embryos development in pueriparous *S. salamandra* is characterized by a set of heterochronic modifications in comparison to the larviparous mode: i) a general acceleration of development; ii) hatching pre-displacement, and iii) precocious and accelerated growth of the anterior part of the body, the cephalic and pharyngeal structures, feeding apparatus, and digestive tract. This results in an asynchronous development among individuals, and enables intrauterine cannibalistic behavior in pueriparous developing embryos, facilitating early intrauterine feeding through oophagy over aborted eggs and adelphophagy by cannibalizing less-developed siblings (see Buckley et al. 2007). Intrauterine active feeding in pueriparous *S. s. bernardezi* leads to functional requirements at early embryonic stages that may entail an impact on musculoskeletal structures involved in feeding (Frederich et al. 2008; Hanken 1989; Kapralova et al. 2015; Kleinteich 2010; Turner 1998). Although metamorphosis may decouple morphological evolution between pre- and post-metamorphic stages (Ivanović et al. 2011; Sherratt et al. 2017), disparity between closely related urodele species seems to remain through metamorphosis in traits such as head shape (Cvijanović et al. 2014; Vučić et al. 2019). Therefore, early functional requirements can affect rates and patterns of ossification and may have long-term consequences on adult salamander morphology (Adriaens and Verraes 1998; Alberch and Blanco 1996; Koyabu and Son 2014; Wake and Hanken 1982). Indeed, qualitative differences in adult head shape have been empirically observed between larviparous *S. s. gallaica* (pointed snout) and pueriparous *S. s. bernardezi* (rounded snout), and used as a diagnostic trait in subspecies delimitation (Bas and Gasser 1994; Velo-Antón and Buckley 2015). However, such differences have never been quantitatively characterized or systematically analysed under an explicit phylogenetic/reproductive framework to identify whether and how they might be associated to reproductive mode.

Here we explore the origin of head shape differentiation within *S. salamandra* and its potential relation to the ontogenetic changes associated to shifts in reproductive modes in the species. First, we characterize and describe, for the first time, main features and differences in head shape in both subspecies and modes of reproduction. Then, we investigate whether different developmental pathways and early-life functional requirements associated to different modes of reproduction are related to head morphological variation in adult salamanders. For this, we performed a detailed comparison of the shape of the snout and jaws (hereafter “snout shape”) – the main head features potentially associated with the feeding implications of different reproductive strategies – across larviparous and pueriparous populations of *S. s. gallaica* and pueriparous populations of *S. s. bernardezi*. Specifically, we hypothesize that 1) shared changes in developmental sequences and in resources used (intrauterine cannibalism) may lead to a convergence of pueriparous salamanders in snout morphology. Alternatively, 2) differences in adult snout shape between *S. s. gallaica* and *S. s. bernardezi* can be independent from the reproductive mode, and rather result from the distinct evolutionary histories of the two subspecies. *S. salamandra* is an ideal model system for studying the eco-evolutionary implications of the emergence of an evolutionary novelty, and this study provides the first quantitative assessment of the potential morphological correlates of the evolution to viviparity.

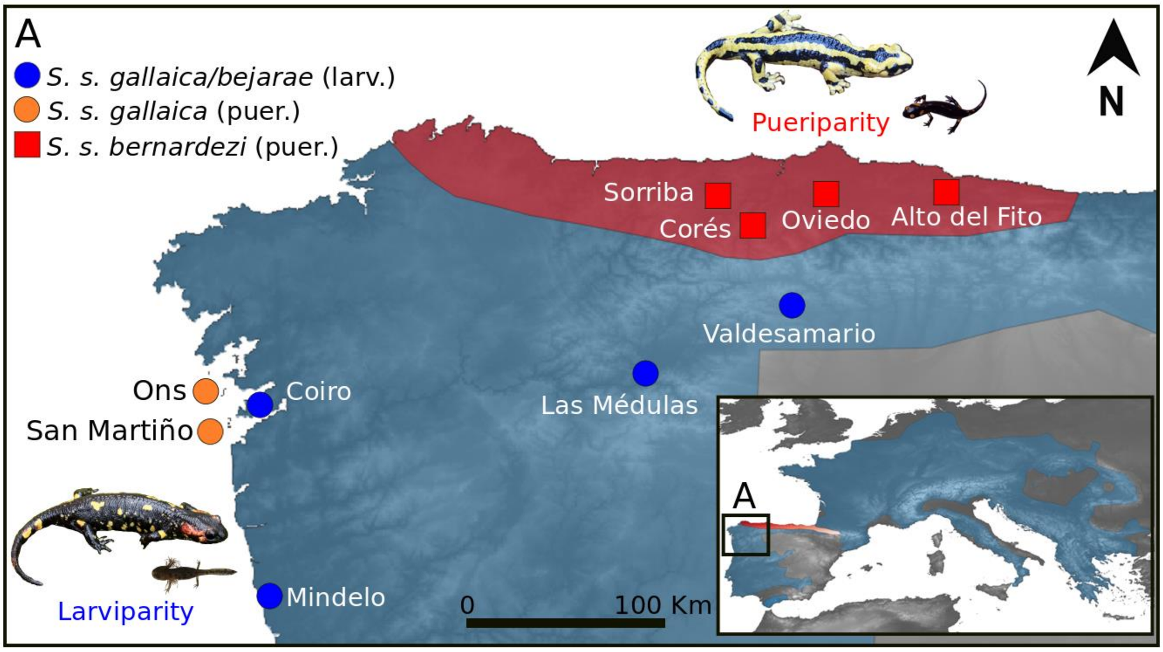


Figure 2.1. Map displaying the location of the studied populations. The inset shows the range of distribution of *S. salamandra* (in blue and red). The blue includes the distribution of all *S. salamandra* subspecies showing larviparity mode. The red west-east gradient corresponds to the area where pueriparity occurs (excluding the two insular populations of *S. s. gallaica*), with darker red representing exclusively pueriparous populations and lighter red those areas where both reproductive modes co-occur. Within A the range of distribution of *S. s. bernardezi* and *S. s. gallaica/bejarae* are represented in red and blue, respectively. The two insular and pueriparous populations of *S. s. gallaica* are represented in orange. Pictures of adult, juveniles and larvae salamanders are not scaled.

MATERIAL AND METHODS

STUDY SYSTEM AND SAMPLING DESIGN

This study is focused on the two *S. salamandra* subspecies that exhibit a pueriparous reproductive strategy: *S. s. bernardezi* and *S. s. gallaica* (Velo-Antón and Buckley 2015). The taxonomic rank of *S. salamandra* subspecies is being re-analysed using nuclear phylogenies since some subspecies description were based solely on mtDNA phylogenies (e.g. (Beukema et al. 2016a), and/or non-informative phenotypic characters. Thus, we exclude *S. s. fastuosa*, which shows a mixed reproductive strategy and similar phenotypic characteristics due to past hybridization and introgression events with *S. s bernardezi* (García-París et al. 2003; Uotila et al. 2013). We also consider *S. s. bejarae* as synonym of *S. s. gallaica* due to the existence of several sublineages intermixed (phenotypically, genetically and ecologically) between the two subspecies, which form a monophyletic lineage (e.g. Beukema et al. 2016b; García-París et al. 2003; Velo-Antón et al. 2007). While *S. s. bernardezi* is strictly pueriparous and occurs along north-central Iberian Peninsula, *S. s. gallaica* is larviparous and widely distributed across western Iberia , with two pueriparous insular populations occurring in south-western Galicia (Velo-Antón et al. 2007, 2012) (Figure 2.1).

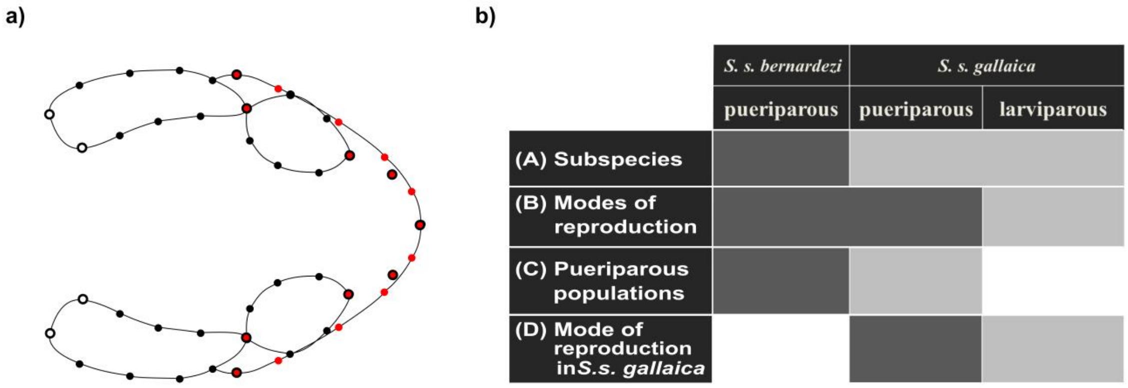
We selected six populations of *S. s. gallaica* (hereafter *gallaica*) including two pueriparous populations in the Ons and San Martiño islands, and four larviparous populations from two geographically separated mainland regions (two coastal, Coiro and Mindelo; and two inland populations, Las Médulas and Valdesamario; Fig. 1). To cover as much phenotypic and genetic diversity as possible within *S. s. bernardezi* (hereafter *bernardezi*), we selected two populations from its western range (Sorriba and Corés) and two eastern populations (Oviedo and Alto del Fito; Fig. 1). We examined a total of 504 live adults: 274 *S. s. gallaica* individuals (102 pueriparous, and 172 larviparous) and 230 of *S. s. bernardezi* (Table S2.1). We sampled individuals in rainy or high-moisture, no-windy nights in March-May and October-November 2015-2017. After collection, animals were transported to laboratory facilities (CIBIO or University of Oviedo, see Table S2.1) and processed following the methodology for image acquisition described in Alarcón-Ríos et al. (2017) (see Chapter 1). Salamanders were captured and measured under collection and ethical permits provided by regional or national governments (Galicia, Ref. 410/2015 and EB016/2018; Portugal Ref. 36490/2016/DRNCN/DGEFF; Castilla y León, EP/CyL/112/2017; Asturias, NºEXPTE:2017/001208; PROAE 10/2017). After recovery from anesthesia (benzocaine; Ethyl 4-aminobenzoate; Sigma-Aldrich, Darmstadt, Germany. Product number: E1501. Ref.: 112909) all the animals were released at the place of capture.

GEOMETRIC MORPHOMETRICS

We applied landmark-based geometric morphometrics (GM), following the methodology described in Alarcón-Ríos et al. (2017) (Chapter 1). These tools have been shown to be accurate for detecting differences at a very fine biological scale (e.g. sexual dimorphism within a population, intra-subspecific shape variation), while maintaining a low, non-systematic measurement error.

We digitized 13 fixed landmarks and 32 sliding semilandmarks on the dorsal view of salamanders' head (Figure 2.2a) using tpsDig2 (Rohlf 2015). Those 45 landmarks provide a comprehensive sampling and capture the most relevant features of salamander head morphology: snout, jaws, eyes and parotid glands (see Figure 1.1). Landmarks not accurately determined (e.g. due to lack of image quality), were recorded as missing data and their location was posteriorly estimated using multivariate regression (Arbour and Brown 2014; Gunz et al. 2009), as implemented in the function *estimate.missing* of *geomorph* R-package (Adams et al. 2017). Then, we obtained head shape variables through a generalized least-squares Procrustes superimposition (GPA; Rohlf 1999; Rohlf and Slice 1990) that standardized the size, translated, and rotated the landmark configurations. Because we were not interested in head asymmetry, we eliminated asymmetry effects by averaging the position of corresponding bilateral landmarks across the midline. As a measure of head size, we used the logarithm of Centroid Size (CS: Dryden and Mardia 2016).

We implemented all GM-operations and statistical analyses using package *geomorph* version 3.0.5 (Adams et al. 2017; Adams and Otárola-Castillo 2013) in the R-language for statistical programming (R Development Core Team, 2016).



	S. s. bernardezi	*S. s. gallaica*	
	pueriparous	pueriparous	larviparous
(A) Subspecies	▓	░	░
(B) Modes of reproduction	▓	▓	░
(C) Pueriparous populations	▓	░	
(D) Mode of reproduction in *S.s. gallaica*		▓	░

Figure 2.2. (a) Scheme of studied landmarks. White/red circles with a black edge are fixed landmarks while the smaller black/red circles are sliding semilandmarks. All of them were used in comparative analysis between subspecies to characterize and describe main features and differences in dorsal head shape. Colored in red are those landmarks used for testing hypotheses of snout shape variation.; (b) table summarizing groupings for testing different origins of snout shape differentiation: individuals grouped according to (A) the subspecies they belong to: *S. s. gallaica* or *S. s. bernardezi*; (B) the reproductive mode: pueriparous (*gallaica* and *bernardezi*) and larviparous (*gallaica*); (C) only pueriparous populations from both subspecies (*bernardezi* vs. pueriparous *gallaica*); and (D) different modes of reproduction within *gallaica* (pueriparous vs. larviparous). Different tones of grey denote the groups being compared in each analysis. For the last two comparisons, the group marked in white was not considered.

HEAD SHAPE CHARACTERIZATION

To characterize head shape of each studied subspecies we used the complete set of 45 digitized landmarks. To obtain a preliminary exploration of the patterns of variability in morphospace we performed a Principal Components Analysis (PCA). Then, we used distance-based permutation ANOVA to test for differences between subspecies in head shape and size, considering population of origin as a factor nested within subspecies. Because we expect differences in size between subspecies (Alcobendas and Castanet 2000), and to explore allometric relationships between head shape and size, we conducted a second Procrustes ANOVA including size as a covariate. Statistical significance was always assessed based on 10,000 random permutations, using Residual Randomization, RRPP (Collyer et al. 2015). To identify distinctive features of head shape in each subspecies, we visualized head shape changes between them using vector plots of Procrustes residuals.

As sexual dimorphism has been described in this species (Alarcón-Ríos et al. 2017; Labus et al. 2013), but was not the main focus of this study, we run preliminary analyses to test whether levels of sexual dimorphism differed between subspecies or modes of reproduction. Because levels of sex differentiation were similar between subspecies and reproductive modes, as indicated by a lack of a significant interaction between sex and these main factors (see Table S2.2), the sex of individuals was not considered for downstream analyses.

SNOUT SHAPE DIFFERENCES

Preliminary exploration of the data showed that most of shape variation is found in the posterior part of the head (parotids, see Results). However, most heterochronic changes in the developmental sequence of pueriparous salamanders involve structures that facilitate early feeding behavior, like the jaws and the snout. As this head region is functionally linked with shape variation due to the different feeding requirements and behavior of the two modes of reproduction, we focused on the snout for a more detailed exploration of our hypotheses. Thus, we reduced the landmarks used (after superimposition of the complete 45-landmarks set) to those from the anterior-most part of the head, including the jaw and eye limits, the nostrils and the snout (Fig. 2a). This provided shape and size variables for the head region of our interest, i.e. the snout.

To test for differences in snout shape between modes of reproduction and between subspecies, we performed four Procrustes ANOVAs, as implemented in the function *procD.lm* of the R package *geomorph* version 3.0.5 (Adams et al. 2017). This approach was necessary due to the biological particularities of the model system, which precludes a full factorial design for subspecies and modes of reproduction, since *S. s. bernardezi* is exclusively pueriparous. For this reason, we conducted a series of complementary ANOVA designs, using different criteria for grouping the data: subspecies, modes of reproduction, and the combination of modes and subspecies (Figure 2.2b). First, to examine the effect of evolutionary history, we grouped individuals according to the subspecies: *gallaica* or *bernardezi*. Second, to explore the potential effect of the reproductive mode, we compared pueriparous *gallaica* and *bernardezi* populations to larviparous *gallaica* populations. Third, to assess the contribution of evolutionary history uninfluenced by reproductive mode, we compared only pueriparous populations within subspecies, that is, pueriparous *gallaica* vs. pueriparous *bernardezi* (Figure 2.2b). Finally, to analyse the effect of reproductive mode regardless of any historical effects, we split the dataset and compared both modes of reproduction in *gallaica* only (pueriparous *gallaica* vs. larviparous *gallaica*, ignoring *bernardezi* individuals). Throughout, we followed the same analytical workflow (see below). In all the analyses, 'population' was nested within subspecies or reproductive mode.

We first performed Procrustes ANOVAs to test for snout shape and size differences between groups and populations in each set of comparisons. To explore shape allometry, and to test whether snout shape differences exist after accounting for size variation, we repeated the permutational Procrustes ANOVA including centroid size as a covariate and all the corresponding interactions with the main factors. This allowed us to investigate the covariation between shape and size, test for common allometric slopes between subspecies and/or modes of reproduction, and among populations, and evaluate group and population differentiation in shape while accounting for size effects. Throughout, we considered effect sizes (i.e. expressed as Z-scores, the standard deviates of F-statistics) to evaluate the contribution of different explanatory factors in describing variation in shape and size within and across different sets of analyses (Collyer et al. 2015; Collyer and Adams 2013). Shape variation between subspecies and modes of reproduction was visualized using vector plots.

RESULTS

HEAD SHAPE

Head shape variation as summarized by a PCA resulted in two first components that cumulatively explained 52.06% of variance. The two subspecies were clearly differentiated across morphospace, while the two modes of reproduction of *S. s. gallaica* highly overlapped (Figure 2.3a). Also within subspecies, populations differed in head shape and size (Table 2.1; Figure 2.3b).

Procrustes ANOVA including size as a covariate revealed that head shape significantly co-varied with head size (Table 2.1). Differences in head shape between subspecies were still present after taking size effects into account, and both of them presented a common allometric slope, as suggested by a non-significant interaction between subspecies and CS. On the other hand, there were differences in allometric slopes among populations within each subspecies (Table 2.1).

As populations differed in their allometric slopes (Size x population term in Table 2.1), size effects on shape variation could not be removed under a common slope. Thus, vector plots to visualize shape variation were calculated with Procrustes residuals, which include population-specific, size-related shape variation. These showed a shorter and more rounded snout in *S. s. bernardezi* as compared to *S. s. gallaica*. The eyes were also slightly more prominent but smaller than in *gallaica*, and positioned anteriorly, closer to the tip of the snout. Because of the shortening of the snout, the nostrils were aligned with the tip of the snout in *bernardezi*, while in *gallaica* they were located in a posterior position. However, the most notorious shape difference was found in the posterior region of the head. While the front part of the head in *bernardezi* was wider (more rounded) than in *gallaica*, the posterior part was narrower and with proportionally larger parotids. In *gallaica*, the relative width of the jaw was generally smaller than that of the parotids, while in *bernardezi*, relative jaw width generally exceeded parotid width. This resulted in an elongated and oval-like head in *bernardezi*, with an approximate constant width from the parotids towards the rounded snout. In *gallaica*, instead, the posterior part was narrower, and more pointed anteriorly (close to the snout), resulting in an arrow-like shape (Figure 2.3c).

Table 2.1. Results of permutational ANOVAs used to test for differences in head shape (a) and size (b) of both *S. s. gallaica* and *S. s. bernardezi* and population of origin (nested within subspecie), and test for size-shape allometric relationship, size-free head shape differentiation between subspecies and localities and common allometric slopes among groups (c). For this analysis, we used the whole data set of 45 landmarks.

	df	**SS**	**F**	**Z**	***P***
a) Shape					
Subspecies	1	0.13696	14.2444	4.6657	0.0001
Population	8	0.07692	7.9957	12.2507	0.0001
Residuals	494	0.59404			
b) Size					
Subspecies	1	6.7524	68.7514	2.1142	0.0003
Population	8	0.7857	5.0227	4.2277	0.0001
Residulas	494	9.6597			
c) Size as covariate					
Size	1	0.0784	67.2959	8.1696	0.0001
Subspecies	1	0.0646	7.2263	3.7008	0.0002
Population	9	0.0865	8.0484	12.3446	0.0001
Size x Subspecies	1	0.0012	0.9528	0.8195	0.2025
Size x Population	9	0.0161	1.6222	4.4621	0.0001
Residuals	496	0.58134			

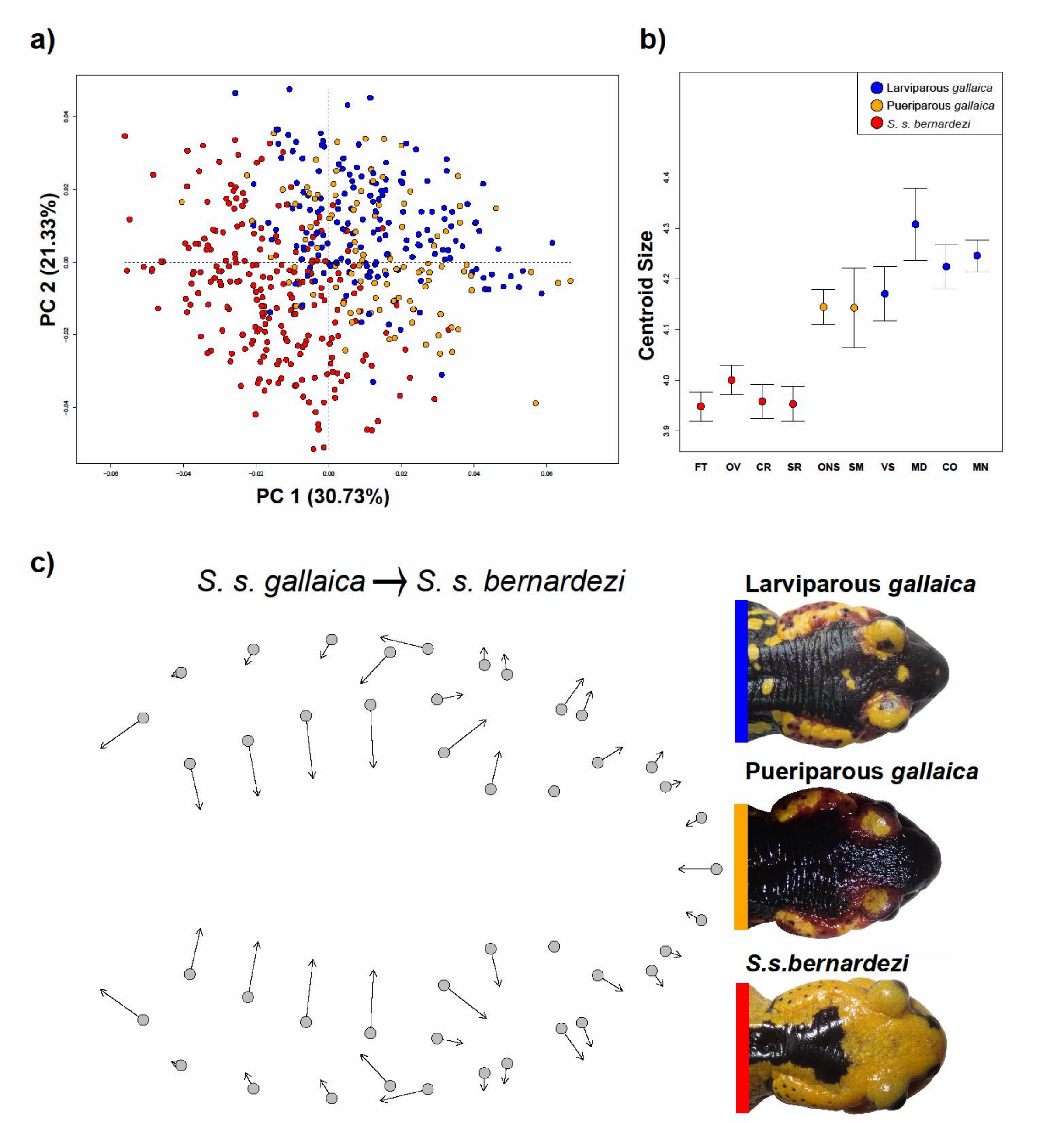
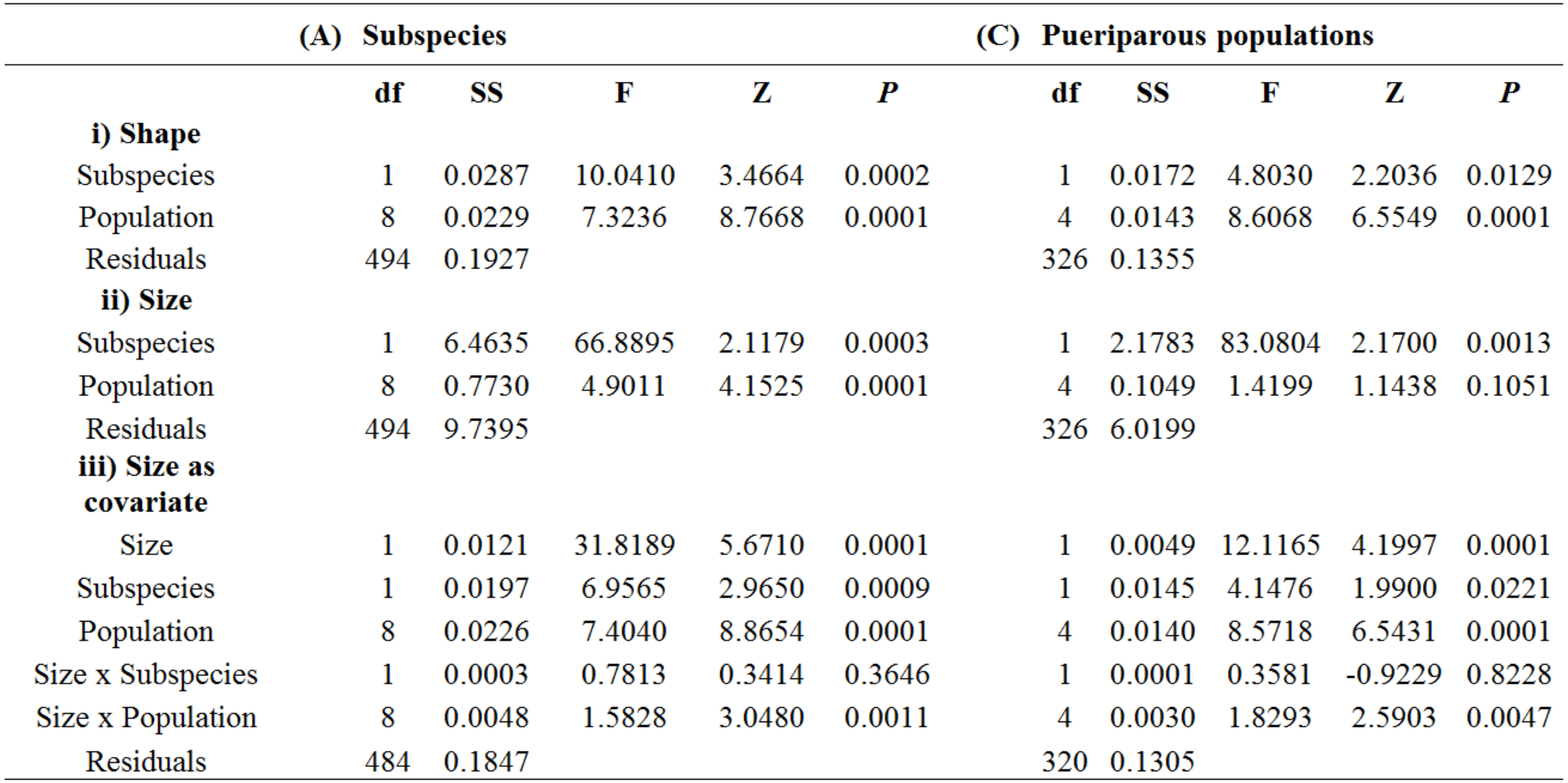


Figure 2.3. (a) Visualization of head shape variation in our sample on the two first principal components of the morphospace. Amount of variance explained by each PC is indicated in the corresponding axis. Different colors denote subspecies differentiated by the reproductive mode in *S. s. gallaica*. Shape data used include size-related shape variation. b) Variation in mean head size (logCS) across all studied populations. Vertical bars denote 95% confidence intervals. **FT**: Alto del Fito; **OV**: Oviedo; **CR**: Corés; **SR**: Sorriba; **Ons**: Illa de Ons; **SM**: San Martiño; **VS**: Valdesamario; **MD**: Las Médulas; **CO**: Coiro; **MN**: Mindelo. (c) Vector plot showing shape change for the dorsal head view between both subspecies reference: *gallaica*, target: *bernardezi*). Shape change has been magnified by a factor of five to facilitate visualization. Shape data used for vector generation includes size-related variation.

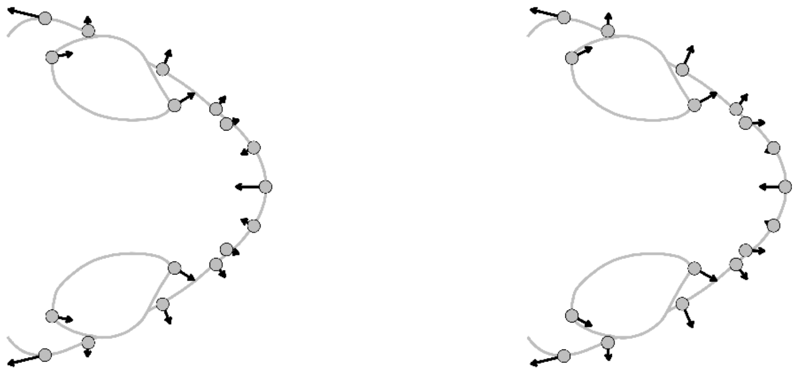
Table 2.2. Results of the four complementary Procrustes ANOVA using different criteria for grouping the data (subspecies or reproductive modes) in order to identify the origin of snout shape differences. In every four cases, we first tested for snout shape and size differences across groups (*i*, *ii* respectively) and then for size-shape covariation, common allometric slopes, and shape variation among selected groups (*iii*). (A) Snout shape and size variation according to subspecie (*S. s. gallaica* vs. *S. s. bernardezi*); (B) reproductive mode (pueriparous vs. larviparous); (C) pueriparous populations from both subspecies (*S. s. bernardezi* vs. pueriparous *gallaica*); (D) and modes within subspecie *gallaica* (pueriparous *gallaica* vs. larviparous *S. s. gallaica*). In all these cases, we used the data set of 17 landmarks.



	(A) Subspecies					**(C) Pueriparous populations**				
	df	**SS**	**F**	**Z**	***P***	**df**	**SS**	**F**	**Z**	***P***
i) Shape										
Subspecies	1	0.0287	10.0410	3.4664	0.0002	1	0.0172	4.8030	2.2036	0.0129
Population	8	0.0229	7.3236	8.7668	0.0001	4	0.0143	8.6068	6.5549	0.0001
Residuals	494	0.1927				326	0.1355			
ii) Size										
Subspecies	1	6.4635	66.8895	2.1179	0.0003	1	2.1783	83.0804	2.1700	0.0013
Population	8	0.7730	4.9011	4.1525	0.0001	4	0.1049	1.4199	1.1438	0.1051
Residuals	494	9.7395				326	6.0199			
iii) Size as covariate										
Size	1	0.0121	31.8189	5.6710	0.0001	1	0.0049	12.1165	4.1997	0.0001
Subspecies	1	0.0197	6.9565	2.9650	0.0009	1	0.0145	4.1476	1.9900	0.0221
Population	8	0.0226	7.4040	8.8654	0.0001	4	0.0140	8.5718	6.5431	0.0001
Size x Subspecies	1	0.0003	0.7813	0.3414	0.3646	1	0.0001	0.3581	-0.9229	0.8228
Size x Population	8	0.0048	1.5828	3.0480	0.0011	4	0.0030	1.8293	2.5903	0.0047
Residuals	484	0.1847				320	0.1305			

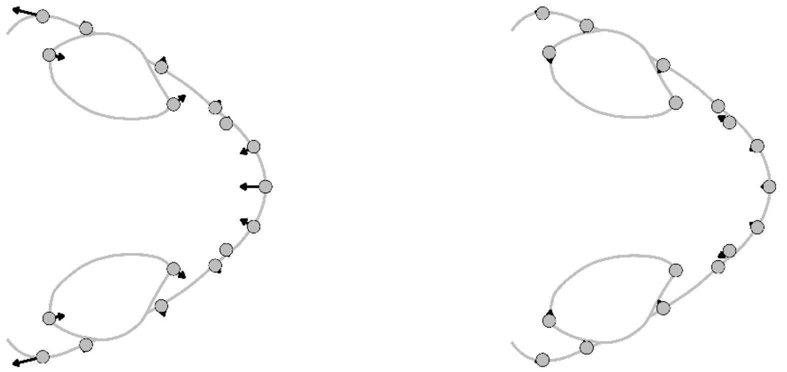
Table 2.2. (Continued)

	(B) Mode of reproduction					**(D) Mode of reproduction within *gallaica***				
	df	**SS**	**F**	**Z**	***P***	**df**	**SS**	**F**	**Z**	***P***
i) Shape										
Mode of reproduction	1	0.0134	2.8085	1.6729	0.0457	1	0.0019	0.8363	-0.0861	0.5383
Population	8	0.0381	12.2242	10.8471	0.0001	4	0.0091	6.1359	5.7030	0.0001
Residuals	494	0.1927				268	0.0988			
ii) Size										
Mode of reproduction	1	4.7212	15.0160	1.5488	0.0102	1	0.4358	7.4418		0.0563
Population	8	2.5153	15.9470	6.0959	0.0001	4	0.2343	2.4548	1.4987	0.0401
Residuals	494	9.7395				268	6.3937			
iii) Size as covariate										
Size	1	0.0121	31.8189	5.6710	0.0001	1	0.0020	5.6608	3.1244	0.0004
Mode of reproduction	1	0.0067	1.4996	0.7895	0.2162	1	0.0019	0.8664	-0.0336	0.5210
Population	8	0.0356	11.6572	10.6577	0.0001	4	0.0089	6.1721	5.7487	0.0001
Size x Mode	1	0.0004	0.9347	0.6039	0.2719	1	0.0002	0.5681	-0.3708	0.6459
Size x Population	8	0.0048	1.5636	3.0036	0.0014	4	0.0020	1.3725	1.4829	0.0719
Residuals	484	0.1847				262	0.0947			



a) Subspecies
S. s. gallaica → *S. s. bernardezi*

c) Pueriparous populations
S. s. gallaica → *S. s. bernardezi*



b) Mode of reproduction
Larviparous → Pueriparous

d) Mode of reproduction within *gallaica*
Larviparous → Pueriparous

Figure 2.4. Vector plots showing snout shape change between: (a) subspecies (reference: *S. s. gallaica*, target: *S. s. bernardezi*), (b) reproductive modes including all studied individuals (reference: larviparous, target: pueriparous), (c) pueriparous *gallaica* (reference) and pueriparous *bernardezi* (target) and, (d) between modes of reproduction within *gallaica* (reference: larviparous *gallaica*, target: pueriparous *gallaica*). Shape change has been magnified by a factor of five to facilitate visualization. Shape data used for vector generation include size-related variation.

SNOUT SHAPE

A more detailed exploration of the sources of variation in snout shape through complementary ANOVAs provided insights to the relative contribution of evolutionary history (i.e. as represented by subspecies) and modes of reproduction in shaping phenotypic patterns in this system. Procrustes ANOVAs revealed significant differences in snout shape and size both between subspecies (all *gallaica* or only pueriparous *gallaica* vs. *bernardezi*) (Table 2.2 A,C), and modes of reproduction (pueriparous *gallaica* and *bernardezi* vs. larviparous *gallaica*) (Table 2.2B). However, differences in snout shape or size between modes of reproduction within *gallaica* were not significant (Table 2.2D).

Population of origin always had a significant effect, both in comparisons conducted within modes of reproduction and within subspecies. On the other hand, snout size did not differ among pueriparous populations of either *gallaica* or *bernardezi* (Table 2.2C). We found a significant allometric relationship between snout shape and size in all performed analyses (Table 2.2). Both subspecies and reproductive modes followed common allometric slopes. However, within each group, population slopes were different, except when considering pueriparous and larviparous *gallaica* populations (Table 2.2D). After including size as a covariate, differences in snout shape between subspecies remained significant, but not between modes of reproduction (Table 2.2). In all the analyses, subspecies exhibited a larger effect sizes (Z-scores) on snout shape and size variation than mode of reproduction (Table 2.2). Similarly, subspecies had a somehow larger effect on snout shape variation when all *gallaica* populations were included than when only pueriparous *gallaica* were included (Table 2.2 A,C).

In accordance with those previous results, the inspection of vector plots showed that snout shape change between subspecies is approximately the same as the change between pueriparous *gallaica* and *bernardezi* (Figure 2.4 a,c). Main changes in snout shape between subspecies consisted in a shortening and widening of the snout, a posterior displacement of the jaws and a more anterior position of the eyes and nostrils. These changes result in a rounder snout with eyes and nostrils closer to the tip and edge of the snout and further from jaws in *bernardezi* individuals. Finally, there were no significant differences in snout shape between modes of reproduction within *gallaica* (ANOVA; Table 2.2D). Both modes of reproduction slightly differed in the position of the nostrils, which were more posterior in pueriparous *gallaica* with respect to the tip of the snout, as well as in the posterior end of the eyes, which were slightly displaced towards the midline of the head in pueriparous individuals (Figure 2.4d).

DISCUSSION

The evolution to pueriparity in *S. salamandra* encompasses a set of developmental and functional innovations at early developmental stages (e.g. precocious development of cephalic and pharyngeal structures that permit intrauterine cannibalism), but our results suggest that those modifications do not translate into distinct patterns of adults' head and snout morphologies. Instead, major differences in head and snout morphology are found between subspecies, constituting a lineage specific trait, and hence highlighting the distinct evolutionary histories of the two subspecies as the main driver of the observed morphological differences in *S. salamandra*.

HEAD SHAPE CHARACTERIZATION IN *S. S. GALLAICA* AND *S. S. BERNARDEZI*

Although differences in snout shape were previously used for subspecies description (see Velo-Antón and Buckley 2015), a formal characterization of head shape using morphometric tools had never been conducted for subspecies and modes of reproduction in *S. salamandra*. Our results clearly indicate morphological differentiation between adults of *S. s. gallaica* and *S. s. benardezi*. Observed head shape patterns seem to result from the deep evolutionary history of *S. salamandra* in the Iberian Peninsula (Antunes et al. 2018; García-París et al. 2003; Pereira et al. 2016; Steinfartz et al. 2000; Velo-Antón et al. 2007). Molecular studies focused on *S. s. bernardezi* and *S. s. gallaica* (Beukema et al. 2016a; García-París et al. 2003; Velo-Antón et al. 2007) support their phylogenetic independent origin and deep genetic structure, particularly within the former. Current subspecies distributions and intrasubspecific structure are likely the result of serial range expansions and contractions in response to glacial and interglacial periods during the Pleistocene, which together with the topographic complexity across its range of distribution, favored allopatric differentiation processes (Beukema et al. 2016a; García-París et al. 2003; Velo-Antón et al. 2007, 2012). Accordingly, we observed remarkably high variation in head shape and size, as well as in allometric trajectories, among populations within each lineage (Table 2.1; Table 2.2A). Previous studies on *S. salamandra* intraspecific diversity described high levels of variation between (polytypism), but also within (polymorphism) each described subspecies (Beukema et al. 2016a; Velo-Antón and Buckley 2015). Indeed, previous intrasubspecific comparative studies between reproductive modes within *S. s. gallaica* highlighted genetic, morphological, demographic, and behavioral differences between pueriparous (insular) and larviparous (coastal and mainland) populations (Lourenço et al. 2018; Velo-Antón et al. 2012; Velo-Antón and Cordero-Rivera 2017), but did not evaluate differences in snout and head shape. According to those studies, pueriparous *S. s. gallaica* present smaller body size than mainland populations, with differences between insular populations, being the San Martiño individuals the smallest. Although we did not find significant differences in head size between modes of reproduction within *S. s. gallaica*, the pueriparous populations appear in the inferior range of head sizes within this subspecies (Figure 2.3b).

Major differences in dorsal head shape between the two subspecies encompass differences in the shape of the eyes and the snout, but also in the posterior head region, with *bernardezi* displaying relatively larger parotids than *gallaica*. Parotid glands in amphibians are macroglands that synthesize, store, and release toxins with a key role in predator avoidance and pathogenic function (Lüddecke et al. 2018 and references therein). Indeed, during antipredator behavior *S. salamandra* individuals adopt a defensive posture lowering their head with the snout pointing to the ground, highlighting the toxin-loaded parotids to warn predators about their noxious poison and unpalatability (Brodie 1983; Stokes et al. 2015). Larger parotids in *bernardezi* presumably imply higher amounts of toxins (see Saporito et al. 2010), maybe resulting from higher predation pressures in this subspecies (see Vences et al. 2014). There are, however, very few studies exploring differences in predation risk between subspecies and its potential relationship with parotids size. High predation levels by a non-native predator over the salamander population in San Martiño island were recently suggested, and this predatory pressure likely resulted in a behavioral plastic shift into diurnal activity (Velo-Antón and Cordero-Rivera 2017) but did not correlate with changes in parotid size (Cordero-Rivera et al. 2007).

ORIGIN OF HEAD SHAPE DIFFERENTIATION IN *S. SALAMANDRA*: REPRODUCTIVE SHIFT VS. PHYLOGENETIC DESCENT

The lack of differences in snout shape between modes of reproduction (after considering size effects) suggests that developmental modifications associated with the evolution of pueriparity in *S. salamandra* is not the main driving force explaining the morphological differentiation in pueriparous *S. s. bernardezi*. Instead, the observed differences seem to be the result of other selective pressures or drift processes linked to the independent evolution of the studied subspecies. Moreover, the lack of differences in snout shape between larviparous and pueriparous *S. s. gallaica* reinforces the idea that heterochronic changes during embryonic development do not shape adult head morphology in *S. salamandra.*

Despite *S. s. bernardezi* and insular *S. s. gallaica* bearing completely metamorphosed terrestrial juveniles with no free aquatic larval stage, pueriparity has evolved independently in these subspecies (Velo-Antón et al. 2007). However, main developmental differences between pueriparous and larviparous fire-salamanders were described using individuals of *S. s. bernardezi* and larviparous *S. s. bejarae/gallaica* only (Buckley et al. 2007), and thus we cannot ensure that developmental mechanisms behind the evolution of pueriparity in *S. s. gallaica* populations are exactly the same as those described for *S. s. bernardezi*. Nonetheless, cannibalized eggs were observed inside the stomach of several new-born juveniles from the Ons population supporting the presence of intrauterine cannibalism phenomena also in pueriparous *gallaica* (Velo-Antón et al. 2015; personal observations). This behavior, together with the smaller broods and heavier juveniles of insular populations of *S. s. gallaica* (Velo-Antón et al. 2015) may suggest the existence of similar heterochronic development of feeding structures and possible precocious embryo hatching as observed in the pueriparous *S. s. bernardezi* (Buckley et al. 2007).

The evolution of distinct trophic morphologies related to the nature, quality, and amount of available food as a result of selective and plastic responses are common in many species of vertebrates (Smith and Skúlason 1996; Wimberger 1994). For example, within amphibians, cannibalism among larvae has been described to cause plastic changes in head shape of cannibals or the development of specialized cannibalistic morphs and structures (Wells 2007), both in anurans (e.g. Levis et al. 2015; Pfennig and Pfenning 1990) and urodeles (e.g. Collins and Cheek 1983). However, previous studies on head shape plasticity within *S. salamandra* in relation to diet suggest that the larvae of this species do not seem to develop diet-specific morphs (Manenti et al. 2018). Accordingly, the results presented here indicate a lack of shape differentiation between reproductive modes within *gallaica,* despite differences in resource use at early stages. Despite commonalities in development, pueriparous groups from different subspecies differ both in snout size and shape, even when considering differences in size and allometric effects. Therefore, it appears that the occurrence of early embryonic intrauterine active feeding in pueriparous populations from both subspecies would not impact adult head morphology, thus contradicting our initial hypothesis about head shape convergence in adult pueriparous *gallaica* and *bernardezi*. However, this convergence could still occur at earlier ontogenetic stages, and then being ‘erased’ during metamorphosis (Ivanović et al. 2011; Moran 1994; Sherratt et al. 2017). Similarly, considering no effects of metamorphosis on levels of head shape disparity (Vučić et al. 2019), new-born pueriparous juveniles from both subspecies may present similar head morphologies that later diverge throughout ontogeny (Adams and Nistri 2010; Urošević et al. 2013). Further studies comparing the developmental trajectories and potential heterochronic processes in pueriparous *gallaica* insular populations are needed to evaluate 1) to what extent both groups of pueriparous populations are comparable, and 2) assess whether early development have stage-specific effects on head morphology. This will contribute to a better understanding of the drivers of extremely high levels of phenotypic variation and diversity within *S. salamandra* and, in general, how individual (e.g., ontogenetic) and population processes relate to the (macro)-evolutionary trends observed among lineages or species.

CONCLUSIONS

Our results clearly supported the ‘phylogenetic hypothesis’ but not the ‘ecological convergence hypothesis’. Heterochronic changes and intrauterine cannibalism that characterize pueriparity in *S. salamandra* do not have a relevant impact on adult head morphology. Instead, head morphological changes are related to the distinct evolutionary histories of *S. s. bernardezi* and *S. s. gallaica*. Moreover, head shape should be considered a discriminant trait between these two subspecies. Further research is needed to understand how integrated or modular are the head anatomical structures, their ontogenetic development, and evolutionary history and potential (evolvability). Such an integrative study would entail considering the hierarchical structure of morphological diversity within and among subspecies, the evolutionary drivers of such structure, and all the potential processes acting at all the scales that can be underlying morphological variation within the species.

REFERENCES

Adams, D. C., M. L. Collyer, A. Kaliontzopoulou, and E. Sherratt. 2017. Geomorph: Software for geometric morphometric analyses. R package version 3.0.5.

Adams, D. C., and A. Nistri. 2010. Ontogenetic convergence and evolution of foot morphology in European cave salamanders (Family: Plethodontidae). BMC Evolutionary Biology 10:216.

Adams, D. C., and E. Otárola-Castillo. 2013. geomorph: An R package for the collection and analysis of geometric morphometric shape data. Methods in Ecology and Evolution 4:393–399.

Adriaens, D., and W. Verraes. 1998. Ontogeny of the osteocranium in the African catfish, *Clarias gariepinus* Burchell (1822) (Siluriformes: Clariidae): ossification sequence as a response to functional demands. Journal of Morphology 235:183–237.

Alarcón-Ríos, L., G. Velo-Antón, and A. Kaliontzopoulou. 2017. A non-invasive geometric morphometrics method for exploring variation in dorsal head shape in urodeles: sexual dimorphism and geographic variation in *Salamandra salamandra*. Journal of Morphology 278:475–485.

Alberch, P. 1980. Ontogenesis and morphological diversification. American Zoologist 20:653–667.

Alberch, P., and M. J. Blanco. 1996. Evolutionary patterns in ontogenetic transformation: from laws to regularities. International Journal of Developmental Biology 40:845–858.

Alcobendas, M., and J. Castanet. 2000. Bone growth plasticity among populations of *Salamandra salamandra*: interactions between internal and external factors. Herpetologica 56:14–26.

Antunes, B., A. Lourenço, G. Caeiro-Dias, M. Dinis, H. Gonçalves, I. Martínez-Solano, P. Tarroso, et al. 2018. Combining phylogeography and landscape genetics to infer the evolutionary history of a short-range Mediterranean relict, *Salamandra salamandra longirostris*. Conservation genetics 19:1411–1424.

Arbour, J., and C. Brown. 2014. Incomplete specimens in geometric morphometric analyses. Methods in Ecology and Evolution 5:16–26.

Bas, S., and F. Gasser. 1994. Polytypism of *Salamandra salamandra* (L.) in north-western Iberia. Mertensiella 4:41–74.

Beukema, W., A. G. Nicieza, A. Lourenço, and G. Velo-Antón. 2016*a*. Colour polymorphism in *Salamandra salamandra* (Amphibia: Urodela), revealed by a lack of genetic and environmental differentiation between distinct phenotypes. Journal of Zoological Systematics and Evolutionary Research 54:127–136.

Beukema, W., J. Speybroeck, and G. Velo-Antón. 2016*b*. Salamandra. Current Biology 26:R696–R697.

Blackburn, D. G. 2015. Evolution of vertebrate viviparity and specializations for fetal nutrition: a quantitative and qualitative analysis. Journal of Morphology 276:961–990.

Brodie, E. D. 1983. Antipredator adaptations of salamanders: evolution and convergence among terrestrial species. Pages 109–133 *in* N. S. Margaris, M. Arianoutsou-Faraggitaki, and R. J. Reiter, eds. Adaptations to terrestrial environments. Springer, Boston.

Bruce, R. C. 2003. Life histories. Pages 477–525 *in* B. Jamieson, ed. Reproductive biology and phylogeny of Urodela (Vol. 1). Science Publishers.

Buckley, D., M. Alcobendas, M. García-París, and M. H. Wake. 2007. Heterochrony, cannibalism, and the evolution of viviparity in *Salamandra salamandra*. Evolution & development 9:105–15.

Carroll, S. B., and S. B. Carrol. 2001. Chance and necessity: the evolution of morphological complexity and diversity. Nature 409:1102.

Collins, J. P., and J. E. Cheek. 1983. Effect of food and density on development of typical and cannibalistic salamander larvae in *Ambystoma tigrinum nebulosum*. American Zoologist 23:77–84.

Collyer, M. L., and D. C. Adams. 2013. Phenotypic trajectory analysis : comparison of shape change patterns in evolution and ecology. Hystrix, Italian Journal of Mammalogy 24:75–83.

Collyer, M. L., D. Sekora, and D. C. Adams. 2015. A method for analysis of phenotypic change for phenotypes described by high- dimensional data. Heredity 115:357–365.

Cordero-Rivera, A., G. Velo-Antón, and P. Galán. 2007. Ecology of amphibians in small coastal Holocene islands: local adaptations and the effect of exotic tree plantations. Munibe 25:94–103.

Cvijanović, M., A. Ivanović, M. L. Kalezić, and M. L. Zelditch. 2014. The ontogenetic origins of skull shape disparity in the *Triturus cristatus* group. Evolution & development 16:306–317.

Donaire, D., and X. Rivera. 2016. La salamandra común *Salamandra salamandra* (Linnaeus, 1758) en el subcantábrico: Origen, dispersión, subespecies y zonas de introgresión. Butlletí Societat Catalana de Herpetología 23:7–38.

Dopazo, H., and P. Alberch. 1994. Preliminary results on optional viviparity and intrauterine siblicide in *Salamandra salamandra* populations from Northern Spain. Mertensiella 4:125–137.

Dryden, I. L., and K. V. Mardia. 2016. Statistical shape analysis with applications in R (2nd Edition). John Wiley & Sons, Ltd, Chichester.

Duellman, W. E., and L. Trueb. 1986. Biology of amphibians. The Johns Hopkins University Press, New York.

Frederich, B., D. Adriaens, and P. Vandewalle. 2008. Ontogenetic shape changes in *Pomacentridae* (Teleostei, Perciformes) and their relationships with feeding strategies: a geometric morphometric approach. Biological Journal of the Linnean Society 95:92–105.

García-París, M., M. Alcobendas, D. Buckley, and D. B. Wake. 2003. Dispersal of viviparity across contact zoes in iberian populatios of fire salamanders (*Salamandra*) inferred from discordance of genetic and morphological traits. Evolution 57:129–143.

Gómez-Mestre, I., R. A. Pyron, and J. J. Wiens. 2012. Phylogenetic analyses reveal unexpected patterns in the evolution of reproductive modes in frogs. Evolution 66:3687–3700.

Gould, S. J. 1977. Ontogeny and phylogeny. Harvard University Press.

Greven, H. 2003. Larviparity and Pueriparity. Pages 447–475 *in* D. M. Sever, ed. Reproductive Biology and Phylogeny of Urodela (Vol. 1). Science Publisher, Inc. Enfield, USA

Gunz, P., P. Mitteroecker, S. Neubauer, G. W. Weber, and F. L. Bookstein. 2009. Principles for the virtual reconstruction of hominin crania. Journal of Human Evolution 57:48–62.

Hanken, J. 1989. Development and evolution in amphibians. American Scientist 77:336–343.

———. 1999. Larvae in amphibian development and evolution. Pages 61–108 *in* B. K. Hall, and M. H. Wake, eds. The origin and evolution of larval forms. Elsevier.

Ivanović, A., M. Cvijanović, and M. L. Kalezić. 2011. Ontogeny of body form and metamorphosis: Insights from the crested newts. Journal of Zoology 283:153–161.

Kapralova, K. H., Z. O. Jónsson, A. Palsson, K. Dudek, S. le Deuff, B. K. Kristjánsson, S. S. S. Snorrason, et al. 2015. Bones in motion: Ontogeny of craniofacial development in sympatric arctic charr morphs. Developmental Dynamics 244:1168–1178.

Kleinteich, T. 2010. Ontogenetic differences in the feeding biomechanics of oviparous and viviparous caecilians (Lissamphibia: Gymnophiona). Zoology 113:283–294.

Koyabu, D., and N. T. Son. 2014. Patterns of postcranial ossification and sequence heterochrony in bats: Life histories and developmental trade-offs. Journal of Experimental Zoology Part B: Molecular and Developmental Evolution 322:607–618.

Labus, N., M. Cvijanović, and T. D. Vukov. 2013. Sexual size and shape dimorphism in *Salamandra salamandra* (Amphibia, Caudata, Salamandridae) from the central Balkans. Arch. Biol. Sci 65:969–975.

Levis, N. A., S. de la Serna Buzón, and D. W. Pfennig. 2015. An inducible offense: carnivore morph tadpoles induced by tadpole carnivory. Ecology and evolution 5:1405–1411.

Lourenço, A., F. Sequeira, D. Buckley, and G. Velo-Antón. 2018. Role of colonization history and species-specific traits on contemporary genetic variation of two salamander species in a Holocene island-mainland system. Journal of Biogeography 45:1054–1066.

Lüddecke, T., S. Schulz, S. Steinfartz, and M. Vences. 2018. A salamander’s toxic arsenal: review of skin poison diversity and function in true salamanders, genus *Salamandra*. The Science of Nature 105:56.

Manenti, R., L. Perreau, G. F. Ficetola, and M. Mangiacotti. 2018. Effects of diet quality on morphology and intraspecific competition ability during development: the case of fire salamander larvae. The European Zoological Journal 85:321–330.

McKinney, M. L. 1988. Heterochrony in evolution. Pages 327–340 *in* M. McKinney, ed. Heterochrony in Evolution. Springer.

McNamara, K. J., and M. L. McKinney. 2005. Heterochrony, disparity, and macroevolution. Paleobiology 31:17–26.

Moran, N. A. 1994. Adaptation and constraint in the complex life cycles of animals. Annual Review of Ecology and Systematics 25:573–600.

Pereira, R. J., I. Martínez-Solano, and D. Buckley. 2016. Hybridization during altitudinal range shifts: nuclear introgression leads to extensive cyto-nuclear discordance in the fire salamander. Molecular ecology 25:1551–1565.

Pfennig, D., and D. Pfenning. 1990. The adaptive significance of an environmentally-cued developmental switch in an anuran tadpole. Oecologia 85:101–107.

R Development Core Team. 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rohlf, F. J. 1999. Shape statistics: Procrustes superimpositions and tangent spaces. Journal of Classification 16:197–223.

———. 2015. TpsDig2, digitize landmarks and outlines. State University of New York.

Rohlf, F. J., and D. E. Slice. 1990. Extensions of the Procrustes method for the optimal superimposition of landmarks. Systematic Biology 39:40–59.

Saporito, R. A., M. Isola, V. C. Maccachero, K. Condon, and M. A. Donnelly. 2010. Ontogenetic scaling of poison glands in a dendrobatid poison frog. Journal of Zoology 282:238–245.

Sears, K. E. 2014. Quantifying the impact of development on phenotypic variation and evolution. journal of experimental biology. Part B: Molecular and Developmental Evolution 322B:643–53.

Sherratt, E., M. Vidal-García, M. Anstis, and J. S. Keogh. 2017. Adult frogs and tadpoles have different macroevolutionary patterns across the Australian continent. Nature Ecology and Evolution 1:1385–1391.

Smith, K. K. 2003. Time’s arrow: heterochrony and the evolution of development. International Journal of Developmental Biology 47:613–621.

Smith, T. B., and S. Skúlason. 1996. Evolutionary significance of resource polymorphisms in fishes, amphibians, and birds. Annual Review of Ecology and Systematics 27:111–133.

Steinfartz, S., M. Veith, and D. Tautz. 2000. Mitochondrial sequence analysis of *Salamandra* taxa suggests old splits of major lineages and postglacial recolonizations of Central Europe from distinct source populations of *Salamandra salamandra*. Molecular Ecology 9:397–410.

Stokes, A. N., A. M. Ray, M. W. Buktenica, B. G. Gall, E. Paulson, D. Paulson, S. S. French, et al. 2015. Otter predation on *Taricha granulosa* and variation in tetrodotoxin levels with elevation. Northwestern Naturalist 13–21.

Turner, C. H. 1998. Three rules for bone adaptation to mechanical stimuli. Bone 23:399–407.

Uotila, E., A. C. Díaz, I. S. Azkue, and X. Rubio Pilarte. 2013. Variation in the reproductive strategies of *Salamandra salamandra* (Linnaeus, 1758) populations in the province of Gipuzkoa (Basque Country). Munibe Ciencias Naturales. Natur zientziak 61.

Urošević, A., K. Ljubisavljević, and A. Ivanović. 2013. Patterns of cranial ontogeny in lacertid lizards: morphological and allometric disparity. Journal of Evolutionary Biology 26:399–415.

Velo-Antón, G., and D. Buckley. 2015. Salamandra común – *Salamandra salamandra* (Linnaeus, 1758). Enciclopedia Virtual de los Vertebrados Españoles 2–12.

Velo-Antón, G., and A. Cordero-Rivera. 2017. Ethological and phenotypic divergence in insular fire salamanders: diurnal activity mediated by predation? Acta Ethologica 20:243–253.

Velo-Antón, G., M. García-París, P. Galán, A. Cordero Rivera, G. Velo-Antón, M. García-París, P. Galán, et al. 2007. The evolution of viviparity in holocene islands: Ecological adaptation versus phylogenetic descent along the transition from aquatic to terrestrial environments. Journal of Zoological Systematics and Evolutionary Research 45:345–352.

Velo-Antón, G., X. Santos, I. Sanmartín-Villar, A. Cordero-Rivera, and D. Buckley. 2015. Intraspecific variation in clutch size and maternal investment in pueriparous and larviparous *Salamandra salamandra* females. Evolutionary Ecology 29:185–204.

Velo-Antón, G., K. R. Zamudio, and A. Cordero-Rivera. 2012. Genetic drift and rapid evolution of viviparity in insular fire salamanders (*Salamandra salamandra*). Heredity 108:410–8.

Vences, M., E. Sanchez, J. S. Hauswaldt, A. Eikelmann, D. Rodríguez, S. Carranza, P. Werner, D. Donarire-Barroso, et al. 2014. Nuclear and mitochondrial multilocus phylogeny and survey of alkaloid content in true salamanders of the genus *Salamandra* (Salamandridae). Molecular phylogenetics and evolution 73:208–216.

Vučić, T., M. Sibinović, T. D. Vukov, N. T. Kolarov, M. Cvijanović, and A. Ivanović. 2019. Testing the evolutionary constraints of metamorphosis: the ontogeny of head shape in *Triturus* newts. Evolution 73: 1253-1264.

Wake, D. B., and J. Hanken. 1996. Direct development in the lungless salamanders: What are the consequences for developmental biology, evolution and phylogenesis? International Journal of Developmental Biology 40:859–869.

Wake, D. B., M. H. Wake, and C. D. Specht. 2011. Homoplasy : from detecting pattern and mechanism of evolution. Science 331:1032–1035.

Wake, M. H. 2003. Reproductive modes , ontogenies , and the evolution of body form. Animal biology 53:209–223.

———. 2015. Fetal adaptations for viviparity in amphibians. Journal of Morphology 276:941–960.

Wake, M. H., and J. Hanken. 1982. Development of the skull of *Dermophis mexicanus* (Amphibia: Gymnophiona), with comments on skull kinesis and amphibian relationships. Journal of Morphology 173:203–223.

Wells, K. D. 2007. The ecology and behavior of amphibians. University of Chicago Press.

West-Eberhard, M. 2003. Developmental plasticity and evolution. Oxford University Press, Oxford, UK.

Wimberger, P. H. 1994. Trophic polymorphisms, plasticity, and speciation in vertebrates. Pages 19-43 *in* D. J. Stouder, K. L. Fresh, R. J. Feller and B. W. Baruch, eds. Theory and application in fish feeding ecology (Volume 1). University of South Carolina Press. USA.

Supplementary Material Chapter 2

Table S2.1: Details of each studied population

Population	Latitude	Longitude	Subspecies	Mode of reproduction	Ntotal	Females	Males	Unknown sex	Laboratory of processing
Oviedo	43.36	-5.87	*S.s.bernardezi*	pueriparity	55	31	24	-	University of Oviedo
Fito	43.44	-5.19	*S.s.bernardezi*	pueriparity	64	32	32	-	University of Oviedo
Corés	43.11	-6.32	*S.s.bernardezi*	pueriparity	59	36	23	-	University of Oviedo
Sorriba	43.28	-6.44	*S.s.bernardezi*	pueriparity	52	15	37	-	University of Oviedo
Valdesamario	42.72	-5.95	*S.s.gallaica/bejarae*	larviparity	39	23	16	-	University of Oviedo
Las Médulas	42.46	-6.77	*S.s.gallaica/bejarae*	larviparity	13	1	12	-	University of Oviedo
Mindelo	41.31	-8.74	*S.s.gallaica*	larviparity	80	42	38	-	CIBIO
Coiro	42.27	-8.77	*S.s.gallaica*	larviparity	40	15	25	-	CIBIO
Ons	42.37	-8.94	*S.s.gallaica*	pueriparity	83	13	20	50	CIBIO
San Martiño	42.20	-8.91	*S.s.gallaica*	pueriparity	19	12	7	-	CIBIO

Table S2.2: Procrustes ANOVAs including sex as a factor.

	HEAD (45 landmarks)					SNOUT (17 landmarks)									
	A) Subspecies					(B) Subspecies					(D) Pueriparous populations				
	Df	**SS**	**F**	**Z**	***P***	**Df**	**SS**	**F**	**Z**	***P***	**Df**	**SS**	**F**	**Z**	***P***
a) Shape															
Sex	1	0.01715	14.9284	5.3958	1.00E-04	1	0.005862	15.583	4.5885	1.00E-04	1	0.004217	10.4127	3.9204	1.00E-04
Subspecies	1	0.1198	14.0852	4.64E+00	1.00E-04	1	0.025441	9.5896	3.3482	1.00E-04	1	0.009382	2.797	1.516	0.0637
Population	8	0.06804	7.4032	1.22E+01	1.00E-04	8	0.021224	7.0518	8.7916	1.00E-04	4	0.013418	8.2832	6.4805	1.00E-04
Sex x Subspecies	1	0.00072	0.6306	-1.31E-02	0.5041	1	0.000212	0.5647	-0.1537	0.5645	1	0.000115	0.2846	-1.2716	0.8972
Sex x Pop	8	0.01206	1.3117	3.3622	5.00E-04	8	0.003678	1.2219	2.0536	0.0226	4	0.002127	1.313	1.6055	0.056
Residuals	434	0.4986				434	0.163275				270	0.109341			
b) Size															
Sex	1	0.5455	32.5427		1.00E-04	1	0.3918	22.9291	1.9934	1.00E-04	1	0.2425	16.0823	1.81332	1.00E-04
Subspecies	1	6.7863	71.5626	2.1105	0.0003	1	6.4337	63.3986	2.0539	0.0002	1	1.1831	38.1483	1.84058	0.0044
Population	8	0.7586	5.6571	4.6636	1.00E-04	8	0.8118	5.9396	4.6895	1.00E-04	4	0.1241	2.0565	1.60232	0.0265
Sex x Subspecies	1	0.0222	1.3225	1.0496	0.0947	1	0.0224	1.313	1.0134	0.101	1	0.0029	0.1953	-0.02809	0.6041
Sex x Pop	8	0.0902	0.6724	0.8792	0.189	8	0.0738	0.5397	0.3914	0.3755	4	0.046	0.762	0.39072	0.3806
Residuals	434	7.2752				434	7.4151				270	4.0718			
c) Size as covariate															
Size	1	0.08001	71.2384	8.3459	1.00E-04	1	0.012141	33.0842	5.7303	1.00E-04	1	0.00256	6.5266	3.1845	0.0004
Sex	1	0.01329	11.8312	5.3713	1.00E-04	2	0.01019	13.8841	6.0171	1.00E-04	1	0.004361	11.1166	4.0324	1.00E-04
Subspecies	1	0.05366	6.5193	3.5539	1.00E-04	1	0.018278	7.1576	2.9732	0.0009	1	0.009307	2.8942	1.5546	0.0589
Size x Sex	1	0.00121	1.0798	0.8566	0.1962	2	0.000636	0.8669	0.3146	0.379	1	0.000231	0.5887	-0.2876	0.6124
Subspecie x Population	8	0.06585	7.3284	12.1476	1.00E-04	8	0.020429	6.9583	8.7373	1.00E-04	4	0.012863	8.1975	6.5238	1.00E-04
Size x Subspecies	1	0.0018	1.6055	1.9079	0.0314	1	0.000374	1.0179	0.8054	0.2103	1	0.000292	0.7451	0.2587	0.403
Sex x Subspecies	1	0.00146	1.2958	1.51E+00	0.0661	1	0.000377	1.0269	0.8217	0.2029	1	0.000241	0.6153	-0.0472	0.5193
Size x Pop	8	0.01523	1.6947	4.8774	1.00E-04	8	0.005722	1.9491	4.0458	1.00E-04	4	0.003631	2.3139	3.2967	0.0005
Sex x Pop	8	0.01078	1.2	3.13E+00	0.0006	8	0.004104	1.3978	2.8102	0.003	4	0.002503	1.5951	2.318	0.0101
Size x Sex x Subsp	1	0.00166	1.4798	1.9016	0.032	1	0.000142	0.3881	-0.6061	0.7295	1	0.000488	1.2434	1.1149	0.1333
Size x Sex x Pop	7	0.00529	0.6729	0.2326	0.4062	7	0.001903	0.7407	3.15E-01	0.3774	4	0.000914	0.5826	-0.5236	0.6933
Residuals	415	0.46612				463	0.169913				258	0.101209			

TTable S2.2. (Continued)

						(C)	Mode of reproduction				(F)	Mode of reproduction within *gallaica*			
	-	-	-	-	-	**Df**	**SS**	**F**	**Z**	***P***	**Df**	**SS**	**F**	**Z**	***P***
a) Shape	-	-	-	-	-										
Sex	-	-	-	-	-	1	0.005862	15.583	4.5885	1.00E-04	1	0.002412	7.0167	3.4225	0.0002
Mode	-	-	-	-	-	1	0.017345	4.7325	2.4155	0.0066	1	0.001307	0.5481	-0.6656	0.7474
Population	-	-	-	-	-	8	0.02932	9.7419	10.1348	1.00E-04	4	0.009541	6.9396	6.1352	1.00E-04
Sex x Mode	-	-	-	-	-	1	0.000157	0.4186	-0.6291	0.739	1	0.00006	0.1754	-2.1306	0.9821
Sex x Pop	-	-	-	-	-	8	0.003733	1.2402	2.1128	0.0177	4	0.002355	1.7129	2.1729	0.0176
Residuals	-	-	-	-	-	434	0.163275				212	0.072868			
b) Size	-	-	-	-	-										
Sex	-	-	-	-	-	1	0.3918	22.9291	1.9934	1.00E-04	1	0.2857	14.0838	1.78821	0.0004
Mode	-	-	-	-	-	1	5.5918	27.0499	1.7673	0.0036	1	0.3427	3.7497	0.96588	0.1356
Population	-	-	-	-	-	8	1.6538	12.0993	5.8241	1.00E-04	4	0.3656	4.5058	2.35406	0.0006
Sex x Mode	-	-	-	-	-	1	0.0218	1.275	1.0062	0.1052	1	0.0023	0.1131	-0.32933	0.7086
Sex x Pop	-	-	-	-	-	8	0.0744	0.5445	0.4105	0.3676	4	0.0443	0.5457	-0.17211	0.6115
Residuals	-	-	-	-	-	434	7.4151				212	4.2999			
c) Size as a covariate	-	-	-	-	-										
Size	-	-	-	-	-	1	1.20E-02	32.9931	5.7964	1.00E-04	1	0.002081	6.3232	3.2173	0.0002
Sex	-	-	-	-	-	1	0.006224	17.0906	4.7915	1.00E-04	1	0.00265	8.0526	3.6625	1.00E-04
Mode	-	-	-	-	-	1	8.63E-03	2.4023	1.4721	0.0694	1	0.00123	0.5289	-0.7201	0.765
Size x Sex	-	-	-	-	-	1	0.00033	0.9067	0.3541	0.3625	1	0.000317	0.9622	0.3456	0.3644
Population	-	-	-	-	-	8	0.02873	9.8605	10.1776	1.00E-04	4	0.009303	7.0683	6.2566	1.00E-04
Size x Mode	-	-	-	-	-	1	0.000425	1.168	1.0027	0.159	1	0.000336	1.0207	0.6422	0.2562
Sex x Mode	-	-	-	-	-	1	0.000209	0.5728	-0.1072	0.549	1	0.000053	0.162	-2.1739	0.9869
Size x Population	-	-	-	-	-	8	0.005664	1.944	4.0519	1.00E-04	4	0.002574	1.9554	2.707	0.0032
Sex x Population	-	-	-	-	-	8	0.004278	1.4684	3.0454	0.0012	4	0.002503	1.9021	2.6475	0.0039
Size x Sex x Mode	-	-	-	-	-	1	0.000057	0.1576	-1.9753	0.9741	1	0.000403	1.2239	1.0411	0.1509
Size x Sex x Population	-	-	-	-	-	7	0.001988	0.7797	0.5808	0.2793	3	0.000956	0.9684	0.8121	0.206
Residuals						415	0.151143				201	0.066138			

Chapter 3

FINE-SCALE GENETIC STRUCTURE AND INTRASPECIFIC PHENOTYPIC DIVERSITY IN A VIVIPAROUS SALAMANDER

ABSTRACT

Integrative approaches that combine information from different sources such as genetic, ecological or phenotypic data are extremely useful for the identification of the evolutionary processes driving diversification among and within species. The fire salamander is an exceptional case of intraspecific diversity, especially within the subspecies *S. s. bernardezi* which present the highest degree of phenotypic diversity and genetic structuration despite its restricted geographic distribution. Thus, it constitutes an ideal model to identify fine-grained patterns of phenotypic and genetic diversity and determine whether genetic and phenotypic structures correlate at a small spatial scale, and proximate the causes leading to such structure. Here, we will explore: (i) whether different coloration morphs within *S. s. bernardezi* distribution differ in two phenotypic traits: head morphology (both shape and size) and body size; (ii) whether genetic diversity present any structure throughout the studied area; if so, (iii) compare the spatial structure of neutral genetic diversity and the distribution of different colorations; and (iv) finally, try to determine what evolutionary mechanisms led to genetic and morphological diversity structuration. Through the combination of coloration morphs diversity and geometric morphometrics tools we found that different colorations within *S. s. bernardezi d*iffer both in body size, with striped colorations being larger than nonstriped ones, and head morphology, may be resulting from different evolutionary pressures acting on each morph. The analysis of neutral genetic markers identified two evolutionary units which were highly concordant with the distribution of coloration morphs, pointing to any mechanism generating a spatial structuration of variation. Finally, while head morphological diversity do not follow a pattern of isolation by distance (IBD), neutral genetic diversity does at both considered scales: across all study area, and within each identified genetic unit, pointing to a reduced connectivity among populations, even at reduced geographic distances.

INTRODUCTION

Patterns of diversity in nature are the result of the combined action of different evolutionary forces (Mitchell-Olds et al. 2007; Grant and Grant 2011). The identification of underlying processes and their relative contribution to differentiation is challenging and has been the focus of several studies (e.g. Giordano et al. 2007; Wang and Summers 2010; Paz et al. 2015). A commonly used approximation is based on integrative approaches combining information from different levels, as for example the comparison of patterns of genetic and phenotypic diversity across populations or species (Storz 2002; Mullen et al. 2009; Zamudio et al. 2016).

The spatial structure of neutral genetic diversity follows a migration-drift equilibrium reflecting the action of stochastic demographic processes in which differentiation is highly dependent on the degree of isolation between populations (i.e. it depends on levels of gene flow). This can lead to different patterns of population structure depending on the underlying mechanisms. Thus, genetic spatial structure can be shaped by geographic distance (isolation-by-distance, Wright 1943; Slatkin 1993; Bohonak 1999; stepping-stone processes, Kimura and Weiss 1964), environmental heterogeneity (isolation-by-environment; Wang and Bradburd 2014; barriers, Castric et al. 2001), or phenotypic traits that affect population connectivity (isolation-by-phenotype; Wang and Summers 2010). In the latter case, neutral genetic structure and traits divergence might present high levels of concordance (Wang and Summers 2010). Similarly, for traits that do not affect population connectivity, if phenotypic divergence follows a model of random accumulation of variation it is expected a parallelism between the spatial patterns of phenotypic and neutral genetic variation (Storz 2002; Hoffman et al. 2006). However, because phenotype is the target of selection and mediates the performance of organisms in different environments, selective processes (i.e. natural or sexual selection) acting differentially throughout the species ranges can also determine the structure of phenotypic diversity within a species (Schemske and Bierzychudek 2007). In such situation, patterns of phenotypic and neutral genetic variation are expected to be inconsistent. Thus, the comparison of patterns of geographic variation in phenotypic and neutral genetic diversity may provide important information about evolutionary processes driving functional diversification at microevolutionary scales (Mullen et al. 2009; Zamudio et al. 2016). This approach is especially interesting in species with high levels of genetic and phenotypic diversity and, at least, some potential for spatial structuring.

The fire salamander, *Salamandra salamandra*, represents an exceptional case of intraspecific diversity, with high levels of genetic, morphological, and reproductive variation (Velo-Antón and Buckley 2015), which makes this species an ideal model to address questions about evolutionary mechanisms triggering intraspecific diversity. More specifically, *S. s. bernardezi*, which is restricted to central northwest of the Iberian Peninsula, is the subspecies with the highest degree of diversity and differentiation, which has been assumed to arise as a result of historical events (García-París et al. 2003). The complex topography of this region coupled with climatic oscillation cycles along

Pleistocene lead to allopatric processes resulting in a highly structuration of genetic diversity and the differentiation of several sublineages across its range of distribution (García-París et al. 2003; Velo-Antón et al. 2007). Regarding the rest of Iberian subspecies, with the exception of its geographic neighbor *S. s. fastuosa*, *S. s. bernardezi* is highly differentiated at the phenotypic level, with different reproductive modes (Dopazo and Alberch 1994), smaller body size (Alcobendas and Castanet 2000), unique coloration patterns (Bass and Gasser 1994; García-París et al. 2003; Beukema et al. 2016), and different head morphology (Bass and Gasser 1994; see Chapter 2). In addition, *S. s. bernardezi* shows profound levels of differentiation within its area of occurrence. Indeed, it is the first documented case of colour polymorphism within the family Salamandridae (Beukema et al. 2016), with up to six diagnosable phenotypes coexisting in some populations from the eastern range of its distribution (Pasmans and Keller 2000; Beukema et al. 2016).

Despite color polymorphism is considered a trait of ecological and evolutionary significance (Gray and McKinnon 2007), potential origins and drivers of such diversity in coloration remains unknown. Within amphibians, color polymorphisms could result from drift processes (Hoffman et al 2006) or a combination of drift and selective processes (e.g. Rudh et al. 2007). However, almost nothing is known about the potential biological or ecological role of the different colorations within *S. s. bernardezi*. Some authors (Pasmans and Keller 2000) suggested that the loss of aposematic colors (i.e. black and yellow) can be associated to a reduction of migratory activity, as a result of the viviparous mode of reproduction, which confers independence from aquatic breeding sites. However, this trend was not observed throughout the remaining viviparous colorations or subspecies. Aside coloration, no further phenotypic traits have been explored across these coloration morphs. For instance, head morphology is highly variable across the salamander distribution, with marked differences between subspecies (Bass and Gasser 1994; Chapter 2), but also among populations within each subspecies (Chapter 2). Because head shape is a highly relevant ecological trait (Brodie 1983; Kästle 1986; Adams and Rohlf 2000), it is likely a target for selection (Trueb 1993; Alarcón-Ríos et al. 2017; Ivanović and Arntzen 2017). However, whether head morphological differentiation arises as a result of drift processes or results from other evolutionary mechanisms is still unknown.

The main aim of this study is to identify fine-grained patterns of phenotypic and genetic diversity and determine whether genetic and phenotypic structures correlate at a small spatial scale. This is a necessary step to understand how high phenotypic diversification can arise over very small spatial scales, and the proximate causes leading to such structure. Therefore, we will address four main questions: first, (i) whether colorations across *S. s. bernardezi* distribution differ in other phenotypic traits with a presumably high evolutionary and adaptive significance (i.e. head morphology and body size); secondly, (ii) whether genetic diversity present any pattern of structuration throughout the studied area; and, if so, (iii) compare the geographic structure of neutral genetic diversity and the occurrence of different colorations; and finally, (iv) we try to determine what evolutionary processes led to genetic and morphological diversity structuration.

MATERIAL AND METHODS

SAMPLING

A total of 18 sampling locations across the eastern part of the range of distribution of the subspecies *S. s. bernardezi* (see Velo-Antón and Buckley 2015; Beukema et al. 2016) were visited in two periods (Figure 3.1). From the first one, which took place between 2006 and 2010, we selected a total of 165 samples (mucosa or tissue samples from toe-clipping) for genetic analyses. In the second one, between 2015 and 2017, we collected a minimum of 12 individuals of either sex from the same 18 locations to obtain phenotypic data on coloration morphotypes and head shape (Table 3.1). After collection, animals were transported to the University of Oviedo laboratory facilities were they were processed following the methodology for image acquisition described in Alarcón-Ríos et al 2017 (see Chapter 1). Animals capture and processing were conducted under collection and ethical permits provided by regional government (Asturias: 2006/000223; 2008/000272; 2010/000371; 2016/001092; 2017/001208; Picos de Europa National Park: CO/09/0007/2006; CO/09/646/2006; CO/09/077/2009; CO/09/0571/2009; CO/09/065/2015; Ethical Committee: PROAE 10/2017). After recovery from anesthesia (benzocaine; Ethyl 4-aminobenzoate; Sigma-Aldrich, Germany. Product number: E1501. Ref.: 112909) all the animals were released at the place of capture. We checked that all sampling points were located more than two kilometers apart as it is the maximum distance of dispersion reported for this species (Hendrix et al. 2017; Lourenço et al. 2018). Therefore, sampling locations can be considered as discrete local populations.

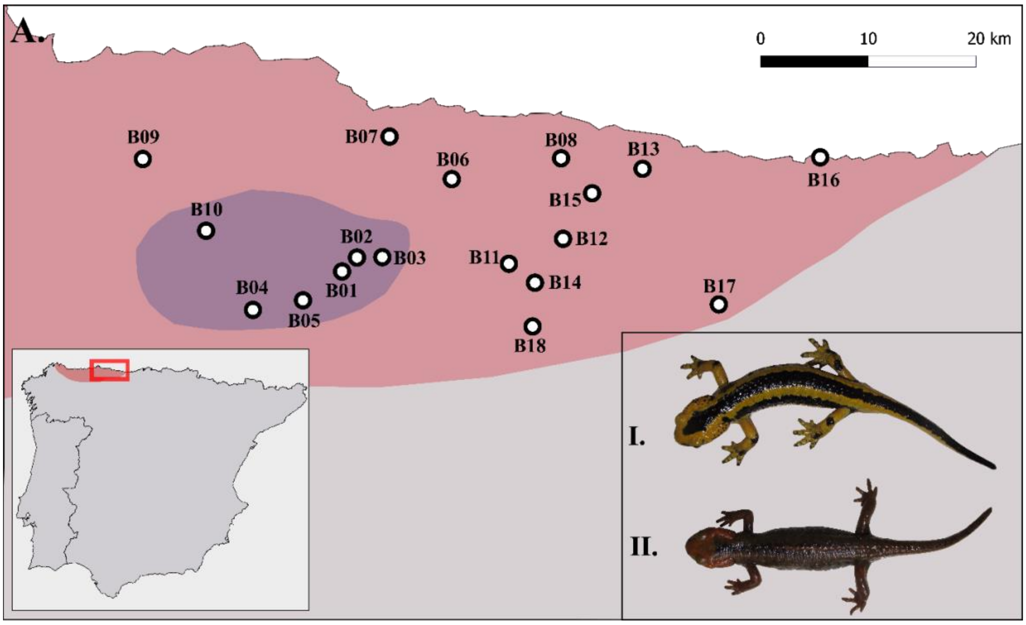


Figure 3.1. Location of the studied populations. The inset shows the range of distribution of *S. s. bernardezi* (red shade). Within A the red shaded area correspond to the area of occurrence of *S. s. bernardezi* and typical 'striped morphs' (Picture I), and the purple-shaded area correspond with the putative area of occurrence of 'unstriped morphs' (Picture II).

Table 3.1. Details on each studied population, and sample size of each of them for genetic analyses ($N_{genotyped}$) and morphological ones (HM_{total}). We also show results for descriptive population genetic statistics: number of alleles (N_A), unbiased allelic richness (A_R), observed heterozygosity (H_o), expected heterozygosity (H_E) and inbreeding coefficient (F_{IS}). Asterisks (*) denote populations excluded for genetic analysis.

Sampling location	Code	$N_{genotyped}$	$HM_{females}$	HM_{males}	HM_{total}	N_A	A_R	H_O	H_E	F_{IS}
Color	B01	7	8	34	42	5.89	3.07	0.68	0.76	0.12
Tendi	B02	15	26	41	67	9.11	3.20	0.73	0.81	0.11
Llerandi	B03	7	11	14	25	6.44	3.06	0.73	0.75	0.02
La Marea	B04	8	11	21	32	5.78	2.93	0.63	0.71	0.16
La Pesanca	B05	9	8	13	21	6.22	2.99	0.70	0.75	0.08
Zardón	B06	7	20	3	23	6.22	3.15	0.66	0.77	0.15
Sueve	B07	12	32	32	64	8.00	3.22	0.66	0.80	0.18
Doradiellu	B08	18	20	23	43	8.22	3.04	0.72	0.78	0.08
Fario	B09	4	6	17	23	3.44	2.36	0.54	0.49	-0.10
Fuensanta	B10	9	14	11	25	6.56	3.10	0.65	0.78	0.16
La Huesera	B11	4	11	14	25	3.00	2.41	0.45	0.55	0.15
Jolagua*	B12*	3	14	17	31	-	-	-	-	-
Mañanagas	B13	13	16	12	28	6.78	2.82	0.49	0.69	0.26
Buferrera	B14	6	9	20	29	5.44	2.94	0.71	0.71	0.00
Las Xareras	B15	4	13	7	20	3.67	2.68	0.61	0.64	0.10
Pimiango	B16	20	5	20	25	8.56	3.02	0.67	0.78	0.16
Valdediezma	B17	16	6	6	12	6.89	2.81	0.59	0.72	0.21
Vegarredonda*	B18*	2	10	9	19	-	-	-	-	-

DATA ACQUISITION

i. Head morphological variation

We applied landmark-based geometric morphometrics (GM), following the methodology described in Alarcón-Ríos et al. 2017 (see Chapter 1). These tools have been shown to be accurate for detecting differences at a very fine biological scale (e.g. sexual dimorphism within a population, intra-subspecific shape variation), which is of special interest in the present study, while maintaining a low, non-systematic measurement error.

To record head shape we digitized 13 fixed landmarks and 32 sliding semilandmarks on the dorsal view of salamanders' head (see Figure 1.1) using tpsDig2 (Rohlf 2015). Those 45 landmarks provide a comprehensive sampling and capture the most relevant features of salamander head shape: snout, jaws, eyes and parotid glands. Some, landmarks were not accurately determined (e.g. due to lack of image quality), and therefore these were recorded as missing data and their location was posteriorly estimated using multivariate regression (Gunz et al. 2009; Arbour and Brown 2014), as implemented in the function estimate.missing of geomorph R-package (Adams et al. 2017). Then, we obtained head shape variables through a generalized least-squares Procrustes superimposition (GPA; Rohlf and Slice 1990, Rohlf 1999) that standardized the size, translated and rotated the landmark configurations. Because head is a bilaterally symmetric structure and we were not interested in head asymmetry, we eliminated asymmetry effects by averaging the position of corresponding bilateral landmarks across the midline. As a measure of head size, we used the logarithm of Centroid Size (CS: Bookstein 1991, Zelditch et al. 2012). We implemented all GM-operations and statistical analyses using package geomorph version 3.0.5 (Adams and Otárola-Castillo 2013; Adams et al. 2017) in the R-language for statistical programming (R Development Core Team 2016).

ii. Morphs classification based on coloration patterns

Sampling locations included all the different coloration morphs described for *S. s. bernardezi* (Beukema et al. 2016) (Figure 3.1). In order to test whether different morphs (i.e. colorations) differ in head morphology we classified individuals following the classification criteria described in Beukema et al. 2016. First, we assigned each individual to a phenotype (types 1 to 6) according to its background and pattern. Because we were interested in exploring whether morphs with well-defined coloration patterns differ in head morphology, we grouped individuals assigned to phenotypes 1 and 2 (yellow background with black dorsolateral stripes) together, hereafter ‘striped’ morph; and morphs 4 (yellow/light brown background and yellow or orange colored head region and tiny irregular lighter flecks covering the body) and 5 (brown background with lateral and/or dorsal black stripes and occasionally lighter parotids), hereafter ‘unstriped’ morph. Morph 3 (yellow background with only dorsal black stripe or also with vestigial lateral stripes) can be attributed to both of these phenotypic groups (Beukema et al. 2016), and therefore we considered it as an intermediate group (hereafter ‘intermediate’ morph). Group 6 (yellow or black background with a pattern not corresponding to groups 1-5) include all those colorations not attributable to any other group (hereafter ‘unassigned’ morph). To test for differences in body size among the four coloration morphs all individuals were weighted with a digital scale (±0.01 g; Mettler Toledo PB3002-S Delta Range®), and measured for snout-vent length, SVL, using electronic calipers (±0.01mm).

iii. Genetic data

Nuclear DNA was extracted using E.Z.N.A® Tissue DNA Kit (Omega Bio-tek) following the manufacturers protocol. Quality and quantity of extracted product was tested by electrophoresing in 0.8% agarose gels. Then, it was used as a template in polymerase chain reaction (PCR) amplifications of nine *Salamandra* specific microsatellite loci (SalE2, SalE5, SalE6, SalE7, SalE8, SalE12, Sal3, Sal23 and Sal29; Steinfartz et al. 2004; Table S3.1) distributed in two optimized multiplexes (Mix 1 and Mix 2). Each multiplex mix contained distilled H2O, fluorescently labelled forward (6-FAM, VIC, NED or PET; Table S3.1 for sequence details) and reverse primers. Each PCR reaction contained a total volume of 10–11 µl: 5 µl of Multiplex PCR Kit Master

Mix (QIAGEN), 3 µl of distilled water, 1 µl of primer multiplex mix and 1–2 µl of DNA extract (~50 ng/µl). To identify possible contaminations, a negative control was employed. PCR touchdown cycling conditions for Mix 1: the reaction started with an initial step at 95 °C for 15 min, 7 cycles at 95 °C for 30 s, 45 s of annealing at 65 °C (decreasing 1 °C each cycle), 72 °C for 45 s, followed by 33 cycles of 95 °C for 30 s, 58 °C for 45 s, 72 °C for 45 s, and ended with a final extension of 30 min at 60 °C. PCR touchdown for Mix 2 was: initial step at 95 °C for 15 min, 11 cycles at 95 °C for 30 s, 45 s of annealing at 60 °C (decreasing 1 °C each cycle), 72 °C for 45 s, followed by 29 cycles of 95 °C for 30 s, 58 °C for 45 s, 72 °C for 45 s, and ended with a final extension of 30 min at 60 °C. To determine the relative size of fragments the DNA Size Standard GeneScan-LIZ 500 was employed. PCR products were separated by capillary electrophoresis on an ABI3130XL Genetic Analyzer (Applied Biosystems). Alleles were scored in GENEMAPPER 4.0 (Applied Biosystems) and manually checked twice.

DATA ANALYSIS

i. Head shape: differences among coloration morphs

To obtain a preliminary exploration of the patterns of variability in morphospace we performed a Principal Component Analysis (PCA). Then, to investigate whether different morphs differ in head shape and size we performed distance-based permutation ANOVAs as implemented in the function *procD.lm* of the R package *geomorph* (Adams et al. 2017) with coloration group as a factor. We performed a second Procrustes ANOVA including head size as a covariate and its interaction with coloration group to explore potential allometric relationships between shape and size. This allowed us to investigate the covariation between head shape and head size, test for common allometric slopes among coloration groups, and evaluate morphological differentiation among groups while taking into account the effect of size variation on head shape. Statistical significance was assessed based on 10,000 random permutations, using Residual Randomization, RRPP (Collyer et al. 2015). To identify differences between specific morphs we performed a post hoc pairwise comparison of group means correcting for size variation and using 10,000 RRPP. To visualize shape variation and distinctive features of head shape across coloration groups we used vector plots based on a), the Procrustes residuals, and b), on size-corrected Procrustes residuals, obtained as the residuals of the regression of shape on log(CS) under a common slope for all groups (see also Results).

Sexual dimorphism has been described in this species (Labus et al. 2013; Alarcón-Ríos et al. 2017). Although it was not the main focus of this study, we run preliminary analyses to test if levels of sexual dimorphism differed between morphs. Because sex differentiation was similar among coloration groups (non-significant interaction between sex and coloration; see Table S3.2), we did not include sex as a factor in subsequent analyses.

Although differences in body size among different colorations have been previously noted (Pasmans and Keller 2000), no formal test have been done to explore if colorations significantly differ in body size. For that purpose, we log-transformed SVL values to meet homoscedasticity and normality assumptions and performed an analysis of variance (ANOVA) to compare SVL among coloration morphs using R software (R Development Core Team 2016).

ii. Genetic structure

The presence of null alleles and scoring errors were estimated with MicroChecker v 2.2.3 (Van Oosterhout et al. 2004) using 9999 replicates and Bonferroni adjusted 95% confidence interval. We used Genepop v 4.2 (Raymond and Rousset 1995) to test for deviations from Hardy-Weinberg Equilibrium (HWE) and Linkage Disequilibirum (LD).

As a measurement of population connectivity we investigated the neutral genetic structure of studied populations of S. s. bernardezi using the Bayesian algorithm implemented in the program Structure v 2.3.4 (Pritchard et al. 2000). We applied admixture and correlate allele models with no prior information regarding population of origin. A burn-in period of 500,000 iterations, followed by a 500,000 iterations was set for ten independent runs for a number of clusters (K) ranging between 1 and 20. The best K was identified with STRUCTURE HARVESTER v 0.6.94 (Earl and vonHoldt 2012) following the criteria of the highest ad hoc statistic ΔK (Evanno et al. 2005). Graphics for the best K were summarized and displayed using the R package *pophelper* v 2.9.9. (Francis 2017). Each sampling population was assigned to a cluster if its proportion of membership to a specific cluster is higher than 70% (Zamudio and Wieczorek 2007).

We calculated descriptive population genetic statistics using the software GenAlEx v 6.5 (number of alleles (N_A), observed heterozygosity (H_O), expected heterozygosity (H_E) and inbreeding coefficient (F_{IS}), Peakall and Smouse 2012). As N_A could be highly biased by differences in sample size we calculated the unbiased allelic richness (AR) using a rarefaction method implemented in HP-RARE (Kalinowski 2005). Finally, we calculated pairwise F_{ST} for all pairs of populations using FSTAT 2.9.4 (Goudet 1995) (but see Results). To test for genetic differentiation we estimated pairwise Nei's Genetic Distance (D) using GenAlex v 6.5 (Peakall and Smouse 2012).

iii. Patterns of genetic and morphological divergence

Finally, for further exploration of patterns of phenotypic and genetic distances, we estimated pairwise morphological divergence among sampling populations as the Procrustes distances between least squares (LS) means for each population. As populations differ in allometric slopes (data not shown), we could not correct shapes by size, so Procrustes distances among populations include size variation.

We conducted a number of Mantel test with 9999 permutations to investigate patterns of Isolation-by-distance (IBD) between genetic distances matrix and geographic distances. We then tested for correlation between all possible combinations of matrices of morphological Procrustes distances (LS), genetic differentiation, and pairwise linear geographical distances (for testing for spatial correlation of head shape and genetic distance). Mantel tests were performed as implemented in the function *mantel* of the R package *ecodist* (Goslee and Urban 2007).

RESULTS

HEAD SHAPE: DIFFERENCES AMONG COLORATION MORPHS

The first two components of the PCA on head shape variation cumulatively explained 45.26% of variance. Despite some overlapping, striped and unstriped morphs clearly segregated across the bidimensional morphospace. In contrast, intermediate and unassigned group overlapped broadly with the remainder groups (Figure 3.2a).

Procrustes ANOVA reveled that coloration groups differed in head shape and size (Table 3.2) (Figure 3.2b). Procrustes ANOVA including head size as a covariate revealed that head shape significantly co-varied with head size (Table 3.2). However, differences in shape between coloration morphotypes were still present after taking into account size effects. As pointed by a non-significant interaction between coloration and head size (CS), all four coloration groups presented common allometric slopes.

Table 3.2. Results of permutational ANOVAs used to test for differences in head shape (a) and head size (b) among different morphs within *S. s. bernardezi,* and test for size-shape allometric relationship, size-free head shape differentiation among colorations and common allometric slopes among groups (c).

	Df	SS	F	Z	*P*
a) Head shape					
Morph	3	0.035	9.182	7.297	<0.0001
Residuals	550	0.689			
b) Head size					
Morph	3	0.287	5.908	2.196	$8 \cdot 10^{-4}$
Residuals	550	8.916			
c) Head size as covariate					
Head Size	1	0.011	8.640	4.521	<0.0001
Morph	3	0.031	8.474	7.078	<0.0001
Size x Morph	3	0.005	1.336	1.314	0.096
Residuals	546	0.676			

A pairwise *post hoc* analysis showed that all coloration groups differ between each other but except for the intermediate and unassigned phenotypes (Table 3.3). Differences in head size paralleled those on body size (ANOVA; SVL: $F_{3,\ 516} = 9.82$, $P = <0.001$) (Figure 3.2c).

Table 3.3. Pairwise *post hoc* analysis among each morph. Values below the diagonal are distances between each morph, and values above the diagonal are the p-values.

	Striped morph	**Unstriped morph**	**Intermediate morph**	**Unassigned morph**
Striped morph		0.0001	0.0001	0.0165
Unstriped morph	0.0184		0.0294	0.0261
Intermediate morph	0.0120	0.0085		0.1468
Unassigned morph	0.0132	0.0134	0.0108	

Inspection of vector plots after size correction revealed similar patterns as those observed for Procrustes residuals. Therefore, only the vector plots from size-corrected data are shown for visualization of shape change (Figure 3.3). These showed that striped morphs differ from unstriped ones in parotids, eyes and snout shape. Morphs assigned to unstriped colorations presented narrower heads and slightly bigger eyes than striped ones, with most variation located in the parotid region (Figure 3.3a). When exploring shape change between these two main groups and the intermediate morph (Figure 3.3b-e) we see that both comparisons indicate changes in the parotid region, but in opposite directions. Meanwhile intermediate morph presents narrower head than striped salamanders (Figure 3.3b), it presents slightly wider parotid region than unstriped ones (Figure 3.3d). Both striped and unstrapped colorations present differences regarding the length, and the posterior part of the head of the unassigned group, eyes and snout (Figure 3.3c-e). Finally, the intermediate and unassigned morphs did not differ significantly in head shape or size (Figure 3.3f).

GENETIC STRUCTURE

Meanwhile no evidences from deviations from LE, two loci (Sal3 and SalE06) consistently present deviation from HWE and evidences of null alleles. Although observed patterns of null alleles probably result from the small sample size in some of the sampling populations, we repeated all genetic structure analyses with the 7 loci with no deviance (see Supplementary Material). Both analyses, with 7 and 9 loci, generated the same results regarding the most likelihood number of clusters (K=2); thus, we only present here the results from the complete dataset (i.e. 9 loci) (Figure 3.4; see Table S3.3). Those sampling populations that presented inconsistencies in the assignation to each cluster, as well as those that could not be assigned to any cluster in more than 70% were discarded for downstream analyses. This resulted in the exclusion of two populations with an extremely low sample size (Vegarredonda (B18), N=2, not clear assignation to any cluster; and Jolagua (B12), N=3, inconsistent assignment to clusters between 7- and 9-loci datasets; Figure 3.4; Table S3.3).

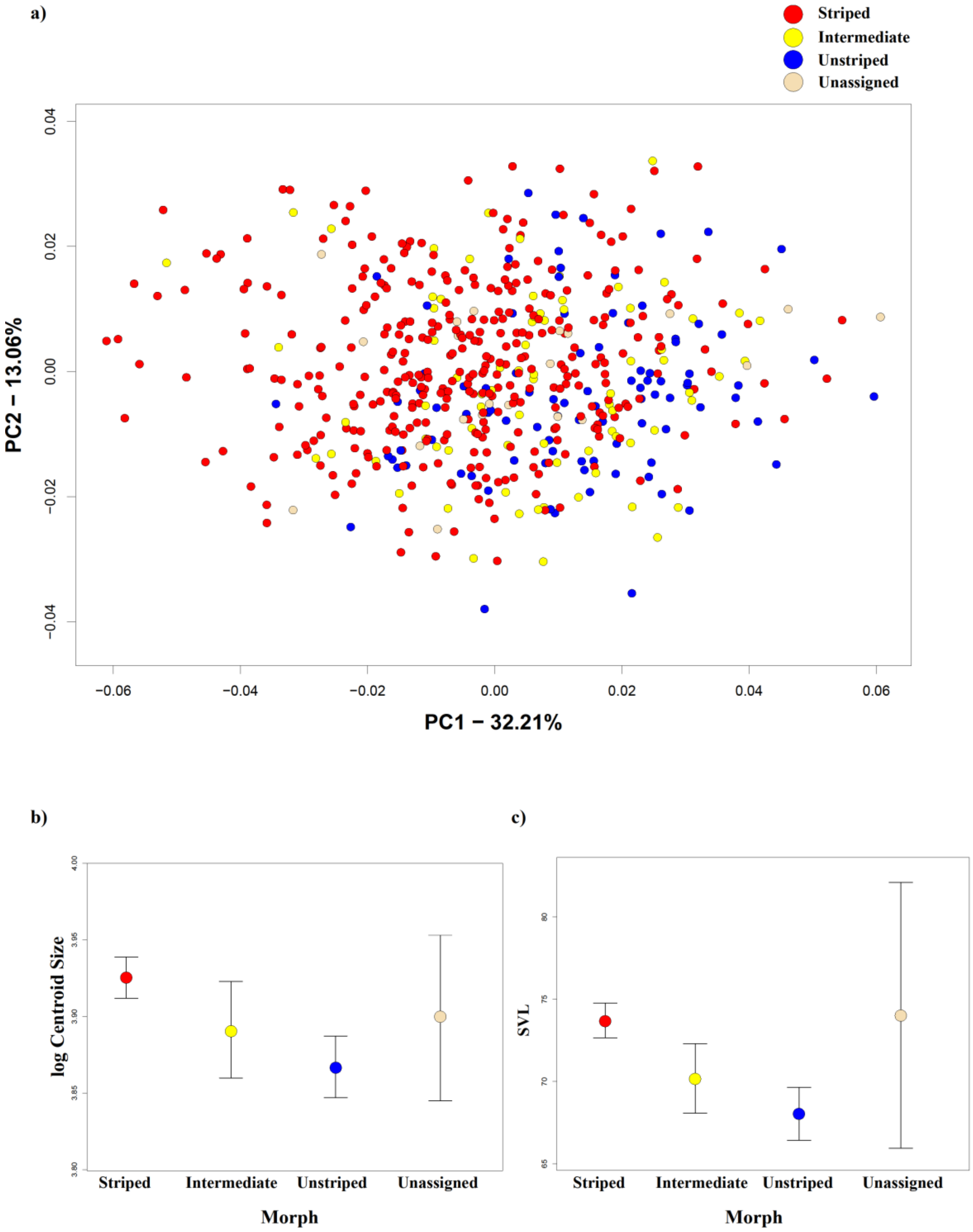


Figure 3.2. (a) Visualization of head shape variation in our sample on the two first principal components of the morphospace. Amount of variance explained by each PC is indicated in the corresponding axis. Different colors denote each considered morph. Shape data used include size-related shape variation. (b) Variation in mean head size (logCS) across morphs. (c) Variation in mean SVL across morphs. Vertical bars in (b) and (c) denote 95% confidence intervals.

The best K following the Evano's method identified two differentiated genetic clusters with eight populations each: the western cluster (B01, B02, B03, B04, B05, B06, B07, B10); and the eastern cluster (B09, B11, B13, B14, B15, B16, B17) (see Table S3.3; Figure 3.4). Overall, populations assigned to both clusters presented similar levels of genetic diversity, between medium and high values: range Na: 3-9.11; range AR: 3-9.11; range H_E: 0.49-0.81; range H_O: 0.45-0.73. Fixation index was low in all the cases: range F_{IS}: -0.1-0.26 (Table 3.1).

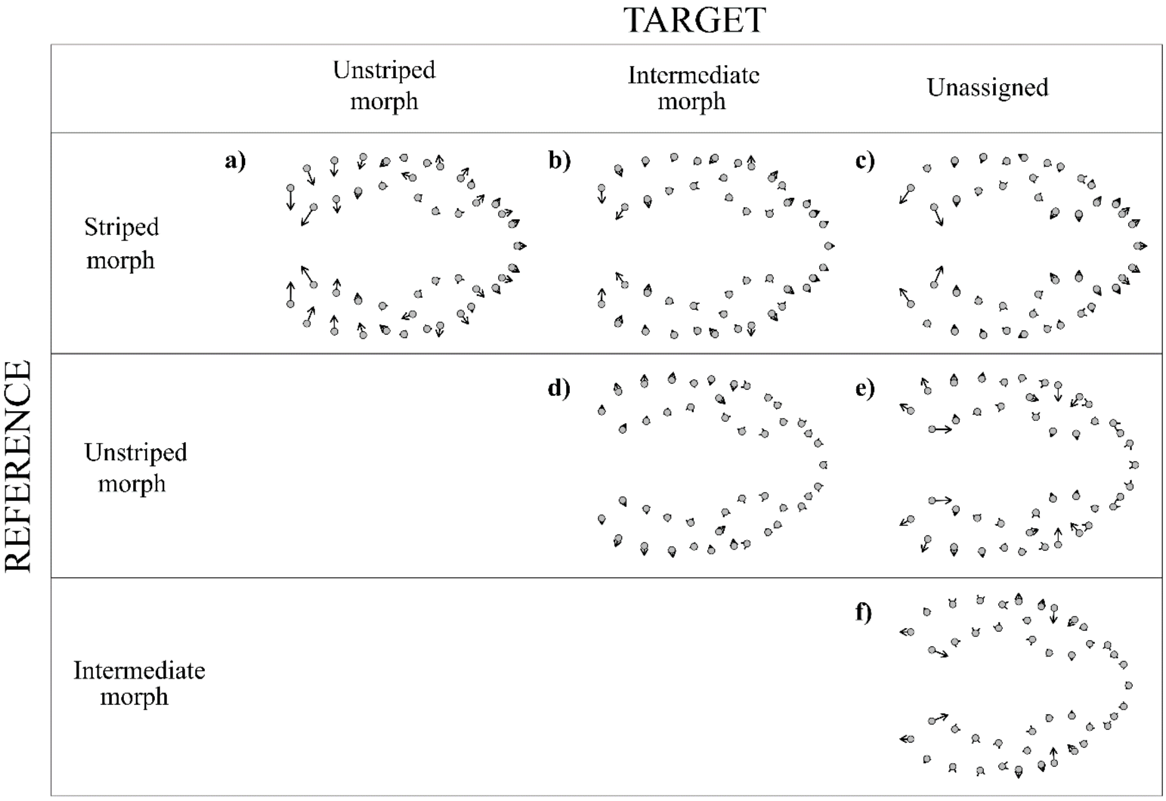


Figure 3.3. Vector plots showing snout pairwise shape change among morphs. Morphs used as reference are denoted in row names, and target in each column. Shape change has been magnified by a factor of seven to facilitate visualization. Shape data used for vector generation is size-corrected.

PATTERNS OF GENETIC AND MORPHOLOGICAL DIVERGENCE

Pairwise values of F_{ST} and D were highly correlated (for 7 loci: r = 0.88, $P < 0.0001$; 9 loci: r = 0.86, $P < 0.0001$), therefore we used only F_{ST} values as a measure of genetic differentiation in subsequent correlation analysis and discussion. In addition, pairwise F_{ST} values from the 7 and 9 loci datasets are also highly correlated (r = 0.95, $P < 0.0001$), therefore, we only present results from the complete, 9 loci, dataset. Genetic differentiation between populations was highly variable ranging from 0.009 to 0.25 F_{ST} values (Table S3.4).

We founded evidence of isolation by distance (IBD) in neutral genetic diversity (Mantel = 0.42, $P = 0.002$), but not in morphological differentiation (Mantel = -0.0006, $P = 0.96$).

Also, patterns of neutral genetic diversity and morphological divergence were not correlated (Mantel = -0.11; *P* = 0.48). The same pattern of IBD in neutral genetic diversity was observed within both identified clusters separately (West: Mantel= 0.42, *P* = 0.03*;* East: Mantel= 0.62, *P* = 0.03). Head morphological differentiation within each cluster neither follow an IBD pattern (West: Mantel= 0.28, *P* = 0.40; East: Mantel = -0.38, *P* =0.09), and the correlation between genetic and morphological diversity patterns within clusters are also not significant (West: Mantel=-0.26, *P* =0.28; East: Mantel=-0.22, *P* =0.34).

DISCUSSION

We used genetic and morphometric data to uncover evolutionary mechanisms linking phenotypic and genetic divergence. Despite the coloration morphotypes analyzed in this study are geographically dispersed (see Beukema et al. 2016; Zamudio et al. 2016), our results unequivocally evidenced differences in head shape and body size among coloration morphotypes. In addition, the analysis of neutral markers identified two evolutionary units revealing a well delineated spatial structure which was consistent with a pattern of clustering in the distribution of some (but not all) coloration phenotypes. Overall, these results suggest the occurrence of mechanisms generating spatial concordance of genetic and phenotypic variation.

MORPHOLOGICAL DIFFERENCES AMONG COLORATION MORPHS

Coloration patterns are one of the most obvious expressions of phenotypic polymorphism, and morphs often differ in other traits, such as morphological, ecological, physiological and behavioral features (McKinnon and Pierotti 2010). Although it is assumed that *S. s. bernardezi* presents a conserved morphology despite high levels of genetic diversity and strong spatial structure within its range (Beukema et al. 2016), our results show that, at the fine scale, morphological differentiation is stronger than previously assumed. More specifically, the different coloration morphs showed strong differentiation in size and head morphology.

The existence of coloration polymorphism within this species has been known for a long time (Barrio and Fonoll 1997), but the potential morphological variation of these phenotypes had not been previously assessed. The smaller body size of local populations presenting dominance of the nonstriped morphs was highlighted by Pasmans and Keller (2000), although that study did no report a formal test to compare body size among colorations. Here we evidenced that nonstriped individuals tend to be smaller than the typical striped morphotypes. It should be pointed that the intermediate morph is closer in body size to the unstriped morph than to the striped one. This is interesting because we found a parallel tendency in head shape: the intermediate morph was in fact closer to nonstriped than to the striped morphs.

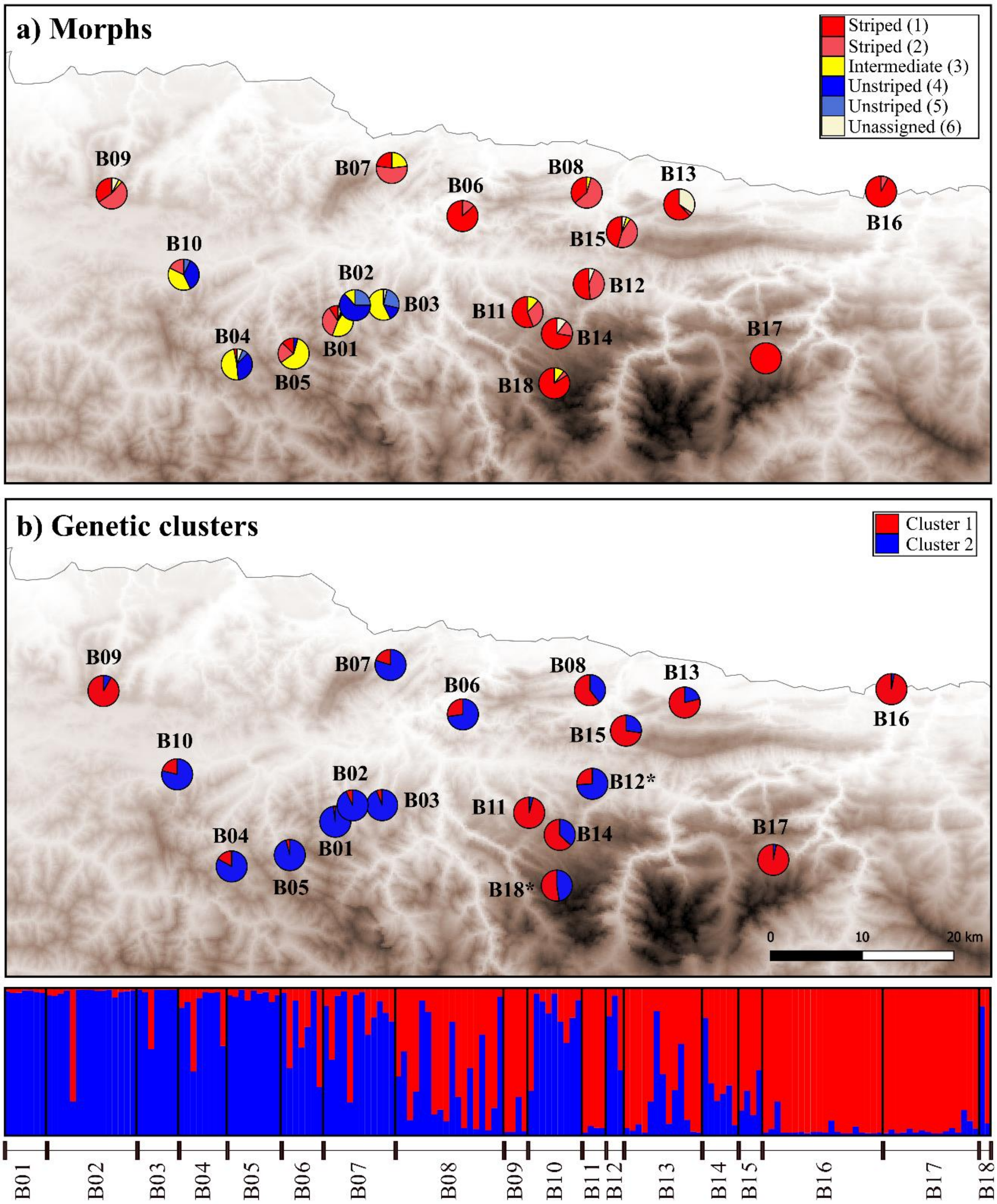


Figure 3.4. (a) Map showing different morphs frequency within each *S. s .bernardezi* sampling population. Different colors in pie charts represent each morph (classification according to Beukema et al 2016). (b) Population pie charts and STRUCTURE barplots (below the map) represent individual cluster membership for K=2.

The relationship between coloration and body size can be influenced by a number of lifestyles, differences in habitat use, reproductive strategies, and predator avoidance strategies (Rudh 2013; Rudh et al. 2013; Winebarger et al. 2018). The black and yellow pattern of adult fire salamanders, together with the presence of toxic steroidal alkaloids (Lüddecke et al. 2018), has been considered an aposematic signal to avoid predation (Cott 1940; Brodie Jr and Smatresk 1990; Sanchez et al. 2019). Despite its nocturnal habits, this coloration remains conspicuous for predators even with dim light (Sanchez et al. 2019), supporting its aposematic function. Some studies related aposematic signal efficacy to larger body sizes, while the loss of aposematic signal were related to a reduction in body size (Hagman and Forsman 2003; Rudh 2013; Hossie et al. 2015). This should imply differences in predators and predation pressures across morphs within *S. s. bernardezi* that led to the selection of different predator avoidance strategies. This is, however, highly improbable as previous studies did not detect environmental or ecological differences among colorations (Beukema et al. 2016; personal observation), and thus we cannot assume differences in predation among morphs.

Here we also identified for the first time differences in head shape among striped and nonstriped phenotypes of *S. s. bernardezi*. Moreover, the allometric relationship of these morphotypes was similar, and thus differences in shape were not due to marked differences in size. In fact, the morphological differentiation of coloration phenotypes persisted after correcting for size variation. Differences in head size among morphs might have some functional and adaptive consequences, even though these differences can reflect variation in whole body size only. For instance, it can favor a change in the trophic niche, due to changes in the potential preys available for each morph and their profitability (Anthony et al. 2008; Scali et al. 2016). At the same time, differences in preys size and robustness may imply adaptive or plastic changes in the biomechanical needs, driving differences in head shape among morphs (Huyghe et al. 2007). On the other hand, main differences in head shape are focused on head width, in the parotid region, with unstriped morph displaying narrower heads and smaller parotids than the striped one (Figure 3.3). Parotids play an important role in defense against predators (Brodie 1983; Stokes et al. 2015). Although those differences may result from differences in the predation pressures between morphs (Vences et al. 2014; see Chapter 2), as set above, differences in predation pressures are not expected due to the lack of niche differences between morphs (Beukema et al. 2016).

PATTERNS OF GENETIC AND PHENOTYPIC DIVERGENCE

A remarkable result of this study was a strong correspondence between the spatial distribution of coloration morphs and the genetic discontinuity revealed by Structure (Figure 3.4). This discontinuity delineates two population units over an east-west axis composed by up to eight populations each, a western cluster, and an eastern one (but one of these, B09, in the western end). In the western cluster, we identified a 'core' of four neighboring populations (B01, B02, B03 and B05) that formed a homogeneous subset. The unstriped morph was restricted to the area occupied by the western cluster (in fact, to six local populations; see Figure 3.4). In contrast, striped morphs were vestigial or rare in four populations and dominant just in two populations of the western cluster, but unambiguously dominant outside the area of the western cluster (see Figure 3.4). It should be pointed out that the intermediate morph was also geographically restricted; these colorations were well represented in all but one of the local populations assigned to the western cluster, but appeared only with marginal or very low frequencies in a few populations of the eastern cluster. Altogether, this suggests a strong consistency of the spatial organization of genetic and phenotypic (color) variation. As we move westwards from our study area only the striped morphs are found (Beukema et al. 2016; personal observation). Thus, the presence of a population belonging to the eastern cluster in the western-most location confirms that unstriped morphs, and probably the genetic unit they belong to, are restricted to a particular geographic area surrounded by striped populations. This pattern of geographic variation in the frequency of different coloration morphs and the observed genetic structure support the independent allopatric evolution of these polymorphisms in a highly restricted geographic area (see García-París et al. 2003; Roulin 2004; Mclean and Stuart-Fox 2014; Beukema et al. 2016).

In addition, the finding of a strong genetic structure, coupled with a nearly parallel structure of coloration morphs, might implicate mechanisms favoring genetic differentiation of these population units. There are several possible explanations for that divergence, as the acquisition of different colorations can have implications in many other biological traits (Rudh and Qvarnström 2013). For instance, population differentiation can be driven by the existence of different morphs and assortative mating (Anthony et al. 2008), selection against migrants (Nosil et al. 2005), or differences in the environmental optima of these morphs (Fisher-Reid et al. 2013; but see Beukema et al. 2016). Nonetheless, further studies on biological, ecological, environmental and population dynamics (i.e. migration, gene flow) would be needed to understand how this polymorphism arose, and its evolutionary and ecological consequences.

Inferring the processes that underlie genetic discontinuities and spatial structure can be fundamental to understand the proximate factors generating diversity in potentially functional traits. Genetic structures can result from an array of factors that constrain gene flow among local populations (i.e., distance, landscape barriers, environmental specialization) (e.g. Wright 1943; Wang and Bradburd 2014), but also from historic processes (i.e., expansion from glacial refuges) or specific population dynamics (e.g., population bottlenecks) (Avise 2000; Stewart and Lister 2001; Pointing et al. 2014). Together with genetic structuration, neutral genetic differentiation among sampling populations, as well as within each genetic cluster, was consistent with a pattern of isolation-by-distance, suggesting that genetic differentiation among the studied populations of *S. s. bernardezi* can be the result of stochastic demographic processes (i.e. mutation, genetic drift), and reduced population connectivity regarding geographic distance. However, differentiation of local populations in head shape, in the whole range and within clusters, did not match an IBD pattern, and neither there was a correlation between genetic and morphometric distances. These results suggest that other evolutionary processes, rather than an accumulation of variance and stochastic demographic processes dependent upon population connectivity, can be responsible of patterns of head morphological differentiation. In that sense, due to the geographic structuration of different morphs we expect differences in head shape among clusters. In addition, studied populations are distributed in a wide altitudinal range (from the sea-level to 1500 masl; Figure 3.4), which is wider in the eastern cluster, while the western cluster is restricted to the lower range (300-900 m). Environmental and microhabitat differences throughout this cline can determine selective biotic and abiotic pressures acting on head morphological variation, such as the availability and characteristics of prey, shelters or predation, among others (Anthony et al. 2008; Naya et al. 2009; Luquet et al. 2015). On the other hand, intraspecific variation can be determined by intrinsic traits of organisms, such as body size and reproductive mode, which influence the degree of connectivity (Pabijan et al. 2012; Paz et al. 2015). For instance, the pueriparous mode of reproduction of *S. s. bernardezi* confers greater independence from water for breeding, avoiding the aquatic larval stage. This can affect the spatial structure of phenotypic variation in different ways. First, independence from water increases the number of potential habitats to be colonized (Lourenço et al. 2017), and therefore, the range of selective pressures among them, favoring divergence among populations. Although the reduction of constraints for reproduction might lead to an increased connectivity and a more continuous distribution, pueriparity in *S. salamandra* do not lead to significant differences in dispersal regarding larviparous populations and females are more philopatric (Lourenço et al. 2018). Indeed, we detected an IBD pattern among populations from both genetic clusters. While the eastern cluster occupies a relatively large area, the western one is restricted to a small area of near 25 kilometers wide, without any potential barrier to dispersion. Those patterns may suggest that dispersal in pueriparous *S. salamandra*, and consequently gene flow, is low even at really small scales, which might increase the divergence among populations and clusters.

Summarizing, *S. s. bernardezi* is a highly diverse polymorphic species in which different morphs differ in body size and head shape. In addition, genetic and phenotypic diversity are highly structured, and present strong geographic concordance. Spatial structuration of genetic and phenotypic diversity might result from a historical isolation scenario in which the polymorphism arose, was positively selected, and later expanded; from evolutionary processes acting differentially over different phenotypes; or a combination of both mechanisms.

REFERENCES

Adams, D. C., M. L. Collyer, A. Kaliontzopoulou, and E. Sherratt. 2017. Geomorph: Software for geometric morphometric analyses. R package version 3.0.5.

Adams, D. C., and E. Otárola-Castillo. 2013. geomorph: An R package for the collection and analysis of geometric morphometric shape data. Methods in Ecology and Evolution 4:393–399.

Adams, D. C., and F. J. Rohlf. 2000. Ecological character displacement in *Plethodon*: biomechanical differences found from a geometric morphometric study. Proceedings of the National Academy of Sciences 97:4106–4111.

Alarcón-Ríos, L., G. Velo-Antón, and A. Kaliontzopoulou. 2017. A non-invasive geometric morphometrics method for exploring variation in dorsal head shape in urodeles: sexual dimorphism and geographic variation in *Salamandra salamandra*. Journal of Morphology 278:475–485.

Alcobendas, M., and J. Castanet. 2000. Bone growth plasticity among populations of *Salamandra salamandra*: interactions between internal and external factors. Herpetologica 56:14–26.

Anthony, C. D., M. D. Venesky, and C. A. M. Hickerson. 2008. Ecological separation in a polymorphic terrestrial salamander. Journal of Animal Ecology 77:646–653.

Arbour, J., and C. Brown. 2014. Incomplete specimens in geometric morphometric analyses. Methods in Ecology and Evolution 5:16–26.

Avise, J. C. 2000. Phylogeography: the history and formation of species. Harvard university press.

Barrio, C. L., and R. Fonoll. 1997. Sobre una población de salamandras *Salamandra salamandr*a con pigmentación anómala. Boletín de la Asociación Herpetológica Española 8:33–36.

Bass, S., and F. Gasser. 1994. Polytipism of *Salamandra salamandra* (L.) in north-western Iberia. Mertensiella 4:41–74.

Beukema, W., A. G. Nicieza, A. Lourenço, and G. Velo-Antón. 2016. Colour polymorphism in *Salamandra salamandra* (Amphibia: Urodela), revealed by a lack of genetic and environmental differentiation between distinct phenotypes. Journal of Zoological Systematics and Evolutionary Research 54:127–136.

Bohonak, A. J. 1999. Dispersal, gene flow, and population structure. The Quarterly Review of Biology 74:21–45.

Bookstein, F. 1991. Morphometric Tools for Landmark Data: Geometry and Biology. Cambridge: Cambridge University Press.

Brodie, E. D. 1983. Antipredator adaptations of salamanders: evolution and convergence among terrestrial species. Pages 109–133 *in* N. S. Margaris, M. Arianoutsou-Faraggitaki, and R. J. Reiter, eds. Adaptations to terrestrial environments. Springer, Boston.

Brodie Jr, E. D., and N. J. Smatresk. 1990. The antipredator arsenal of fire salamanders: spraying of secretions from highly pressurized dorsal skin glands. Herpetologica 1–7.

Castric, V., F. Bonney, and L. Bernatchez. 2001. Landscape structure and hierarchical genetic diversity in the brook charr, *Salvelinus fontinalis*. Evolution 55:1016–1028.

Collyer, M. L., D. Sekora, and D. C. Adams. 2015. A method for analysis of phenotypic change for phenotypes described by high- dimensional data. Heredity 115:357–365.

Cott, H. B. 1940. Adaptive coloration in animals. Methuen & Co., Ltd., London.

Dopazo, H., and P. Alberch. 1994. Preliminary results on optional viviparity and intrauterine siblicide in *Salamandra salamandra* populations from Northern Spain. Mertensiella 4:125–137.

Earl, D. A., and B. M. vonHoldt. 2012. STRUCTURE HARVESTER: A website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetics Resources 4:359–361.

Evanno, G., S. Regnaut, and J. Goudet. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. Molecular Ecology 14:2611–2620.

Fisher-Reid, M. C., T. N. Engstrom, C. A. Kuczynski, P. R. Stephens, and J. J. Wiens. 2013. Parapatric divergence of sympatric morphs in a salamander: Incipient speciation on Long Island? Molecular Ecology 22:4681–4694.

Francis, R. M. 2017. pophelper: an R package and web app to analyse and visualize population structure. Molecular ecology resources 17:27–32.

García-París, M., M. Alcobendas, D. Buckley, and D. B. Wake. 2003. Dispersal of viviparity across contact zones in Iberian populations of fire salamanders (*Salamandra*) inferred from discordance of genetic and morphological traits. Evolution; international journal of organic evolution 57:129–43.

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 macrodactulym*). Molecular Ecology 16:1625–1637.

Goslee, S., and D. Urban. 2007. ecodist: Dissimilarity-based functions for ecological analysis. R package version 1.

Goudet, J. 1995. FSTAT (Version 1.2):A Computer Program to Calculate F-Statistics. Journal of Heredity 86:485–486.

Grant, P. R., and B. R. Grant. 2011. How and why species multiply: the radiation of Darwin’s finches. Princeton University Press.

Gray, S. M., and J. S. McKinnon. 2007. Linking color polymorphism maintenance and speciation. Trends in Ecology and Evolution 22:71–79.

Gunz, P., P. Mitteroecker, S. Neubauer, G. W. Weber, and F. L. Bookstein. 2009. Principles for the virtual reconstruction of hominin crania. Journal of Human Evolution 57:48–62.

Hagman, M., and A. Forsman. 2003. Correlated evolution of conspicuous coloration and body size in poison frogs (Dendrobatidae). Evolution 57:2904–2910.

Hendrix, R., B. R. Schmidt, M. Schaub, E. T. Krause, and S. Steinfartz. 2017. Differentiation of movement behaviour in an adaptively diverging salamander population. Molecular ecology 26:6400–6413.

Hoffman, E. A., F. W. Schueler, A. G. Jones, and M. S. Blouin. 2006. An analysis of selection on a colour polymorphism in the northern leopard frog. Molecular Ecology 15:2627–2641.

Hossie, T. J., J. Skelhorn, J. W. Breinholt, A. Y. Kawahara, and T. N. Sherratt. 2015. Body size affects the evolution of eyespots in caterpillars. Proceedings of the National Academy of Sciences 112:6664–6669.

Huyghe, K., B. Vanhooydonck, A. Herrel, Z. Tadić, and R. Van Damme. 2007. Morphology, performance, behavior and ecology of three color morphs in males of the lizard *Podarcis melisellensis*. Integrative and Comparative Biology 47:211–220.

Ivanović, A., and J. W. Arntzen. 2017. Evolution of skull shape in the family Salamandridae (Amphibia: Caudata). Journal of Anatomy.

Kalinowski, S. T. 2005. hp-rare 1.0: a computer program for performing rarefaction on measures of allelic richness. Molecular Ecology Notes 5:187–189.

Kästle, W. 1986. Rival combats in *Salamandra salamandra*. Studies in herpetology 525–528.

Kimura, M., and G. H. Weiss. 1964. The stepping stone model of population structure and the decrease of genetic correlation with distance. Genetics 49:561.

Labus, N., M. Cvijanović, and T. D. Vukov. 2013. Sexual size and shape dimorphism in *Salamandra salamandra* (Amphibia, Caudata, Salamandridae) from the central Balkans. Archives of Biological Science 65:969–975.

Lourenço, A., D. Álvarez, I. J. Wang, and G. Velo-Antón. 2017. Trapped within the city: integrating demography, time since isolation and population-specific traits to assess the genetic effects of urbanization. Molecular Ecology 26:1498–1514.

Lourenço, A., B. Antunes, I. J. Wang, and G. Velo-Antón. 2018. Fine-scale genetic structure in a salamander with two reproductive modes: Does reproductive mode affect dispersal? Evolutionary Ecology 32:699–732.

Lüddecke, T., S. Schulz, S. Steinfartz, and M. Vences. 2018. A salamander's toxic arsenal: review of skin poison diversity and function in true salamanders, genus *Salamandra*. Science of Nature 105:56.

Luquet, E., J.-P. Léna, C. Miaud, and S. Plénet. 2015. Phenotypic divergence of the common toad (*Bufo bufo*) along an altitudinal gradient: evidence for local adaptation. Heredity 114:69.

McKinnon, J. S., and M. E. R. Pierotti. 2010. Colour polymorphism and correlated characters: Genetic mechanisms and evolution. Molecular Ecology 19:5101–5125.

McLean, C. A., and D. Stuart-Fox. 2014. Geographic variation in animal colour polymorphisms and its role in speciation. Biological Reviews 89:860–873.

Mitchell-Olds, T., J. H. Willis, and D. B. Goldstein. 2007. Which evolutionary processes influence natural genetic variation for phenotypic traits? Nature Reviews Genetics 8:845.

Mullen, L. M., S. N. Vignieri, J. A. Gore, and H. E. Hoekstra. 2009. Adaptive basis of geographic variation: Genetic, phenotypic and environmental differences among beach mouse populations. Proceedings of the Royal Society B: Biological Sciences 276:3809–3818.

Naya, D. E., C. Veloso, and F. Bozinovic. 2009. Gut size variation among *Bufo spinulosus* populations along an altitudinal (and dietary) gradient. Annales Zoologici Fennici 46:16–20.

Nosil, P., T. H. Vines, and D. J. Funk. 2005. Reproductive isolation caused by natural selection against immigrants from divergent habitats. Evolution 59:705–719.

Pabijan, M., K. C. Wollenberg, and M. Vences. 2012. Small body size increases the regional differentiation of populations of tropical mantellid frogs (Anura: Mantellidae). Journal of evolutionary biology 25:2310–2324.

Pasmans, F., and H. Keller. 2000. Morphological variation in neighbouring populations of *Salamandra salamandra bernardezi* in northern Spain. Zeitschr Feldherp 7:77–84.

Paz, A., R. Ibáñez, K. R. Lips, and A. J. Crawford. 2015. Testing the role of ecology and life history in structuring genetic variation across a landscape: A trait-based phylogeographic approach. Molecular Ecology 24:3723–3737.

Peakall, R., and P. E. Smouse. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. Bioinformatics 28, 2537e2539.

Pointing, S. B., B. Bollard-Breen, and L. N. Gillman. 2014. Diverse cryptic refuges for life during glaciation. Proceedings of the National Academy of Sciences 111:5452–5453.

Pritchard, J. K., M. Stephens, and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics 155:945–959.

Raymond, M., and F. Rousset. 1995. GENEPOP on the Web (Version 3.4). URL: http://wbiomed. curtin. edu. au/genepop/Updated from Raymond & Rousset.

R Development Core Team. 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rohlf, F. 2015. tpsDig2 Software. Stony Crook, NY: State Univ of New York.

Rohlf, F. J. 1999. Shape statistics: Procrustes superimpositions and tangent spaces. Journal of Classification 16:197–223.

Rohlf, F. J., and D. E. Slice. 1990. Extensions of the Procrustes method for the optimal superimposition of landmarks. Systematic Biology 39:40–59.

Roulin, A. 2004. The evolution, maintenance and adaptive function of genetic colour polymorphism in birds. Biological Reviews 79:815–848.

Rudh, A. 2013. Loss of conspicuous coloration has co-evolved with decreased body size in populations of poison dart frogs. Evolutionary Ecology 27:755–767.

Rudh, A., M. F. Breed, and A. Qvarnström. 2013. Does aggression and explorative behaviour decrease with lost warning coloration? Biological Journal of the Linnean Society 108:116–126.

Rudh, A., and A. Qvarnström. 2013. Adaptive colouration in amphibians. Seminars in Cell & Developmental Biology 24: 553–561.

Rudh, A., B. Rogell, and J. Höglund. 2007. Non-gradual variation in colour morphs of the strawberry poison frog *Dendrobates pumilio*: Genetic and geographical isolation suggest a role for selection in maintaining polymorphism. Molecular Ecology 16:4284–4294.

Sanchez, E., H. Pröhl, T. Lüddecke, S. Schulz, S. Steinfartz, and M. Vences. 2019. The conspicuous postmetamorphic coloration of fire salamanders, but not their toxicity, is affected by larval background albedo. Journal of Experimental Zoology Part B: Molecular and Developmental Evolution 332:26–35.

Scali, S., R. Sacchi, M. Mangiacotti, F. Pupin, A. Gentilli, C. Zucchi, M. Sannolo, et al. 2016. Does a polymorphic species have a “polymorphic” diet? A case study from a lacertid lizard. Biological Journal of the Linnean Society 117:492–502.

Schemske, D. W., and P. Bierzychudek. 2007. Spatial differentiation for flower color in the desert annual Linanthus parryae: Was Wright right? Evolution 61:2528–2543.

Slatkin, M. 1993. Isolation by distance in equilibrium and non-equilibrium populations. Evolution 47:264–279.

Steinfartz, S., D. Kusters, and D. Tautz. 2004. Isolation and characterization of polymorphic tetranucleotide microsatellite loci in the Fire salamander *Salamandra salamandra* (Amphibia: Caudata). Molecular Ecology Notes 4:626–628.

Stewart, J. R., and A. M. Lister. 2001. Cryptic northern refugia and the origins of the modern biota. Trends in Ecology & Evolution 16:608–613.

Stokes, A. N., A. M. Ray, M. W. Buktenica, B. G. Gall, E. Paulson, D. Paulson, S. S. French, et al. 2015. Otter Predation on *Taricha granulosa* and Variation in Tetrodotoxin Levels with Elevation. Northwestern Naturalist 96:13–21.

Storz, J. F. 2002. Contrasting patterns of divergence in quantitative traits and neutral DNA markers: analysis of clinal variation. Molecular Ecology 11:2537–2551.

Trueb, L. 1993. Patterns of cranial diversity among the Lissamphibia. Pages 255–343 *in* J. Hanken, and B. K. Hall. The skull. Volume 2. Uiversity of Chicago Press.

Van Oosterhout, C., W. F. Hutchinson, D. P. M. Wills, and P. Shipley. 2004. MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Notes 4:535–538.

Velo-Antón, G., and D. Buckley. 2015. Salamandra común – *Salamandra salamandra* (Linnaeus, 1758). Enciclopedia Virtual de los Vertebrados Españoles 2–12.

Velo-Antón, G., M. García-París, P. Galán, and A. Cordero-Rivera. 2007. The evolution of viviparity in holocene islands: ecological adaptation versus phylogenetic descent along the transition from aquatic to terrestrial environments. Journal of Zoological Systematics and Evolutionary Research 45:345–352.

Vences, M., E. Sanchez, J. S. Hauswaldt, A. Eikelmann, D. Rodríguez, S. Carranza, P. Werner, D. Donarire-Barroso, et al. 2014. Nuclear and mitochondrial multilocus phylogeny and survey of alkaloid content in true salamanders of the genus *Salamandra* (Salamandridae). Molecular phylogenetics and evolution, 73:208–216.

Wang, I. J., and G. S. Bradburd. 2014. Isolation by environment. Molecular Ecology 23:5649–5662.

Wang, I. J., and K. Summers. 2010. Genetic structure is correlated with phenotypic divergence rather than geographic isolation in the highly polymorphic strawberry poison-dart frog. Molecular Ecology 19:447–458.

Winebarger, M. M., M. W. Pugh, M. M. Gangloff, M. S. Osbourn, and L. Siefferman. 2018. Body size is positively correlated with conspicuous coloration in Ambystoma salamanders, but negatively correlated with conspicuous coloration in Plethodon salamanders. Frontiers in Ecology and Evolution 6:1–8.

Wright, S. 1943. Isolation by distance. Genetics 28:114.

Zamudio, K. R., R. C. Bell, and N. A. Mason. 2016. Phenotypes in phylogeography: Species' traits, environmental variation, and vertebrate diversification. Proceedings of the National Academy of Sciences 113:8041–8048.

Zamudio, K. R., and A. M. Wieczorek. 2007. Fine-scale spatial genetic structure and dispersal among spotted salamander (*Ambystoma maculatum*) breeding populations. Molecular Ecology 16:257–274.

Zelditch, M. L., D. L. Swiderski, and D. H. Sheets. 2012. Geometric Morphometrics for biologists. A primer (Second Edition). Elsevier.

Supplementary Material Chapter 3

Table S3.1. Details of the 9 microsatellites used in this study and information on multiplex arrangement. Original published primers forward and reverse sequences, fluorescently labelled oligonucleotides used as template for modified forward primers and the concentration of primer mix used to construct multiplex and on 10 µl PCR reactions are displayed.

Locus	**Multiplex**	**Label***	**Primer forward (5' – 3')**	**Primer reverse (5' – 3')**	**Primer Mix multiplex/PCR (µM)**
SalE8	Mix 1	6-FAM	GCAAAGTCCATGCTTTCCCTTTCTC	GACATACCAAAGACTCCAGAATGGG	0.8 / 0.08
SalE6	Mix 1	6-FAM	GGACTCATGGTCACCCAGAGGTTCT	ATGGATTGTGTCGAAATAAGGTATC	1.2 / 0.12
SalE5	Mix 1	VIC	CCACATGATGCCTACGTATGTTGTG	CTCCTGTTTACGCTTCACCTGCTCC	0.6 / 0.06
Sal29	Mix 1	NED	CTCTTTGACTGAACCAGAACCCC	GCCTGTCGGCTCTGTGTAACC	8.0 / 0.8
Sal3	Mix 1	PET	CTCAGACAAGAAATCCTGCTTCTTC	ATAAATCTGTCCTGTTCCTAATCAG	3.6/0.36
Sal23	Mix 2	6-FAM	TCACTGTTTATCTTTGTTCTTTTAT	AATTATTTGTTTGAGTCGATTTTCT	2/0.2
SalE2	Mix 2	VIC	CACGACAAAATACAGAGAGTGGATA	ATATTTGAAATTGCCCATTTGGTA	1.2/0.12
SalE7	Mix 2	PET	TTTCAGCACCAAGATACCTCTTTTG	CTCCCTCCATATCAAGGTCACAGAC	1/0.1
SalE12	Mix 2	NED	CTCAGGAACAGTGTGCCCCAAATAC	CTCATAATTTAGTCTACCCTCCCAC	0.8 / 0.08

*An extra number of base pairs were added at the 5' end of the original sequence of forward primers in order to allow binding of four different fluorescent labelled oligonucleotides (6-FAM - TGT AAA ACG ACG GCC AGT; VIC - TAA TAC GAC TCA CTA TAG GG; NED - TTT CCC AGT CAC GAC GTT G; PET - GAT AAC AAT TTC ACA CAG G)

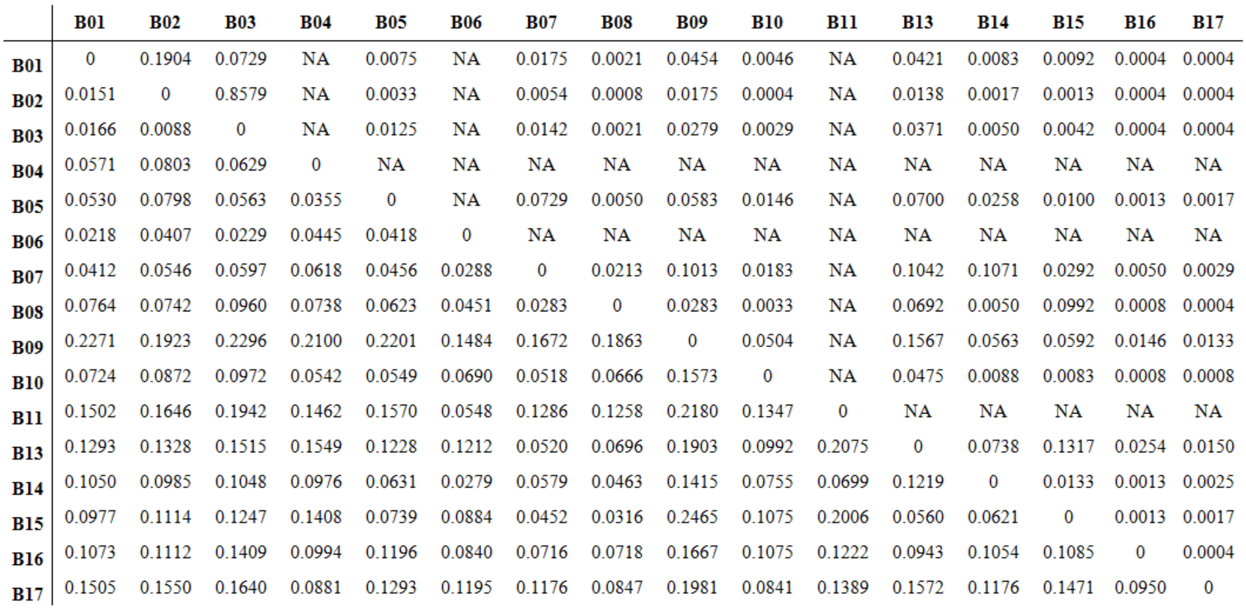
Table S3.2. Procrustes ANOVAs including sex as a factor. As there are no differences in levels of sexual dimorphism we did not include it in main analyses.

	Df	**SS**	**F**	**Z**	***P***
a) Shape					
Sex	1	0.024	19.561	6.145	0.000
Morph	3	0.032	8.829	7.422	0.000
Sex x Morph	3	0.003	0.840	-0.164	0.564
Residuals	546	0.664			
b) Size					
Sex	1	0.959	65.586	2.484	0.000
Morph	3	0.241	5.503	2.247	0.000
Sex x Morph	3	0.024	0.545	-0.111	0.604
Residuals	546	7.979			
c) Size as covariate					
Size	1	0.011	8.929	4.587	0.000
Sex	1	0.020	17.012	5.878	0.000
Morph	3	0.032	8.846	7.417	0.000
Size x Sex	1	0.002	1.706	1.544	0.065
Size x Morph	3	0.005	1.484	1.801	0.038
Sex x Morph	3	0.003	0.727	-0.589	0.720
Size x Sex x Morph	3	0.006	1.606	2.119	0.020
Residuals	538	0.645			

Table S3.3. Proportion of assignation to each cluster (K=2) for 7 and 9 loci datasets

Sampling location	**Code**	**$N_{genotyped}$**	**9 loci**		**7 loci**	
			Cluster 1	**Cluster 2**	**Cluster 1**	**Cluster 2**
Color	B01	7	0.025	0.975	0.043	0.957
Tendi	B02	15	0.075	0.925	0.046	0.954
Llerandi	B03	7	0.069	0.931	0.049	0.951
La Marea	B04	8	0.17	0.83	0.379	0.621
La Pesanca	B05	9	0.046	0.954	0.357	0.644
Zardón	B06	7	0.279	0.721	0.434	0.566
Sueve	B07	12	0.205	0.795	0.537	0.464
Doradiellu	B08	18	0.606	0.394	0.771	0.229
Fario	B09	4	0.916	0.084	0.880	0.120
Fuensanta	B10	9	0.216	0.784	0.386	0.614
La Huesera	B11	4	0.954	0.046	0.910	0.090
Jolagua*	B12*	3	0.26	0.74	0.705	0.295
Mañanagas	B13	13	0.782	0.218	0.891	0.109
Buferrera	B14	6	0.63	0.37	0.749	0.251
Las Xareras	B15	4	0.722	0.278	0.838	0.162
Pimiango	B16	20	0.965	0.035	0.923	0.077
Valdediezma	B17	16	0.962	0.038	0.876	0.124
Vegarredonda*	B18*	2	0.521	0.479	0.532	0.468

Table S3.4. Pairwise F_{ST} values (9 loci). Values above de diagonal are the p-values.



	B01	**B02**	**B03**	**B04**	**B05**	**B06**	**B07**	**B08**	**B09**	**B10**	**B11**	**B13**	**B14**	**B15**	**B16**	**B17**
B01	0	0.1904	0.0729	NA	0.0075	NA	0.0175	0.0021	0.0454	0.0046	NA	0.0421	0.0083	0.0092	0.0004	0.0004
B02	0.0151	0	0.8579	NA	0.0033	NA	0.0054	0.0008	0.0175	0.0004	NA	0.0138	0.0017	0.0013	0.0004	0.0004
B03	0.0166	0.0088	0	NA	0.0125	NA	0.0142	0.0021	0.0279	0.0029	NA	0.0371	0.0050	0.0042	0.0004	0.0004
B04	0.0571	0.0803	0.0629	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
B05	0.0530	0.0798	0.0563	0.0355	0	NA	0.0729	0.0050	0.0583	0.0146	NA	0.0700	0.0258	0.0100	0.0013	0.0017
B06	0.0218	0.0407	0.0229	0.0445	0.0418	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
B07	0.0412	0.0546	0.0597	0.0618	0.0456	0.0288	0	0.0213	0.1013	0.0183	NA	0.1042	0.1071	0.0292	0.0050	0.0029
B08	0.0764	0.0742	0.0960	0.0738	0.0623	0.0451	0.0283	0	0.0283	0.0033	NA	0.0692	0.0050	0.0992	0.0008	0.0004
B09	0.2271	0.1923	0.2296	0.2100	0.2201	0.1484	0.1672	0.1863	0	0.0504	NA	0.1567	0.0563	0.0592	0.0146	0.0133
B10	0.0724	0.0872	0.0972	0.0542	0.0549	0.0690	0.0518	0.0666	0.1573	0	NA	0.0475	0.0088	0.0083	0.0008	0.0008
B11	0.1502	0.1646	0.1942	0.1462	0.1570	0.0548	0.1286	0.1258	0.2180	0.1347	0	NA	NA	NA	NA	NA
B13	0.1293	0.1328	0.1515	0.1549	0.1228	0.1212	0.0520	0.0696	0.1903	0.0992	0.2075	0	0.0738	0.1317	0.0254	0.0150
B14	0.1050	0.0985	0.1048	0.0976	0.0631	0.0279	0.0579	0.0463	0.1415	0.0755	0.0699	0.1219	0	0.0133	0.0013	0.0025
B15	0.0977	0.1114	0.1247	0.1408	0.0739	0.0884	0.0452	0.0316	0.2465	0.1075	0.2006	0.0560	0.0621	0	0.0013	0.0017
B16	0.1073	0.1112	0.1409	0.0994	0.1196	0.0840	0.0716	0.0718	0.1667	0.1075	0.1222	0.0943	0.1054	0.1085	0	0.0004
B17	0.1505	0.1550	0.1640	0.0881	0.1293	0.1195	0.1176	0.0847	0.1981	0.0841	0.1389	0.1572	0.1176	0.1471	0.0950	0

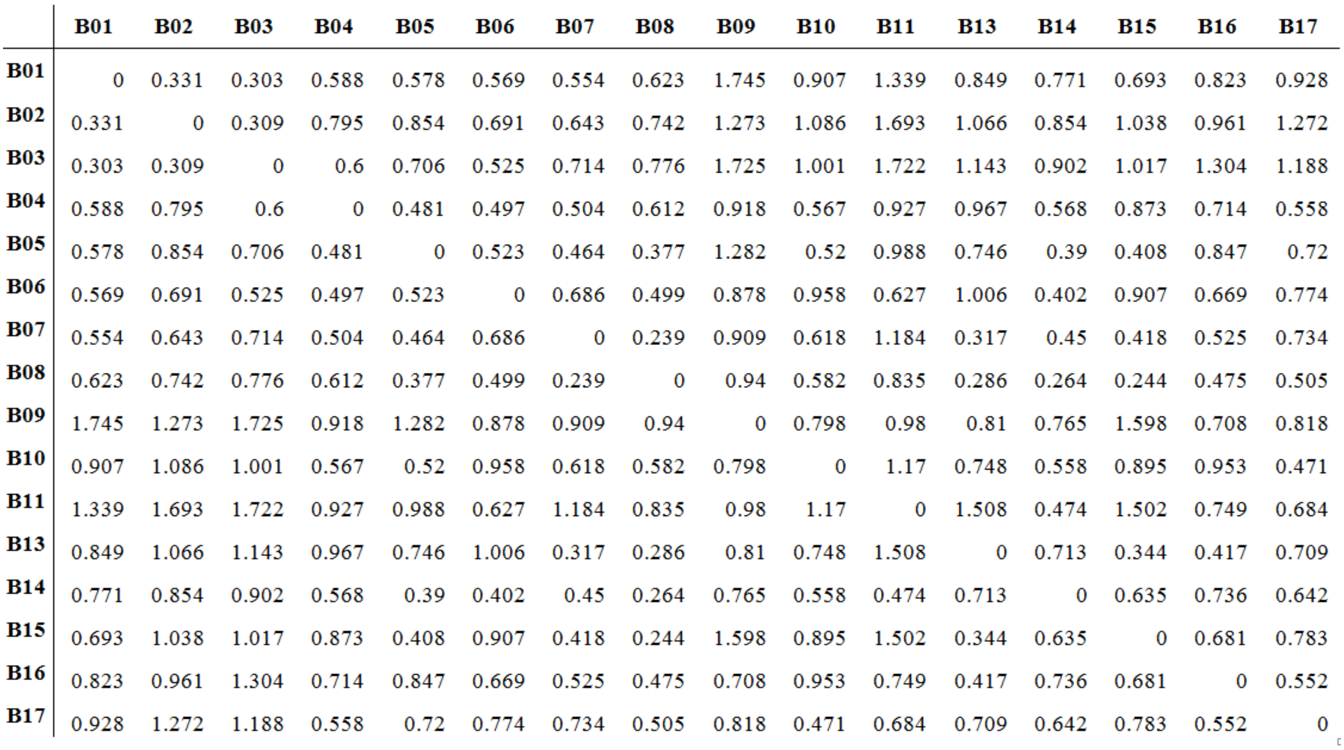
Table S3.5. Pairwise F_{ST} values (7 loci). Values above de diagonal are the p-values.

	B01	B02	B03	B04	B05	B06	B07	B08	B09	B10	B11	B13	B14	B15	B16	B17
B01	0	0.3500	0.3463	0.0100	0.0004	0.0571	0.0013	0.0004	0.0113	0.0004	NA	0.0017	0.0038	0.0208	0.0004	0.0004
B02	0.0080	0	0.6967	0.0025	0.0004	0.0100	0.0004	0.0004	0.0046	0.0004	NA	0.0004	0.0004	0.0017	0.0004	0.0004
B03	-0.0046	0.0139	0	0.0204	0.0013	0.1142	0.0008	0.0004	0.0100	0.0008	NA	0.0013	0.0013	0.0071	0.0004	0.0004
B04	0.0443	0.0829	0.0645	0	0.0092	0.0308	0.0550	0.0033	0.0563	0.0154	NA	0.0058	0.0092	0.0321	0.0017	0.0025
B05	0.0501	0.0948	0.0819	0.0485	0	0.0154	0.0167	0.0013	0.0096	0.0013	NA	0.0004	0.0054	0.0267	0.0004	0.0004
B06	0.0237	0.0570	0.0356	0.0276	0.0430	0	0.0117	0.0033	0.0263	0.0054	NA	0.0025	0.0988	0.0125	0.0004	0.0008
B07	0.0330	0.0598	0.0665	0.0391	0.0424	0.0487	0	0.0154	0.0054	0.0004	NA	0.0013	0.1058	0.0788	0.0004	0.0004
B08	0.0651	0.0887	0.0955	0.0778	0.0460	0.0491	0.0144	0	0.0013	0.0004	NA	0.0004	0.0988	0.3004	0.0004	0.0004
B09	0.1969	0.1747	0.2266	0.1629	0.1998	0.1361	0.1345	0.1649	0	0.0083	NA	0.0038	0.0133	0.0313	0.0013	0.0017
B10	0.0772	0.1041	0.1027	0.0511	0.0534	0.0824	0.0554	0.0720	0.1285	0	NA	0.0004	0.0004	0.0054	0.0004	0.0004
B11	0.1619	0.1877	0.2136	0.1408	0.1564	0.0748	0.1479	0.1414	0.2150	0.1564	0	NA	NA	NA	NA	NA
B13	0.1286	0.1554	0.1739	0.1577	0.1357	0.1472	0.0503	0.0567	0.1855	0.1280	0.2500	0	0.0004	0.0704	0.0004	0.0004
B14	0.0706	0.0909	0.1045	0.0603	0.0333	0.0162	0.0330	0.0171	0.1466	0.0541	0.0652	0.1284	0	0.0104	0.0004	0.0004
B15	0.0688	0.1196	0.1320	0.1167	0.0378	0.0997	0.0285	0.0114	0.2625	0.1081	0.2423	0.0502	0.0866	0	0.0008	0.0013
B16	0.0836	0.1064	0.1345	0.0883	0.1082	0.0686	0.0587	0.0681	0.1292	0.1095	0.1242	0.0837	0.0918	0.0931	0	0.0004
B17	0.1165	0.1510	0.1536	0.0841	0.1139	0.1017	0.1030	0.0878	0.1654	0.0682	0.1299	0.1492	0.0982	0.1273	0.0936	0

Table S3.6. Pairwise Nei's Genetic Distance (9 loci).

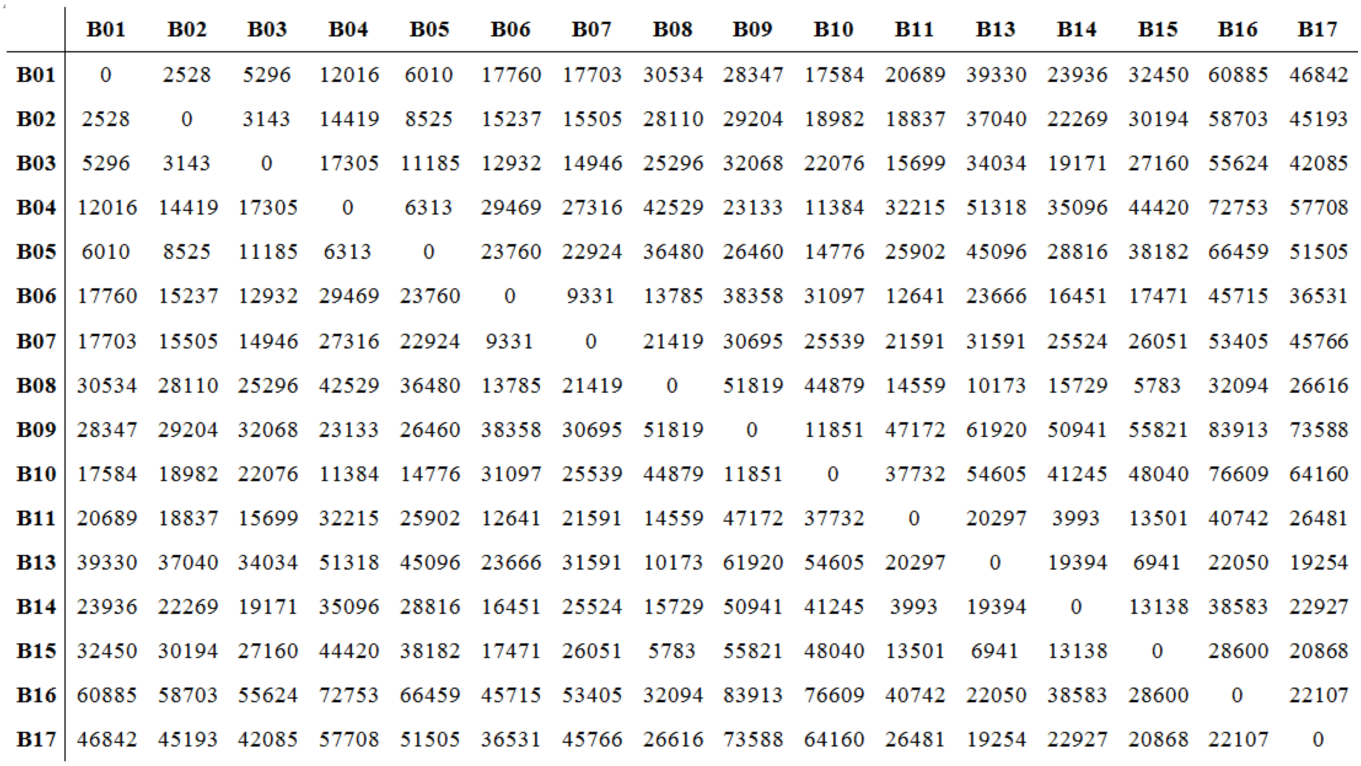
	B01	B02	B03	B04	B05	B06	B07	B08	B09	B10	B11	B13	B14	B15	B16	B17
B01	0	0.361	0.419	0.637	0.586	0.552	0.603	0.709	1.767	0.835	1.23	0.978	1.037	0.887	1.002	1.252
B02	0.361	0	0.306	0.786	0.767	0.612	0.661	0.66	1.397	0.973	1.533	1.026	0.979	1.001	1.06	1.418
B03	0.419	0.306	0	0.64	0.583	0.523	0.731	0.856	1.624	1.057	1.773	1.18	0.986	1.05	1.462	1.403
B04	0.637	0.786	0.64	0	0.45	0.662	0.718	0.623	1.199	0.642	1.104	1.155	0.858	1.166	0.803	0.59
B05	0.586	0.767	0.583	0.45	0	0.59	0.553	0.528	1.435	0.6	1.177	0.831	0.602	0.636	1.023	0.894
B06	0.552	0.612	0.523	0.662	0.59	0	0.621	0.545	0.913	0.957	0.651	1.034	0.536	0.938	0.848	0.986
B07	0.603	0.661	0.731	0.718	0.553	0.621	0	0.369	1.135	0.692	1.181	0.443	0.682	0.57	0.669	0.928
B08	0.709	0.66	0.856	0.623	0.528	0.545	0.369	0	1.115	0.621	0.864	0.437	0.456	0.372	0.529	0.518
B09	1.767	1.397	1.624	1.199	1.435	0.913	1.135	1.115	0	0.96	0.993	0.922	0.696	1.305	0.928	0.995
B10	0.835	0.973	1.057	0.642	0.6	0.957	0.692	0.621	0.96	0	1.167	0.73	0.787	0.993	1.002	0.609
B11	1.23	1.533	1.773	1.104	1.177	0.651	1.181	0.864	0.993	1.167	0	1.479	0.589	1.363	0.822	0.809
B13	0.978	1.026	1.18	1.155	0.831	1.034	0.443	0.437	0.922	0.73	1.479	0	0.845	0.474	0.561	0.895
B14	1.037	0.979	0.986	0.858	0.602	0.536	0.682	0.456	0.696	0.787	0.589	0.845	0	0.589	0.872	0.799
B15	0.887	1.001	1.05	1.166	0.636	0.938	0.57	0.372	1.305	0.993	1.363	0.474	0.589	0	0.822	0.967
B16	1.002	1.06	1.462	0.803	1.023	0.848	0.669	0.529	0.928	1.002	0.822	0.561	0.872	0.822	0	0.574
B17	1.252	1.418	1.403	0.59	0.894	0.986	0.928	0.518	0.995	0.609	0.809	0.895	0.799	0.967	0.574	0

Table S3.7. Pairwise Nei's Genetic Distance (7 loci).



	B01	B02	B03	B04	B05	B06	B07	B08	B09	B10	B11	B13	B14	B15	B16	B17
B01	0	0.331	0.303	0.588	0.578	0.569	0.554	0.623	1.745	0.907	1.339	0.849	0.771	0.693	0.823	0.928
B02	0.331	0	0.309	0.795	0.854	0.691	0.643	0.742	1.273	1.086	1.693	1.066	0.854	1.038	0.961	1.272
B03	0.303	0.309	0	0.6	0.706	0.525	0.714	0.776	1.725	1.001	1.722	1.143	0.902	1.017	1.304	1.188
B04	0.588	0.795	0.6	0	0.481	0.497	0.504	0.612	0.918	0.567	0.927	0.967	0.568	0.873	0.714	0.558
B05	0.578	0.854	0.706	0.481	0	0.523	0.464	0.377	1.282	0.52	0.988	0.746	0.39	0.408	0.847	0.72
B06	0.569	0.691	0.525	0.497	0.523	0	0.686	0.499	0.878	0.958	0.627	1.006	0.402	0.907	0.669	0.774
B07	0.554	0.643	0.714	0.504	0.464	0.686	0	0.239	0.909	0.618	1.184	0.317	0.45	0.418	0.525	0.734
B08	0.623	0.742	0.776	0.612	0.377	0.499	0.239	0	0.94	0.582	0.835	0.286	0.264	0.244	0.475	0.505
B09	1.745	1.273	1.725	0.918	1.282	0.878	0.909	0.94	0	0.798	0.98	0.81	0.765	1.598	0.708	0.818
B10	0.907	1.086	1.001	0.567	0.52	0.958	0.618	0.582	0.798	0	1.17	0.748	0.558	0.895	0.953	0.471
B11	1.339	1.693	1.722	0.927	0.988	0.627	1.184	0.835	0.98	1.17	0	1.508	0.474	1.502	0.749	0.684
B13	0.849	1.066	1.143	0.967	0.746	1.006	0.317	0.286	0.81	0.748	1.508	0	0.713	0.344	0.417	0.709
B14	0.771	0.854	0.902	0.568	0.39	0.402	0.45	0.264	0.765	0.558	0.474	0.713	0	0.635	0.736	0.642
B15	0.693	1.038	1.017	0.873	0.408	0.907	0.418	0.244	1.598	0.895	1.502	0.344	0.635	0	0.681	0.783
B16	0.823	0.961	1.304	0.714	0.847	0.669	0.525	0.475	0.708	0.953	0.749	0.417	0.736	0.681	0	0.552
B17	0.928	1.272	1.188	0.558	0.72	0.774	0.734	0.505	0.818	0.471	0.684	0.709	0.642	0.783	0.552	0

Table S3.8. Pairwise linear geographic distances among sampling populations (meters).



	B01	B02	B03	B04	B05	B06	B07	B08	B09	B10	B11	B13	B14	B15	B16	B17
B01	0	2528	5296	12016	6010	17760	17703	30534	28347	17584	20689	39330	23936	32450	60885	46842
B02	2528	0	3143	14419	8525	15237	15505	28110	29204	18982	18837	37040	22269	30194	58703	45193
B03	5296	3143	0	17305	11185	12932	14946	25296	32068	22076	15699	34034	19171	27160	55624	42085
B04	12016	14419	17305	0	6313	29469	27316	42529	23133	11384	32215	51318	35096	44420	72753	57708
B05	6010	8525	11185	6313	0	23760	22924	36480	26460	14776	25902	45096	28816	38182	66459	51505
B06	17760	15237	12932	29469	23760	0	9331	13785	38358	31097	12641	23666	16451	17471	45715	36531
B07	17703	15505	14946	27316	22924	9331	0	21419	30695	25539	21591	31591	25524	26051	53405	45766
B08	30534	28110	25296	42529	36480	13785	21419	0	51819	44879	14559	10173	15729	5783	32094	26616
B09	28347	29204	32068	23133	26460	38358	30695	51819	0	11851	47172	61920	50941	55821	83913	73588
B10	17584	18982	22076	11384	14776	31097	25539	44879	11851	0	37732	54605	41245	48040	76609	64160
B11	20689	18837	15699	32215	25902	12641	21591	14559	47172	37732	0	20297	3993	13501	40742	26481
B13	39330	37040	34034	51318	45096	23666	31591	10173	61920	54605	20297	0	19394	6941	22050	19254
B14	23936	22269	19171	35096	28816	16451	25524	15729	50941	41245	3993	19394	0	13138	38583	22927
B15	32450	30194	27160	44420	38182	17471	26051	5783	55821	48040	13501	6941	13138	0	28600	20868
B16	60885	58703	55624	72753	66459	45715	53405	32094	83913	76609	40742	22050	38583	28600	0	22107
B17	46842	45193	42085	57708	51505	36531	45766	26616	73588	64160	26481	19254	22927	20868	22107	0

Table S3.9. Pairwise matrix of morphological Procrustes distances (LS)

	B01	B02	B03	B04	B05	B06	B07	B08	B09	B10	B11	B13	B14	B15	B16	B17
B01	0	0.007397	0.014413	0.011320	0.021376	0.044176	0.018371	0.030477	0.024040	0.016837	0.020900	0.025483	0.015233	0.035411	0.018527	0.029302
B02	0.007397	0	0.016122	0.012786	0.021596	0.046012	0.018312	0.031315	0.026080	0.017394	0.022387	0.027330	0.014711	0.036383	0.019059	0.031644
B03	0.014413	0.016122	0	0.012313	0.022306	0.044754	0.015981	0.028591	0.020291	0.014165	0.015347	0.025208	0.016634	0.031740	0.017551	0.031136
B04	0.011320	0.012786	0.012313	0	0.019375	0.046899	0.016684	0.031717	0.023755	0.013503	0.018272	0.025518	0.016505	0.035528	0.020541	0.030274
B05	0.021376	0.021596	0.022306	0.019375	0	0.037920	0.014891	0.021781	0.021176	0.013833	0.014701	0.018525	0.020305	0.026333	0.023356	0.019991
B06	0.044176	0.046012	0.044754	0.046899	0.037920	0	0.035008	0.021641	0.028042	0.038063	0.038436	0.035008	0.040144	0.023959	0.033225	0.025380
B07	0.018371	0.018312	0.015981	0.016684	0.014891	0.035008	0	0.018628	0.014009	0.007878	0.012460	0.020904	0.016676	0.022838	0.015215	0.022178
B08	0.030477	0.031315	0.028591	0.031717	0.021781	0.021641	0.018628	0	0.016477	0.021245	0.020888	0.021624	0.026400	0.013641	0.021323	0.018152
B09	0.024040	0.026080	0.020291	0.023755	0.021176	0.028042	0.014009	0.016477	0	0.016907	0.015237	0.020860	0.018492	0.020598	0.014537	0.021481
B10	0.016837	0.017394	0.014165	0.013503	0.013833	0.038063	0.007878	0.021245	0.016907	0	0.011889	0.018736	0.016266	0.024287	0.017825	0.022780
B11	0.020900	0.022387	0.015347	0.018272	0.014701	0.038436	0.012460	0.020888	0.015237	0.011889	0	0.017463	0.018943	0.025079	0.018911	0.024670
B13	0.025483	0.027330	0.025208	0.025518	0.018525	0.035008	0.020904	0.021624	0.020860	0.018736	0.017463	0	0.023731	0.022399	0.023579	0.023526
B14	0.015233	0.014711	0.016634	0.016505	0.020305	0.040144	0.016676	0.026400	0.018492	0.016266	0.018943	0.023731	0	0.031719	0.014214	0.029732
B15	0.035411	0.036383	0.031740	0.035528	0.026333	0.023959	0.022838	0.013641	0.020598	0.024287	0.025079	0.022399	0.031719	0	0.027301	0.023133
B16	0.018527	0.019059	0.017551	0.020541	0.023356	0.033225	0.015215	0.021323	0.014537	0.017825	0.018911	0.023579	0.014214	0.027301	0	0.026707
B17	0.029302	0.031644	0.031136	0.030274	0.019991	0.025380	0.022178	0.018152	0.021481	0.022780	0.024670	0.023526	0.029732	0.023133	0.026707	0

Chapter 4

THE EVOLUTION OF VIVIPARITY INCREASES MULTIPLE PATERNITY IN A REPRODUCTIVE POLYMORPHIC SPECIES WITH INTRAUTERINE CANNIBALISM

ABSTRACT

The acquisition of a viviparous mode of reproduction generally entails a reduction in fecundity. Polygamous mating strategies are among the mechanisms favored by evolution to counteract the potential drawbacks of a lowered fecundity on reproductive success and genetic diversity. Here, we use the reproductive polymorphic species *Salamandra salamandra* to explore the relationship between multiple paternity and reproductive strategies. Throughout its distribution, it displays two reproductive modes that differ in the birth product and brood size: larviparity (big clutches of free aquatic larvae), and pueriparity or viviparity (small clutches of terrestrial juveniles), which have evolved independently in two subspecies. Larviparous populations present multiple paternity, but such pattern has never been tested in viviparous ones. Present study aimed to determine paternity patterns in pueriparous *S. salamandra* and compare them between both independent origins of pueriparity, gestation stages, modes of reproduction and test for the potential benefits of polyandry. We conducted paternity analysis of 18 families from three different pueriparous populations using 11 microsatellite loci. Our results provide the first evidence of multiple paternity in pueriparous salamanders. Although both pueriparous subspecies present multipaternity they differ in the incidence and number of sires, which are higher in the insular population of the subspecies *S. s. gallaica*. We also found that viviparity did not reduce, but maintains, and even increase, the occurrence of multiple paternity and number of sires regarding the larviparous strategy. Thus, although we did not detect any benefit on offspring genetic diversity, multipaternity seems to be a mechanism highly related to the pueriparous mode of reproduction, maybe as a mechanism of assurance of reproduction success avoiding fertilization failures with special relevance in isolated populations.

INTRODUCTION

The evolution of viviparity has repeatedly occurred in the animal kingdom (Blackburn 1999; Avise 2013). The acquisition of viviparous strategy entails several physiological and morphological shifts, as well as important evolutionary and ecological consequences (Blackburn 2015; Blackburn and Starck 2015). Viviparity generally implies an increased parental investment, larger and more developed offspring (which often confers an increased fitness), but also a reduction in broods size (Wourms and Lombardi 1992). This reduction in fecundity may impact genetic diversity within a species, which is largely determined by the demographic history, but also by specific life-history traits and reproductive strategies (Romiguier et al. 2014). In that sense, there is a trade-off between offspring production (fecundity) and offspring condition (in terms of size or parental care). Moreover, species with relative high fecundity (R-strategy) tend to be genetically more diverse than species with a small number of relatively larger offspring (K-strategy) (Ellegren and Galtier 2016). Maintenance of high levels of genetic diversity is considered essential for species viability, as it determines their adaptive potential to respond to changing environmental pressures (Hughes et al. 2008; Jump et al. 2009). Thus, live-bearing is hypothesized to be favored by evolution when the associated benefits, such as increased offspring quality, are higher than costs of a reduced fecundity or female's survival (e.g. reduced locomotor performance) (Shine 2003; Furness et al. 2015). For instance, within amphibians, in which viviparity arose in the three extant orders (Wells 2007), the removal of the free larval aquatic stage confers higher independence from water, and the avoidance of a stage with high levels of mortality and uncertainty, with important ecological and evolutionary implications (Magnusson and Hero 1991; Crump 2015).

Evolution has favored mechanisms that can counteract the potential drawbacks of a lowered fecundity on reproductive success and genetic diversity such as polygamous mating strategies (Tregenza and Wedell 2002). In polyandrous systems (i.e. when females mate with multiple mates) direct benefits are obtained, like the increase of paternal contribution to egg production, parental care, or the assurance of fertilization avoiding genetic incompatibilities or infertile males; but also indirect benefits, by increasing the offspring fitness and/or their genetic diversity (Slatyer et al. 2012; Parker and Birkhead 2013). While polyandrous strategy has inherent costs, such as time allocated in mating, higher predation risk and disease infection, and increased chances of physical and physiological harm (Arnqvist 1989; Ashby and Gupta 2013; Parker and Birkhead 2013), it is considered a successful strategy widespread in nature (Arnqvist and Nilsson 2000; Tennessen and Zamudio 2003; Uller and Olsson 2008; Griffith et al. 2002; Avise et al. 2002; Aloise King et al. 2013).

Polyandry is an obvious prerequisite for multiple paternity, but multiple mating does not always lead to mixed paternity broods. In species with internal fertilization, this bias can result from post-copulatory sexual competitive and selective processes (Parker and Birkhead 2013), that comprise different male's sperm competition for ova fertilization

(Parker 1970; Simmons 2005) or cryptic female choice (Eberhard 1996; Birkhead and Pizzari 2002). In addition, when embryos are retained within the mother's reproductive tract during all or part of development, as occur in viviparous species, other processes acting beyond fertilization can have profound effects on paternity outcomes, such as female reallocation of nutrients towards viable embryos (Zeh and Zeh 1997) or intrauterine cannibalism among siblings or half-siblings. However, the study of the effects of intrauterine cannibalism on the distribution of parentage and post-copulatory sexual selection processes is constrained by the limited occurrence of those events in nature (Gilmore et al. 2005; Exbrayat 2006; Buckley et al. 2007).

The urodele *Salamandra salamandra* (Linnaeus, 1758) is a reproductive polymorphic species representing an unusual system for the study of the implications of the evolution of new reproductive modes at several levels of biological organization (Buckley et al. 2007; Velo-Antón et al. 2015). It is a live-bearing species with internal fertilization that present two discrete modes of reproduction across its range of distribution: an ancestral and more widespread reproductive mode, larviparity, in which females lay free aquatic larvae, and a viviparous mode of reproduction, or pueriparity (sensu Greven 2003), in which females give birth to fully metamorphosed terrestrial juveniles (Dopazo and Korenblum 2000; Buckley et al. 2007; Velo-Antón et al. 2007, 2015). This reproductive strategy independently evolved in two subspecies from the north-west Iberian Peninsula (*S. s. bernardezi* and *S. s. gallaica*) (García-París et al. 2003; Velo-Antón et al. 2007, 2012), and it is characterized by a reduced number of descendants (between 1-35 metamorphosed terrestrial juveniles) regarding the larviparous strategy (between 20-80 larvae) (Dopazo et al. 1998; Dopazo and Korenblum 2000; Buckley et al. 2007; Velo-Antón et al. 2015). The smaller brood sizes arise from several heterochronic processes in the pueriparous strategy: i) incomplete fertilization of the ovulated eggs; ii) an accelerated and asynchronous rates of development of developing embryos; iii) intrauterine active feeding over unfertilized eggs (oophagy), but also, over less developed siblings (adelphophagy or intrauterine cannibalism) (Buckley et al. 2007). Similar heterochronic processes are thought to mediate pueriparity evolution in the two insular populations of *S. s. gallaica* from the north-west Iberian coast (Velo-Antón et al. 2015), which are isolated since the Holocene (ca. 8,000 years ago; see Velo-Antón et al 2007) and show lower genetic diversity values and higher levels of inbreeding compared to their continental counterparts as a result of their demographic history (i.e. founder effect) and genetic drift (Velo-Antón et al. 2007, 2012; Lourenço et al. 2018b).

Previous studies evidenced *S. salamandra* as polygynandrous species, which also shows multiple paternity in larviparous populations from Germany (Steinfartz et al. 2006; Caspers et al. 2014). Sperm of multiple mates are accumulated in the spermatheca in a *topping off* mechanism (Jones et al. 2002), allowing sperm competition processes, but with a 'priority' effect by which first-mates sire the highest proportion of a female's clutch (Caspers et al. 2014). Surprisingly, cryptic female choice processes have been suggested as females choose mates genetically closer to them (Caspers et al. 2014). However, about the mating system of the pueriparous mode of reproduction nothing is known yet.

Here, we explore for the first time the presence of multiple paternity in pueriparous salamanders. First, we study multiple paternity in populations from both pueriparous nuclei of *S. salamandra* and compare patterns between them. We hypothesize that pueriparous insular *S. s. gallaica* nuclei will display higher incidence of multiple paternity and more fathers regarding *S. s. bernardezi* due to its independent evolutionary history, historical demographic processes, larger body sizes, and the more recent evolution of viviparity, which may led to the retention of larviparous traits, such as larger broods. To reinforce our analysis, we analyzed and compared the data obtained from natural births and data obtained from dissections at early developmental stages, expecting that at early developmental stages there would be embryos fathered by more males than in natural births (either because of selective cannibalisms linked to priority effects, or any other source of variation among males). Second, to evaluate direct and indirect benefits of potential multiple paternity, we test whether females fecundity and offspring genetic diversity levels are influenced by the number of fathers. Specifically, we hypotesize that 1) a higher number of effective fathers will result in a higher number of offspring per female, and 2) clutches sired by a greater number of males will be genetically more diverse than clutches sired by one or a lower number of males. Finally, we will compare multipaternity levels between modes of reproduction in accordance to previous results on multiple paternity in larviparous populations of *S. salamandra* (Steinfartz et al. 2006; Caspers et al. 2014). We hypothesize that the number of sires will be lower in pueriparous than in larviparous populations due either to their smaller brood size, the *topping off* mechanisms of fertilization in the species, or the presence of intrauterine cannibalism.

MATERIAL AND METHODS

STUDY SYSTEM AND SAMPLING

Our study focuses on the two *S. salamandra* subspecies that independently evolve to pueriparism: *S. s. bernardezi*, which inhabits in the north-center of the Iberian Peninsula (Cantabrian Range) and in which pueriparism is the only reproductive mode (García-París et al. 2003); and *S. s. gallaica*, which is larviparous, with two insular pueriparous populations in north-west of Iberia (Velo-Antón et al. 2007, 2012). We selected three local populations from both pueriparous nuclei: two *S. s. bernardezi* (Oviedo and Somiedo) populations and one pueriparous *S. s. gallaica* population (Ons Island) (Table 4.1). Initially, we planned to include females from the other pueriparous S. s. gallaica population, the rare and small insular population of San Martiño (Velo-Antón and Cordero-Rivera 2017), but unfortunately it was no possible to obtain gravid females during the study. During the reproductive periods between 2015 and 2017 we collected a total of six gravid females of *S. s. bernardezi* (Oviedo and Somiedo; three from each population) and four gravid females from *S. s gallaica* (Ons) (Table 4.1). We transported them to laboratory facilities at the University of Oviedo (*S. s. bernardezi*) and CIBIO (*S. s. gallaica*) and placed them in individual terraria (60x30x40; LxWxH) provided with coconut fiber as substrate, a container with water, moss, and shelters (bricks or barks).

Table 4.1. Data of each studied female and clutches. Results on multiple paternity: N_{father}: number of fathers in each clutch; %Sired: proportion of the most successful male, in brackets the proportion of the second most successful male when values are similar. Analyses and genetic diversity within each clutch. H_o: observed heterozygosity; N_A: mean number of alleles; AR: unbiased allelic richness; $R_{offspring}$: mean pairwise relatedness within each clutch.

Subspecies	**Population**	**Female**	**Sample**	**$N_{juv.}$**	**N_{larvae}**	**$N_{embryos}$**	**$N_{offspring}$**	**N_{father}**	**%Sired**	**H_o**	**N_A**	**AR**	**$R_{offspring}$**
S. s. bernardezi	Oviedo	L25	Birth	13	0	0	13	2	77	0.72	3.18	1.91	0.64
S. s. bernardezi	Oviedo	L191	Birth	11	1	0	12	3	67	0.50	2.91	1.81	0.64
S. s. bernardezi	Oviedo	L52	Birth	9	0	0	9	1	100	0.77	2.91	2.02	0.55
S. s. bernardezi	Oviedo	L01	Dissection	0	5	5	10	2	90	0.68	2.36	1.81	0.76
S. s. bernardezi	Oviedo	L12	Dissection	0	12	0	12	1	100	0.69	2.64	1.86	0.68
S. s. bernardezi	Somiedo	L64	Birth	5	5	0	10	2	90	0.67	3.00	1.90	0.60
S. s. bernardezi	Somiedo	L75	Birth	6	4	0	10	1	100	0.85	3.18	2.13	0.54
S. s. bernardezi	Somiedo	L87	Birth	6	0	0	6	1	100	0.77	2.91	2.02	0.58
S. s. bernardezi	Somiedo	L192	Disection	3	0	0	3	2	67	0.80	3.27	2.26	0.35
S. s. bernardezi	Somiedo	L209	Disection	3	1	0	4	2	75	0.61	2.82	1.92	0.62
S. s. gallaica	Ons	L97	Birth	9	0	0	9	4	44	0.60	2.73	3.48	0.56
S. s. gallaica	Ons	L107	Birth	18	0	0	18	3	56	0.56	2.46	1.7	0.65
S. s. gallaica	Ons	L137	Birth	19	1	0	20	7	30 (20)	0.67	3.91	1.94	0.43
S. s. gallaica	Ons	L157	Birth	27	0	0	27	6	40 (37)	0.43	2.64	1.62	0.64
S. s. gallaica	Ons	L128	Birth+ disection	26	8	0	34	7	70	0.54	3.27	1.71	0.59
S. s. gallaica	Ons	L131	Birth+ disection	13	1	0	14	3	79	0.49	2.91	1.7	0.62
S. s. gallaica	Ons	L134	Disection	12	1	0	13	2	77	0.62	2.55	1.79	0.61
S. s. gallaica	Ons	L227	Disection	7	6	0	13	1	100	0.52	2.27	1.7	0.64

We fed them twice a week with crickets (*Acheta* sp.) or flour worms (*Tenebrio* sp.). After parturition (1-4 weeks after capture), we collected tissue samples for DNA analysis from a toe clip in the case of females, and a tail-clip from juveniles. Finally, both females and their offspring were returned to the place of capture and released.

As we were interested in exploring whether the number of fathers involved changes across gestation stages because of cannibalistic behaviour, we additionally collected and sacrificed eight females (two from Oviedo, two from Somiedo and four from Ons) to examine paternity patterns in early gestation stages. Determining whether a female is pregnant at early stages or if it just has ovulated is challenging. Therefore, we used a seasonal criteria; we collected females for dissections in late summer and early spring, during mating season, trying to obtain females in the earliest stage of gestation. Once captured, we sacrificed them by an overdose of anaesthesia (benzocaine; Ethyl 4-aminobenzoate; Sigma-Aldrich, Darmstadt, Germany. Product number: E1501. Ref.: 112909). From the offspring obtained after dissections we recorded the uterus (i.e. right or left) and, the stage of development (i.e. embryo, larvae, juvenile), and stored each individual in pure ethanol for DNA analysis. Salamanders were captured and sacrificed under collection and ethical permits provided by regional or national governments (Galicia, Ref. 410/2015 and EB016/2018; Asturias, NºEXPTE: 2016/001092, 2017/001208; PROAE 10/2017).

LABORATORY PROCEDURES

Genomic DNA was extracted from tissue samples using the EasySpin® Genomic DNA Tissue Kit (Citomed, Lisbon, Portugal), following the manufacturer's protocol. The quantity and quality of extracted DNA were assessed by electrophoresis in a 0.8% agarose gel. A total of 11 microsatellites (Sal29, SalE12, SalE7, SalE5, SalE2, SalE06, Sal3, SalE08, Steinfartz et al. 2004; SST-B11, SST-C3, SST-G9, Hendrix et al. 2010), distributed in three optimized multiplexes (panels S2, S3, S4) (Table S4.1 for details), were amplified through polymerase chain reaction (PCR) following the conditions described in Lourenço et al. 2018a. Each multiplex mix contained distilled H_2O, fluorescently labelled forward (6-FAM, VIC, NED or PET; Table S4.1 for sequence details) and reverse primers. Each PCR reaction contained a total volume of 10–11 µl: 5 µl of Multiplex PCR Kit Master Mix (QIAGEN), 3 µl of distilled water, 1 µl of primer multiplex mix and 1–2 µl of DNA extract (~50 ng/µl). To identify possible contaminations, a negative control was employed. PCR touchdown cycling conditions were equal in all multiplexes reactions: the reaction started with an initial step at 95 °C for 15 min, 19 cycles at 95 °C for 30 s, 90 s of annealing at 65 °C (decreasing 0.5 °C each cycle), 72 °C for 40 s, followed by 25 cycles of 95 °C for 30 s, 56 °C for 60 s, 72 °C for 40 s, and ended with a final extension of 30 min at 60 °C. To determine the relative size of fragments the DNA Size Standard LIZ 500 DSMO-100 (MCLAB) was employed. Quality PCR products were verified on a 2% agarose gel run on an ABI3130XL capillary sequencer (Applied Biosystems). Alleles were scored in GENEMAPPER 4.0 (Applied Biosystems). To reduce the potential influence of allele dropout and false alleles, we scored only alleles exhibiting clear fluorescence peaks higher than 100 relative fluorescent units. To increase the likelihood of amplification we amplified in duplexes reactions those females (mothers) samples in which any microsatellite marker failed to amplify or exhibited dubious allelic profiles (e.g. peak artifacts). Cycling conditions are the same as those described for multiplexes. Then, we manually checked for correspondences between females' and their offspring's genotype. If any incongruence was detected (e.g. any descendant did not present any allele form the mother at any loci), we re-amplified females' loci in uniplex. If incongruences persisted, we re-amplified incongruent loci of the offspring in duplexes or uniplexes. Finally, if re-amplified loci (from both the female and offspring) were still incongruent, we recorded those offspring genotypes as missing data.

PARENTAGE ANALYSIS

We first manually recorded the number of non-maternal alleles as a preliminary analysis to estimate multiple paternity, which was assumed only when more than two alleles not inherited from the mother occurred in more than one locus of the offspring. Then, to estimate the most likely number of fathers of each population separately we used the software COLONY 2.0.6.4. (Jones and Wang 2010). We applied the maximum likelihood approach with high likelihood precision and two very long length runs. We were very conservative during allele scoring and we re-amplified a number of samples to check for possible errors; therefore, we assumed a minimum error rate of 0.0001. We assumed polygamy for both sexes with the maternal genotype known and no candidate father genotype included. We neither provided known population allele frequency nor used sib-ship size prior. Although we accounted for a low error rate to decrease the potential of genotyping errors or mutations, fathers siring only a single individual within a clutch were considered only when they differ from other fathers in the same clutch at least in two or more distinct loci.

Finally, we compared mean size and number of sires among populations using R (R Development Core Team 2016), using non-parametric statistics when data did not fit the normality or homoscedasticity assumptions.

MEASUREMENT OF GENETIC DIVERSITY

We calculated several measures of genetic diversity for each brood: mean heterozygosity (H_o), mean number of alleles (N_A), and mean relatedness across offspring per female ($R_{offsspring}$) using GenAlex v 6.503 (Peakall and Smouse 2012). As N_A could be highly affected by differences in brood size we calculated the unbiased allelic richness (AR) using a rarefaction method implemented in HP-RARE (Kalinowski 2005). Finally, for the evaluation of the effect of multiple paternity on patterns of genetic diversity, we analyzed the potential effects of an increased number of fathers on offspring size ($N_{offspring}$) and different measures of genetic diversity (H_o, AR, $R_{offspring}$) in four separate generalized linear models (GLM) in R (R Development Core Team 2016) for each population, including number of fathers (determined by paternity analysis) as explanatory variable.

RESULTS

MULTIPLE PATERNITY

We obtained a total of 237 individuals from 18 females. All offspring were unambiguously assigned to their mothers. We found multiple paternity in all populations, with one male always siring the largest proportion of the clutch (Table 4.1; Figure 4.1). We also found clutches sired by just one male in both subspecies (Table 4.1). Both *S. s. bernardezi* populations present similar clutch sizes (Mann–Whitney U test, P = 0.06) and mean number of sires (Mann–Whitney U test, P = 0.8174). Thus, we grouped them to compare mean number of sires and clutch sizes in *S. s. bernardezi* against Ons population, which present significantly larger broods (Mann–Whitney U test, P = 0.004), higher number of sires (Mann–Whitney U test, P = 0.013), and higher incidence of multiple paternity (7 out of 8 populations; Table 4.1). There were no substantial differences in the number of descendants from natural births and dissections in neither population (Table 4.1; Figure 4.1). Indeed, the highest number of fathers was found in a natural birth from Ons islands and a dissection (females L137 and L128) while some dissections are single-fathered (L12 and L227, Table 4.1).

EFFECTS OF MULTIPLE PATERNITY ON OFFSPRING NUMBER AND GENETIC DIVERSITY

Somiedo population offspring showed the highest levels of heterozygosity (H_o) and allelic richness (AR), in comparison with Oviedo and Ons (Table 4.1). Conversely, levels of relatedness among siblings within a clutch ($R_{offspring}$) are higher in Oviedo population, meanwhile Ons and Somiedo present similar values of relatedness among sibling within each family (Table 4.1).

We founded a significant positive effect of the number of fathers on females fecundity ($N_{offspring}$) in Ons population, but not in any of *S. s. bernardezi* populations (Table 4.2). Nonetheless, none of considered measures of genetic diversity were significantly affected by the number of fathers in neither population (Table 4.2).

Table 4.2. Results of the four performed GLM in each populations evaluating the potential effects of the number of sires in each clutch over number of offspring (direct benefit) or offspring genetic diversity values (indirect benefits).

	$N_{offspring}$			H_o			AR			$R_{offspring}$		
$N_{fathers}$	**Estimate**	**t-value**	***P***	**Estimate**	**t-value**	***P***	**Estimate**	**t-value**	***P***	**Estimate**	**t-value**	***P***
Oviedo	0.786	0.756	0.50	-0.103	-2.693	0.07	-0.067	-1.448	0.24	0.023	0.450	0.68
Somiedo	-2.333	-0.731	0.52	-0.117	-1.490	0.23	-0.048	-0.309	0.78	-0.037	-0.324	0.77
Ons	2.725	2.784	0.03	0.002	0.173	0.87	0.004	0.038	0.97	-0.018	-1.709	0.14

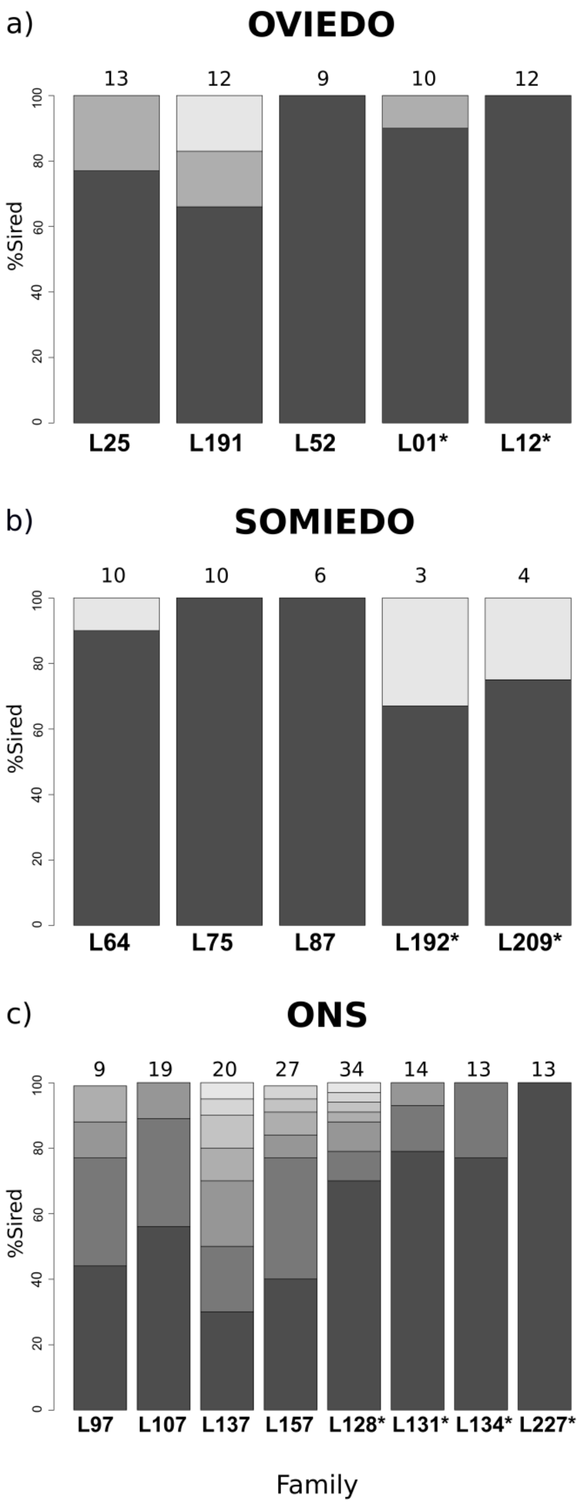


Figure 4.1. Number of fathers per clutch and percentage of the offspring sired by each father in each brood in the three studied populations. Numbers in the top of the barplots refers to the number of offspring in each clutch. Asterisks (*) denote families that came from dissections.

DISCUSSION

This study provides the first evidence of multiple paternity in pueriparous salamanders. We found that both pueriparous nuclei of the species *S. salamandra* present multiple paternity, although the frequency of occurrence as well as the number of fathers involved differ between the two groups, being remarkably high in the insular population of the subspecies *S. s. gallaica*. Although we did not detect any benefit of multiple paternity on offspring genetic diversity, it seems to be a mechanism highly related to the pueriparous mode of reproduction. Indeed, despite the reduced brood size compared to larviparous fire salamanders, we unveil how the shift to viviparity maintains and even increases, both the frequency of multiple paternity and the number of sires.

MULTIPLE PATERNITY IN PUERIPAROUS URODELES

The pueriparous Alpine salamander, *S. atra*, was identified as polygamous (Häfeli 1971; Helfer et al. 2012; Trochet et al. 2014), while mating system and patterns of paternity in pueriparous Lanza's Alpine Salamander, *S. lanzai*, the North African fire salamander *S. algira* (Dinis and Velo-Antón 2017), and *Lyciasalamandra* spp. (Veith et al. 2016) has never been investigated. Alike in larviparous *S. salamandra* (Steinfartz et al 2006; Caspers et al 2014), our study confirms pueriparous *S. salamandra* (*S. s. bernardezi* and insular *S. s. gallaica*) present multiple paternity. However, despite the remarkable differences in the number of descendants per female between larviparous (ca. 20-90 larvae, Velo-Antón et al. 2015, data) and pueriparous populations (1-35 juveniles; Velo-Antón et al. 2015; unpublished data), pueriparous salamanders present an equal, or even higher, (present study, Oviedo: 61%; Somiedo: 60%; Ons: 88%) incidence of multiple paternity than in larviparous populations (Steinfartz et al 2006: 52%; Caspers et al 2014: 37.5%). As in larviparous populations, there is a general reproductive skew towards a dominant male in each brood (see Table 1; Figure 1), probably resulting from the *topping-off* mechanism of sperm storage (Jones et al. 2002; Caspers et al. 2014). Yet, comparisons between modes of reproduction have to be conducted with caution due to differences in the statistical procedures used to infer multiple paternity. In these studies, it was estimated the minimum number of fathers on a clutch (GERUD software, Jones 2005), while our approximation estimates the most-likely number of fathers, and therefore we cannot discard a methodological bias contributing partially to the observed differences, at least in the exact number of sires per female (see for example results in Rovelli et al. 2015; Sandberger-Loua et al. 2016).

While the incidence of multiple paternity is expected to be higher in larger broods, our results were consistent with the pattern reported at the interspecific level (i.e. ‘no pattern’). For instance, in oviparous urodeles (with both aquatic larvae or direct development), multiple paternity is not associated with clutch size, as species with large clutches present similar or even lower incidence of multiple paternity than species with smaller clutches (Jones et al. 2002; Gopurenko et al. 2006; Liebgold et al. 2006; Rovelli et al. 2015).

BENEFITS OF MULTIPLE PATERNITY IN PUERIPAROUS FIRE SALAMANDERS

Salamander males do not provide parental care or extra resources to females or their offspring. Thus, it is expected that females mainly obtain genetic benefits from polyandry, through the reduction of genetic incompatibilities or infertile males and thus, the increase of female reproductive success (Thornhill and Alcock 1983; Jennions and Petrie 2000; Wolff and Macdonald 2004).

It is important to note that reproductive modes can be crucial in favoring the evolution of polyandry (Zeh and Zeh 2001). In the viviparous strategy, female investment per individual offspring is higher than in egg-lying modes, and reproductive failures due to genetic incompatibilities are by far much more costly (Zeh and Zeh 2001; Liu and Avise 2011). Our study shows that higher rates of multiple paternity and the number of fathers involved in fertilization seem to be related to the pueriparous mode of reproduction. Whereas *S. salamandra* larviparous progeny relies solely on their yolk provisions, in the pueriparous populations nutrient provisioning to offspring occurs through the availability of arrested eggs or less developed siblings. Thus, females “sacrifice” their fecundity in favor of a lower number of fitter and more ‘valuable’ descendants (Dopazo and Korenblum 2000; Buckley et al. 2007). In addition, offspring genetic diversity (indirect genetic benefits) is non-affected by number of males (Table 4.2). Thus, our results favour the hypothesis that multiple mating, which can prevent or reduce the costs of mating with sterile males and genetic incompatibilities (genetic direct benefits), would be selected in pueriparous fire salamanders.

Another possible, direct benefit of polyandry for females is an increased of clutch size (Fitze et al. 2005). Though a benefit regarding the number of descendants was found in larviparous populations (Caspers et al 2014), we only detected a relationship between number of sires and number of newborns in Ons population, but not in *S. s. bernardezi* poulations (Table 4.2). However, we should consider two singularities of the pueriparous mode of reproduction that may affect both clutch size and final number of fathers. First, as set before, although the high production of aborted or unfertilized eggs are generally considered as fertilization failures, in pueriparous individuals it is considered as a matrotrophic strategy, with which provide an extra source of nourishment that allows a faster growth and development, favoring the success of the offspring and resulting in heavier newborns (Dopazo and Korenblum 2000; Velo-Antón et al. 2015). Second, the total number of delivered juveniles may not represent the actual number of successful fertilizations nor the actual number of males involved, as they could be modified by means of intrauterine cannibalism (Buckley et al. 2007), as occur in the sand tiger shark (Chapman et al. 2013), and maybe, in the Alpine salamander (Guex and Greven 1994). However, we did not detect substantial differences between both developmental stages, although we cannot discard that the lack of differences resulted from the advanced stage of development of some dissections.

DIFFERENCES IN MULTIPLE PATERNITY BETWEEN PUERIPAROUS *S. S. BERNARDEZI* AND *S. S. GALLAICA*

The frequency of polyandry and number of sires in the study *S. s. bernardezi* populations were within the range observed in larviparous populations. They were, however, noticeably high within the *S. s. gallaica* insular population, in which we found the highest number of males fathering a single litter throughout all paternity studies performed in *S. salamandra*, even when compared across internally fertilizing vertebrates (Uller and Olsson 2008; Avise and Liu 2011; Liu and Avise 2011).

The highest level of multiple paternity observed in this insular population might be explained by a mechanism of genetic compensation to maintain, or increase, the levels of genetic diversity and N_e, which would also reduce deleterious effects of inbreeding depression such as genetic incompatibilities or reduced embryos viability (Tregenza and Wedell 2002; Pearse and Anderson 2009; Michalczyk et al. 2011). This mechanism has been suggested to explain the high N_e/N ratio in a small and isolated pueriparous population of *S. s. bernardezi* (Álvarez et al. 2015), and as the mechanism maintaining levels of genetic diversity in an overexploited population of rockfish (Gao et al. 2018). However, although we did not find an association between the number of males and offspring genetic diversity in any pueriparous origin (Table 4.2), lower levels of genetic diversity and higher levels of inbreeding observed in Ons populations (Velo-Antón et al. 2012b; Lourenço et al. 2018b) may increase the risk of reproductive failures. Altogether, our results suggest that multiple mating in viviparous systems might be a mechanism to enhance female's reproductive success (Zeh and Zeh 2001; Liu and Avise 2011), rather than one increasing offspring genetic diversity.

Despite the potential benefits that multiple mating provide to the mother, the offspring and the population, the high values of multiple paternity observed in Ons can also be explained by a relaxation of the potential constraints determining mates acquisition (e.g. population density, territoriality) (Avise and Liu 2011). In Ons population, *S. salamandra* is remarkable abundant along the eastern side of the island, protected from seawinds (Velo-Antón and Cordero-Rivera 2017), which can increase encounter rates and enhance multiple mating (Soucy and Travis 2003; Avise and Liu 2011; Avise et al. 2011). Moreover, multiple mating can also be a by-product of mating behavior (e.g. female harassment by the male), without any benefits, in which costs of avoiding mating are higher than costs of accepting it (Thornhill and Alcock 1983). The courtship behavior of the *S. salamandra* involves active search of females by males, which push their snout and rub their nares against the females´ bodies (Arnold 1987). Thus, while the high incidence of multiple paternity in Ons island could result from mating forced by males, without any benefit nor for females neither their progeny (Fitze et al. 2005; Trontti et al. 2006), it should be investigated through behavioral studies.

Summarizing, exploring patterns of paternity as well as investigating their potential implications in a reproductively polymorphic species help to understand how reproductive strategies evolve to account for the trade-off of increasing offspring quality (i.e. fitness) and associated costs on fecundity. Our study shows that the shift to viviparity maintains or even increases multiple paternity levels in *S. salamandra*, despite the marked reduction in clutch size. While we did not find a strong association between genetic diversity and multiple paternity, the maintenance of multiple paternity strategy in pueriparous S. salamandra can be a way of ensuring reproduction success avoiding fertilization failures resulting from high levels of isolation and inbreeding.

REFERENCES

Aloise King, E. D., P. B. Banks, and R. C. Brooks. 2013. Sexual conflict in mammals: consequences for mating systems and life history. Mammal Review 43:47–58.

Álvarez, D., A. Lourenço, D. Oro, and G. Velo-Antón. 2015. Assessment of census (N) and effective population size (N_e) reveals consistency of N_e single-sample estimators and a high N_e/N ratio in an urban and isolated population of fire salamanders. Conservation Genetics Resources 7:705–712.

Arnold, S. J. 1987. The comparative ethology of courtship in salamandrid salamanders. *Salamandra* and *Chioglossa*. Ethology 74:133–145.

Arnqvist, G. 1989. Multiple mating in a water strider: mutual benefits or intersexual conflict? Animal Behaviour 38:749–756.

Arnqvist, G., and T. Nilsson. 2000. The evolution of polyandry: multiple mating and female fitness in insects. Animal behaviour 60:145–164.

Ashby, B., and S. Gupta. 2013. Sexually transmitted infections in polygamous mating systems. Philosophical Transactions of the Royal Society B: Biological Sciences 368:20120048.

Avise, J. C. 2013. Evolutionary perspectives on pregnancy. Columbia University Press.

Avise, J. C., A. G. Jones, D. Walker, J. A. DeWoody. 2002. Genetic mating systems and reproductive natural histories of fishes: lessons for ecology and evolution. Annual review of genetics 36:19–45.

Avise, J. C., and J. X. Liu. 2011. Multiple mating and its relationship to brood size in pregnant fishes versus pregnant mammals and other viviparous vertebrates. Proceedings of the National Academy of Sciences 108:7091–7095.

Avise, J. C., A. Tatarenkov, and J.-X. Liu. 2011. Multiple mating and clutch size in invertebrate brooders versus pregnant vertebrates. Proceedings of the National Academy of Sciences 108:11512–11517.

Birkhead, T. R., and T. Pizzari. 2002. Evolution of sex: postcopulatory sexual selection. Nature reviews genetics 3:262.

Blackburn, D. G. 1999. Viviparity and oviparity - Evolution and strategies. Encyclopedia of Reproduction Volume 4 4:994–1003.

Blackburn, D. G. 2015. Evolution of vertebrate viviparity and specializations for fetal nutrition: a quantitative and qualitative analysis. Journal of Morphology 276:961–990.

Blackburn, D. G., and J. M. Starck. 2015. Morphological specializations for fetal maintenance in viviparous vertebrates: An introduction and historical retrospective. Journal of Morphology 276:E1–E16.

Buckley, D., M. Alcobendas, M. García-París, and M. H. Wake. 2007. Heterochrony, cannibalism, and the evolution of viviparity in *Salamandra salamandra*. Evolution & development 9:105–15.

Caspers, B. A., E. T. Krause, R. Hendrix, M. Kopp, O. Rupp, K. Rosentreter, and S. Steinfartz. 2014. The more the better - Polyandry and genetic similarity are positively linked to reproductive success in a natural population of terrestrial salamanders (*Salamandra salamandra*). Molecular Ecology 23:239–250.

Chapman, D. D., S. P. Wintner, D. L. Abercrombie, J. Ashe, A. M. Bernard, M. S. Shivji, and K. A. Feldheim. 2013. The behavioural and genetic mating system of the sand tiger shark, *Carcharias taurus*, an intrauterine cannibal. Biology letters 9:20130003.

Crump, M. L. 2015. Anuran reproductive modes: evolving perspectives. Journal of Herpetology 49:1–16.

Dinis, M., and G. Velo-Antón. 2017. How little do we know about the reproductive mode in the north African salamander, *Salamandra algira*? Pueriparity in divergent mitochondrial lineages of *S. a. tingitana*. Amphibia-Reptilia 38:540–546.

Dopazo, H., L. Boto, and P. Alberch. 1998. Mitochondrial DNA variability in viviparous and ovoviviparous populations of the urodele *Salamandra salamandra*. Journal of Evolutionary Biology 2:365–378.

Dopazo, H., and M. Korenblum. 2000. Viviparity in *Salamandra salamandra* (Amphibia: Salamandridae): adaptation or exaptation? Herpetologica 144–152.

Eberhard, W. 1996. Female control: sexual selection by cryptic female choice. Princeton University Press.

Ellegren, H., and N. Galtier. 2016. Determinants of genetic diversity. Nature Reviews Genetics 17:422.

Exbrayat, J.-M. 2006. Reproductive Biology and Phylogeny of Gymnophiona (Caecilians). Science Publishers Incorporated.

Fitze, P. S., J. F. Le Galliard, P. Federici, M. Richard, and J. Clobert. 2005. Conflict over multiple-partner mating between males and females of the polygynandrous common lizards. Evolution 59:2451–2459.

Furness, A. I., K. R. Morrison, T. J. Orr, J. D. Arendt, and D. N. Reznick. 2015. Reproductive mode and the shifting arenas of evolutionary conflict. Annals of the New York Academy of Sciences 1360:75–100.

Gao, T., K. Ding, N. Song, X. Zhang, and Z. Han. 2018. Comparative analysis of multiple paternity in different populations of viviparous black rockfish, *Sebastes schlegelii*, a fish with long-term female sperm storage. Marine Biodiversity 48:2017–2024.

García-París, M., M. Alcobendas, D. Buckley, and D. B. Wake. 2003. Dispersal of viviparity across contact zoes in iberian populatios of fire salamanders (*Salamandra*) inferred from discordance of genetic and morphological traits. Evolution 57:129–143.

Gilmore, R. G., O. Putz, and J. W. Dodrill. 2005. Oophagy, intrauterine cannibalism and reproductive strategy in lamnoid sharks. Pages 435-462 *in* W. C. Hamlett, ed. Reproductive biology and phylogeny of Chondrichthyes: sharks, batoids and chimaeras. Science Publishers, Enfield, New Hampshire, USA.

Gopurenko, D., R. N. Williams, C. R. McCormick, and J. A. DeWoody. 2006. Insights into the mating habits of the tiger salamander (*Ambystoma tigrinum tigrinum*) as revealed by genetic parentage analyses. Molecular Ecology 15:1917–1928.

Greven, H. 2003. Larviparity and Pueriparity. Pages 447–475 *in* D. M. Sever, ed. Reproductive Biology and Phylogeny (Vol. 1). Enfield, New Hampshire.

Griffith, S. C., I. P. F. Owens, and K. A. Thuman. 2002. Extra pair paternity in birds: a review of interspecific variation and adaptive function. Molecular ecology 11:2195–2212.

Guex, G.-D., and H. Greven. 1994. Structural and physiological aspects of viviparity in *Salamandra atra*. Mertensiella 4:161–208.

Häfeli, H. P. 1971. Reproductive biology of the alpine salamander (*Salamandra atra* Laur). Revue suisse de zoologie; annales de la Societe zoologique suisse et du Museum d'histoire naturelle de Geneve 78:235–293.

Helfer, V., T. Broquet, and L. Fumagalli. 2012. Sex-specific estimates of dispersal show female philopatry and male dispersal in a promiscuous amphibian, the alpine salamander (*Salamandra atra*). Molecular Ecology 21:4706–4720.

Hendrix, R., J. Susanne Hauswaldt, M. Veith, and S. Steinfartz. 2010. Strong correlation between cross-amplification success and genetic distance across all members of "True Salamanders" (Amphibia: Salamandridae) revealed by *Salamandra salamandra*-specific microsatellite loci. Molecular Ecology Resources 10:1038–1047.

Hughes, A. R., B. D. Inouye, M. T. J. Johnson, N. Underwood, and M. Vellend. 2008. Ecological consequences of genetic diversity. Ecology letters 11:609–623.

Jennions, M. D., and M. Petrie. 2000. Why do females mate multiply? A review of the genetic benefits. Biological Reviews 75:21–64.

Jones, A. G. 2005. GERUD 2.0: a computer program for the reconstruction of parental genotypes from half-sib progeny arrays with known or unknown parents. Molecular Ecology Notes 5:708–711.

Jones, A. G., E. M. Adams, and S. J. Arnold. 2002. Topping off: A mechanism of first-male sperm precedence in a vertebrate. Proceedings of the National Academy of Sciences 99:2078–2081.

Jones, O. R., and J. Wang. 2010. COLONY: a program for parentage and sibship inference from multilocus genotype data. Molecular ecology resources 10:551–555.

Jump, A. S., R. Marchant, and J. Peñuelas. 2009. Environmental change and the option value of genetic diversity. Trends in plant science 14:51–58.

Kalinowski, S. T. 2005. hp-rare 1.0: a computer program for performing rarefaction on measures of allelic richness. Molecular Ecology Notes 5:187–189.

Liebgold, E. B., P. R. Cabe, R. G. Jaeger, and P. L. Leberg. 2006. Multiple paternity in a salamander with socially monogamous behaviour. Molecular Ecology 15:4153–4160.

Liu, J. X., and J. C. Avise. 2011. High degree of multiple paternity in the viviparous Shiner Perch, *Cymatogaster aggregata*, a fish with long-term female sperm storage. Marine Biology 158:893–901.

Lourenço, A., B. Antunes, I. J. Wang, and G. Velo-Antón. 2018a. Fine-scale genetic structure in a salamander with two reproductive modes: Does reproductive mode affect dispersal? Evolutionary ecology 32:699–732.

Lourenço, A., F. Sequeira, D. Buckley, and G. Velo-Antón. 2018b. Role of colonization history and species-specific traits on contemporary genetic variation of two salamander species in a Holocene island-mainland system. Journal of Biogeography 45:1054–1066.

Magnusson, W. E., and J.-M. Hero. 1991. Predation and the evolution of complex oviposition behaviour in Amazon rainforest frogs. Oecologia 86:310–318.

Michalczyk, Ł., A. L. Millard, O. Y. Martin, A. J. Lumley, B. C. Emerson, T. Chapman, and M. J. G. Gage. 2011. Inbreeding promotes female promiscuity. Science 333:1739–1742.

Parker, G. A. 1970. Sperm competition and its evolutionary consequences in the insects. Biological Reviews 45:525–567.

Parker, G. A., and T. R. Birkhead. 2013. Polyandry: The history of a revolution. Philosophical Transactions of the Royal Society B: Biological Sciences 368.

Peakall, R., and P. E. Smouse. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. Bioinformatics 28, 2537e2539.

Pearse, D. E., and E. C. Anderson. 2009. Multiple paternity increases effective population size. Molecular Ecology 18:3124–3127.

R Development Core Team. 2016. R: A language and environ- ment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Romiguier, J., P. Gayral, M. Ballenghien, A. Bernard, V. Cahais, A. Chenuil, Y. Chiari, et al. 2014. Comparative population genomics in animals uncovers the determinants of genetic diversity. Nature 515:261.

Rovelli, V., E. Randi, F. Davoli, D. Macale, M. A. Bologna, and L. Vignoli. 2015. She gets many and she chooses the best: Polygynandry in *Salamandrina perspicillata* (Amphibia: Salamandridae). Biological Journal of the Linnean Society 116:671–683.

Sandberger-Loua, L., H. Feldhaar, R. Jehle, and M. O. Rödel. 2016. Multiple paternity in a viviparous toad with internal fertilisation. The Science of Nature 103: 51.

Shine, R. 2003. Effects of pregnancy on locomotor performance: an experimental study on lizards. Oecologia 136:450–456.

Simmons, L. W. 2005. The evolution of polyandry: sperm competition, sperm selection, and offspring viability. Annual Review of Ecology, Evolution, and Systematics 36:125–146.

Slatyer, R. A., B. S. Mautz, P. R. Y. Backwell, and M. D. Jennions. 2012. Estimating genetic benefits of polyandry from experimental studies: a meta-analysis. Biological Reviews 87:1–33.

Soucy, S., and J. Travis. 2003. Multiple paternity and population genetic structure in natural populations of the poeciliid fish, *Heterandria formosa*. Journal of Evolutionary Biology 16:1328–1336.

Steinfartz, S., D. Kusters, and D. Tautz. 2004. Isolation and characterization of polymorphic tetranucleotide microsatellite loci in the Fire salamander *Salamandra salamandra* (Amphibia: Caudata). Molecular Ecology Notes 4:626–628.

Steinfartz, S., K. Stemshorn, D. Kuesters, and D. Tautz. 2006. Patterns of multiple paternity within and between annual reproduction cycles of the fire salamander (*Salamandra salamandra*) under natural conditions. Journal of Zoology 268:1–8.

Tennessen, J. A., and K. R. Zamudio. 2003. Early male reproductive advantage, multiple paternity and sperm storage in an amphibian aggregate breeder. Molecular Ecology 12:1567–1576.

Thornhill, R., and J. Alcock. 1983. The evolution of insect mating systems. Harvard University Press.

Tregenza, T., and N. Wedell. 2002. Polyandrous females avoid costs of inbreeding. Nature 415:71.

Trochet, A., S. Moulherat, O. Calvez, V. M. Stevens, J. Clobert, and D. S. Schmeller. 2014. A database of life-history traits of European amphibians. Biodiversity Data Journal.

Trontti, K., N. Thurin, L. Sundström, and S. Aron. 2006. Mating for convenience or genetic diversity? Mating patterns in the polygynous ant *Plagiolepis pygmaea*. Behavioral Ecology 18:298–303.

Uller, T., and M. Olsson. 2008. Multiple paternity in reptiles: patterns and processes. Molecular ecology 17:2566–2580.

Veith, M., B. Göçmen, K. Sotiropoulos, S. Kieren, O. Godmann, and S. Steinfartz. 2016. Seven at one blow: the origin of major lineages of the viviparous Lycian salamanders (*Lyciasalamandra* Veith and Steinfartz, 2004) was triggered by a single paleo-historic event. Amphibia-Reptilia 37:373–387.

Velo-Antón, G., and A. Cordero-Rivera. 2017. Ethological and phenotypic divergence in insular fire salamanders: diurnal activity mediated by predation? Acta Ethologica 20:243–253.

Velo-Antón, G., M. García-París, P. Galán, and A. Cordero-Rivera. 2007. The evolution of viviparity in holocene islands: ecological adaptation versus phylogenetic descent along the transition from aquatic to terrestrial environments. Journal of Zoological Systematics and Evolutionary Research 45:345–352.

Velo-Antón, G., X. Santos, I. Sanmartín-Villar, A. Cordero-Rivera, and D. Buckley. 2015. Intraspecific variation in clutch size and maternal investment in pueriparous and larviparous *Salamandra salamandra* females. Evolutionary Ecology 29:185–204.

Velo-Antón, G., K. R. Zamudio, and A. Cordero-Rivera. 2012a. Genetic drift and rapid evolution of viviparity in insular fire salamanders (*Salamandra salamandra*). Heredity 108:410–8.

Velo-Antón, G., K. R. Zamudio, and A. Cordero-Rivera. 2012b. Genetic drift and rapid evolution of viviparity in insular fire salamanders (*Salamandra salamandra*). Heredity 108:410.

Wells, K. D. 2007. The ecology and behavior of amphibians. University of Chicago Press.

Wolff, J. O., and D. W. Macdonald. 2004. Promiscuous females protect their offspring. Trends in Ecology & Evolution 19:127–134.

Wourms, J. P., and J. Lombardi. 1992. Reflections on the evolution of piscine viviparity. American Zoologist 32:276–293.

Zeh, J. A., and D. W. Zeh. 1997. The evolution of polyandry II: Post-copulatory defences against genetic incompatibility. Proceedings of the Royal Society B: Biological Sciences 264:69–75.

Zeh, J. A., and D. W. Zeh. 2001. Review: Reproductive mode and the genetic benefits of polyandry. Animal Behaviour 61: 1051–1063.

Supplementary Material Chapter 4

Table S4.1. Details on 11 microsatellites used in the present study, multiplex arrangement, original published primers and fluorescently labelled oligonucleotides used as template for modified forward primers is displayed. Table adapted from Appendix 2 from Lourenço et al 2018*a*. The forward and reverse primers were concentrated at 10 µM and 100 µM respectively. [1] Steinfartz et al 2004; [2] Hendrix et al 2010.

Locus	**Multiplex**	**Label***	**Primer forward (5' – 3')**	**Primer reverse (5' – 3')**	**PF concentration multiplex/PCR (µM)**	**PR concentration multiplex/PCR (µM)**
Sal29 [1]	Panel S2	6-FAM	CTCTTTGACTGAACCAGAACCCC	GCCTGTCGGCTCTGTGTAACC	0.8 / 0.08	8.0 / 0.8
SST-B11 [2]	Panel S2	PET	TCAAACGGTGCCAAAGTTATTAG	TTAATTGGCAGTTTTCTTTCCAG	0.2 / 0.02	2.0 / 0.2
SalE12 [1]	Panel S2	VIC	CTCAGGAACAGTGTGCCCCAAATAC	CTCATAATTTAGTCTACCCTCCCAC	0.08 / 0.008	0.8 / 0.08
SST-C3 [2]	Panel S3	PET	CCGTTTGAGTCACTTCTTTCTTG	TTGCTTTACCAACCAGTTATTGTC	0.14 / 0.014	1.4 / 0.14
SalE7 [1]	Panel S3	NED	TTTCAGCACCAAGATACCTCTTTTG	CTCCCTCCATATCAAGGTCACAGAC	0.08 / 0.008	0.8 / 0.08
SalE5 [1]	Panel S3	6-FAM	CCACATGATGCCTACGTATGTTGTG	CTCCTGTTTACGCTTCACCTGCTCC	0.06 / 0.006	0.6 / 0.06
SalE2 [1]	Panel S3	VIC	CACGACAAAATACAGAGAGTGGATA	ATATTTGAAATTGCCCATTTGGTA	0.3 / 0.03	3.0 / 0.3
SalE06 [1]	Panel S4	VIC	GGACTCATGGTCACCCAGAGGTTCT	ATGGATTGTGTCGAAATAAGGTATC	0.12 / 0.012	1.2 / 0.12
Sal3 [1]	Panel S4	6-FAM	CTCAGACAAGAAATCCTGCTTCTTC	ATAAATCTGTCCTGTTCCTAATCAG	0.12 / 0.012	1.2 / 0.12
SalE8 [1]	Panel S4	NED	GCAAAGTCCATGCTTTCCCTTTCTC	GACATACCAAAGACTCCAGAATGGG	0.08 / 0.008	0.8 / 0.08
SST-G9 [2]	Panel S4	NED	CCTCGTCAGGGGTTGTAGG	CTTTCCAGGAAGAAACTGAGATG	0.08 / 0.008	0.8 / 0.08

*An extra number of base pairs were added at the 5' end of the original sequence of forward primers in order to allow binding of four different fluorescent labelled oligonucleotides (6-FAM - TGT AAA ACG ACG GCC AGT; VIC - TAA TAC GAC TCA CTA TAG GG; NED - TTT CCC AGT CAC GAC GTT G; PET - GAT AAC AAT TTC ACA CAG G)

General Discussion

General Discussion

Understanding the potential consequences of the evolution of new reproductive modes require integrative approaches combining information from different sources. We are still far from a comprehensive knowledge of the implications of reproductive transitions. However, the available natural diversity can provide invaluable advances in the understanding of the processes that lead to the observed patterns in nature. In that sense, the urodele *Salamandra salamandra* has been proven to be an exceptional and almost unique study system to address a number of unsolved questions about the implications of the evolution of a new reproductive mode at the intraspecific level.

Summarizing, the results of this thesis show that the acquisition of viviparity in *S. salamandra* and the associated developmental modifications do not have an impact on adults head morphological diversity, which constitutes a differential and highly variable trait subjected to a number of evolutionary forces among and within subspecies. On the other hand, evolution has favored mechanisms to ensure reproductive success of viviparous females, balancing the reduction in brood size though increasing multiple mating behavior. Finally, results herein add to the bunch of evidences consolidating the fire salamander system as an exceptional model to address different questions about the origin, evolution and consequences of the acquisition of viviparity.

DEVELOPMENT, MODES OF REPRODUCTION AND MORPHOLOGY

The evolution of the wide diversity of life-histories and modes of reproduction across taxa are often tightly linked to modifications in developmental sequences and ontogenetic processes (Hanken 1989; Smith 2002; Bruce 2003; Buckley et al. 2007). Such internal mechanisms are keystone in the generation, constrain or channeling of phenotypic variation both, at the inter- and intra-specific level (Alberch 1980, 1982; Hanken and Hall 1993; Jernvall 2000; Fusco 2001; Beldade et al. 2002; Wake 2003; Richardson and Chipman 2003; Jaekel and Wake 2007; Smith et al. 2015; Bonett and Blair 2017). For instance, at the macroevolutionary scale, in marsupial mammals, highly precocious newborns survival depends upon their ability to crawl to the teat, where they complete their development (see Sears 2014). The strong selective pressure over effective crawling has resulted in developmental modifications leading to early ontogenetic formation of forelimbs and shoulder girdles which in turn constraints the degree of diversification of forelimbs morphology in this group regarding the placental mammals (e.g. bats wings, felines paws or whales flippers) (Cooper and Steppan 2010; Kelly and Sears 2011). On the other hand, within amphibians, family Plethodontidae constitutes the most ecologically and phenotypically diverse family across salamanders. Some species display a biphasic life-cycle, and others direct development (Wells 2007) and both modes of reproduction differ in the ontogenetic origin of some structures. As such, in species with larval stage, adult feeding system (tongue) originates from the hyobranchial larval apparatus during metamorphosis remodeling, meanwhile in direct development species it directly develops from embryo structures (Wake 1982) and display higher diversity in function and structure than in those that experienced metamorphosis. Therefore, the depletion of the larval stage and larval structures change the embryonic patterning and relaxes the developmental constraints imposed on the adult morphology, allowing for the appearance of novel adult structures and diversification (Roth and Wake 1985; Hanken 1989; Deban and Marks 2002; Wake and Hanken 2004; Wake 2009; Bonett and Blair 2017).

At the intraspecific level, phenotypic consequences of developmental modifications related to changes in life-histories seem to be system-specific, making the use of non-model groups increasingly valuable in the study of patterns and processes underlying changes (Bolker 1995; Wake 2003; Jenner and Wills 2007; Sears 2014). The singular design displayed by viviparous populations within *S. salamandra* allowed us to carry out comparative studies to explore the potential implications of diverging ontogenies on adult morphology and life-history traits. As explained in previous section of this thesis, developmental sequence of each mode of reproduction in *S. salamandra* differ from one another by heterochronic changes in development and ontogenetic processes (Buckley et al. 2007). Heterochronies in early ontogeny can have important morphological implications later in life (Holtmeier 2001; Parichy 2006) and even subtle changes in development of specific structures may affect the whole trait shape (Hanken and Hall 1993; Hallgrímsson et al. 2014). Furthermore, ontogenetic modifications associated to the acquisition of viviparity in *S. salamandra* mainly focus in facilitating early intrauterine feeding, which could be considered a strong selective pressure that might have an impact on cephalic structures (Mabee et al. 2000) affecting head morphological diversity in subsequent developmental stages. However, contrary to our expectations, we did not find a convergent shape between both pueriparous nuclei, at least, in the adult stage (Chapter 2). Conversely, head shape arises as a lineage-specific feature that has been shaped by different evolutionary histories. Indeed, head morphology seem to be highly robust to developmental modifications in each evolutionary lineage, specially within *S. s. gallaica*, as differences in developmental sequences between reproductive modes do not result in morphological differentiation.

Nonetheless, at this point, it is important to assume some shortcomings of the original design. Whereas adult specimens from each subspecies examined herein clearly differ in head morphology with no apparent effect of developmental differences between reproductive modes, we cannot ignore ontogenetic trajectories from metamorphosis to the adult stage, along which, both selection and development, may interact to shape adult morphology (Adams and Nistri 2010; Ivanović et al. 2011; Cvijanović et al. 2014). Thus, any potential effect on morphology of pueriparous development may be blurred in adults by the action of other forces on head morphology throughout growth, which can either enhance or reduce morphological differences. In addition, subspecies differ in rates and offset of growth (Alcobendas and Castanet 2000) which have been seen to generate morphological differences in fish (Holtmeier 2001). Finally, it is important to mention the potential role of metamorphosis on the correlations between larval and adults phenotypes (Raff 1987; Hanken 1992; Moran 1994; Deban and Marks 2002; Rose 2003; Crean et al. 2011). Despite delivering fully terrestrial newborns, juveniles of pueriparous salamanders still present complex life-cycle, undergoing a (intrauterine) larval stage and metamorphosis (Buckley et al. 2007). In pueriparous salamanders hatchling occurs in a precocious embryonic stage as well as the onset of active feeding, meanwhile in larviparous larvae hatching and exogenous feeding starts once they have been delivered in the aquatic environment. Due to the profound transformations and remodeling that occur during metamorphosis (Duellman and Trueb 1986; Alberch 1987; Rose 2003) it may be possible that any modification in head structures introduced by early developmental processes in pueriparous developmental sequence is, somehow, 'reset' during metamorphosis, and thus, absent in the adult stage, with both stages morphologically decoupled (Ivanović et al. 2011; Sherratt et al. 2017). However, the effect of metamorphosis on the decoupling of the stages seem to be trait specific and depend on the functional demands in each phase as well as in the amount of transformation experienced across metamorphosis (Ivanović et al. 2011; Cvijanović et al. 2014; Vučić et al. 2019). For instance, morphological divergence in head shape among close related species of the urodele *Triturus* seem to appear during embryonic development and maintains across metamorphosis (Vučić et al. 2019). If salamanders followed the same pattern, any difference derived from early embryonic modifications would be detected between delivered juveniles and metamorphic stages.

VIVIPARITY AND PHENOTYPIC AND GENETIC DIVERSITY

Intraspecific differentiation is determined by extrinsic (e.g. environment, biogeograpahic history) and intrinsic factors (e.g. specific life-history traits) (Wagner et al. 2012; April et al. 2013; Fluker et al. 2014; Wollenberg Valero 2015). Different reproductive strategies (e.g. Vences et al. 2002; Zimkus et al. 2012) and different developmental pathways (Porter and Johnson 2002) have been seen to influence rates of species diversification. In that sense, the acquisition of viviparous reproduction in *S. salamandra* possibly plays a relevant role in the degree of inter-population differentiation by different ways.

Adult head morphology in *S. salamandra* consolidated as a reliable trait for the differentiation of subspecies (Chapter 2). Although we cannot completely discard morphological consequences derived from the acquisition of viviparity in earlier stages (see previous section), adults head morphology seems to result from differences in the evolutionary histories among subspecies. Different subspecies or evolutionary lineages have undergone, and probably currently do, different evolutionary forces and processes, leading to differentiated morphologies. However, head shape is also a highly variable trait within the pueriparous subspecies *S. s. bernardezi*, and differ among coloration morphs (Chapter 3). Phenotypic diversity within this subspecies is spatially concordant with genetic structure, and variation in head morphology among morphs maybe results from evolutionary processes acting differentially on each of them. The structuration of phenotypic diversity is not surprising, as *S. s. bernardezi* demonstrated to be the group with highest levels of intra-lineage diversity (Dopazo et al. 1998; García-París et al. 2003; Velo-Antón et al. 2007; Beukema et al. 2016; unpublished data). Together with the complex topography across its range of distribution, biogeographic history, morphologically differentiated morphotypes, and the variety of environments in which it appears (Velo-Antón and Buckley 2015), the presence of a pueriparous (viviparous) mode of reproduction may also have an effect on inter-population divergence. Among amphibians, one important feature derived from the acquisition of alternative modes of reproduction like viviparity or direct development is to gain independence from aquatic environments. This independence in all the stages of the life-cycle, even to the accomplishment of successful reproduction, presumably reduces the habitat occupancy constraints (Van Bocxlaer et al. 2010; Gómez-Mestre et al. 2012; Jiménez-Robles et al. 2017; Lion et al. 2019). This constraints relaxation could allow the colonization of new niches and environments (e.g. Lourenço et al. 2017) where they will face differential selective and ecological pressures, favoring diverse adaptive responses (Gómez-Mestre et al. 2012; Zimkus et al. 2012; Müller et al. 2013; Liedtke et al. 2017). Therefore, if we consider viviparity as an innovation that allow the colonization of a wide diversity of new environments, this situation may lead to an array of adaptive processes that potentially explains observed diversity in head shape across pueriparous subspecies (Wainwright and Reilly 1994). Nonetheless, it is advisable to take into account that ecological (microhabitat) diversification not necessarily triggers morphological evolution, as occur in plethodontids (Blankers et al. 2012), although this study did not consider head morphology, which is a trait highly related to ecological processes in urodeles (Adams 2004, 2010). On the other hand, this broadness of the potential niches may also affect the genetic diversity structuration (Burney and Brumfield 2009; Wang 2013) as reproductive strategies that favor a higher degree of terrestriality may also affect dispersal abilities, and consequently levels of connectivity (i.e. gene flow) and structuration among populations (Duminil et al. 2007; Measey et al. 2007; Paz et al. 2015; Tilley 2016) even adding to explaining species phylogeographic patterns (Paz et al. 2015). However, in the particular case of *S. salamandra* no differences were observed between dispersal abilities of different reproductive modes (Lourenço et al. 2018) and our results support a structuration of neutral genetic diversity following an isolation-by-distance pattern within the pueriparous *S. s. bernardezi* (Chapter 3).

On the other hand, in viviparous species the relationship between mother and developing offspring becomes tighter than in oviparous (or larviparous) ones due to internal development and the existence of matrotrophy. Female's investment on each offspring is also higher; thus, reproductive failures are much more costly in viviparous species (Liu and Avise 2011). Therefore, mechanisms to reduce the risk of unsuccessful reproduction or mate incompatibilities are supposed to be favored by evolution, which, according to Viviparity Driven Conflict (VDC) hypothesis (Zeh and Zeh 2000, 2008), may result in some degree of inter-population reproductive isolation. This hypothesis posits that reproductive mode influences the rate at which postzygotic reproductive isolation evolves. According to VDC hypothesis viviparous species would experience higher postzygotic divergence than oviparous ones due to the more intense interactions between mother and embryos which result in intense genomic conflicts (Furness et al. 2015). The antagonistic coevolution resulting from those prenatal conflicts would reduce the potential for the generation of viable hybrids, enhancing reproductive isolation among populations (Schrader and Travis 2009). In addition, due to the generation of genetic incompatibilities, polyandry is favored in order to reduce the costs of mating to potentially incompatible mates (Zeh and Zeh 2000; Coleman et al. 2009). At the same time, this higher incidence of multiple paternity result in lower relatedness among siblings, reinforcing among-siblings conflicts, and, in turn, reproductive isolation in polyandrous species (Schrader and Travis 2008).

Reproductive isolation is the first step for speciation; therefore the rapid evolution of postzygotic isolation (viable hybrids) in viviparous species regarding oviparous ones may result in higher diversification of viviparous clades (Fitzpatrick 2004; Mank and Avise 2006). Observed morphological and genetic structuration among populations of *S. s. bernardezi* seem to result from different evolutionary (selective or not) processes acting across its distribution and a limited connectivity among populations, but besides, it would be interesting to consider the potential role of reproductive mode on inter-population differentiation. In that sense, the presence of a pueriparous mode of reproduction would have enhanced the divergence among population by means of inter-population reproductive isolation. It is important to consider that despite considerable smaller broods than larviparous populations, we found that pueriparous salamanders present high incidence of multiple paternity. Furthermore, it seems that that polyandry might arise as a mechanism to ensure successful fertilization, as we did not find any evidence of genetic benefits of polyandry for the offspring (increased genetic diversity) (Chapter 4). Nonetheless, further studies integrating information from different levels (genetic, developmental, plasticity, phylogenetic and ecological) should be performed in order to reach a full understanding of processes underlying morphological differentiation within *S. salamandra* and the role, if any, that the acquisition of a viviparous mode of reproduction plays.

NEW AVENUES FOR EXPLORING DIVERSITY PATTERNS

Morphological variation within the urodele family Plethodontidae (Blankers et al. 2012), as well as skull morphology in caecilians (Sherratt et al. 2014; Herrel et al. 2019) show a strong phylogenetic signal, and it seems to be the pattern for *S. salamandra* (see Chapter 2 and Future directions), in which head shape has been demonstrated to be a relevant trait for subspecies delimitation. Recent molecular and morphological studies (Beukema et al. 2016) showed the need for a taxonomic re-evaluation of described subspecies (Velo-Antón and Buckley 2015). For this purpose, integrative studies combining molecular, historical, and morphological information are especially valuable, and head shape can be a significant trait to take into consideration in this re-evaluation. However, although intraspecific diversity in head shape within *S. salamandra* was pointed by other authors (Bass and Gasser 1994; García-París et al. 2003), we presented herein the first quantitative methodology to explore head shape variation in this species (Chapter 1). It has demonstrated to be accurate enough to detect patterns at fine-scales, such as sexual dimorphism within populations. Indeed, giving its non-invasive nature it can be applied on individuals from natural populations (Chapter 1), but also on museum collection specimens, although the accuracy when working with this sort of samples should be properly tested (see Chapter 1). Finally, together with its applications in taxonomy, this methodology also allows addressing several unsolved evolutionary questions regarding morphological evolution and diversification within studied system.

WHAT ABOUT CONSERVATION?

Amphibians are declining worldwide and are the most threatened group of vertebrates (Stuart et al. 2004). Among causes responsible of global amphibians decline we found overexploitation, introduced species, land use change, contaminants, climate-change, and infectious disease (Collins and Storfer 2003; Collins 2010). Species displaying different reproductive modes would present different ecological requirements throughout their lives, determining in what extent they are threatened by different disturbances (Becker et al. 2007; Loyola et al. 2008). In addition, as showed herein, shifts in the reproductive mode presents further effects in biodiversity at different levels, such as diversity patterns, reproductive traits and evolutionary trends. Therefore, it is important to highlight the relevance of considering specific life-history traits of species for the development of effective conservation practices (Becker et al. 2010).

Unlike amphibians with biphasic life-cycle, the ‘monophasic’ pueriparous *S. salamandra*, in terms of an ontogenetic habitat shift, will be only exposed to threats of the terrestrial phase, avoiding those specific from aquatic environments (Hero et al. 2005; Lips et al. 2006; Becker et al. 2007). Nonetheless, fire salamander is currently highly endangered by the chytrid fungus *Batrachochytrium salamandrivorans* (Martel et al. 2013, 2014), which is causing a dramatic decline (99.9% in the Netherlands) and local extinction of several populations across Europe (Spitzen-van der Sluijs et al. 2016; Stegen et al. 2017). As seen herein as well as in many previous studies, *S. salamandra* arise as a fruitful system to address a number of ecological, reproductive, and evolutionary questions. Therefore, it becomes necessary to be aware of the need to take steps to ensure the persistence of not only this unique system, but also all the knowledge it can provide.

FUTURE DIRECTIONS

From each solved question, many more ones arise. Thus, it is worth pointing out some possible future questions that might be addressed:

- **Hybrid/contact zones.** Contact zones between lineages/subspecies where hybridization events take place constitute important sources of information about the evolutionary processes acting in speciation and species diversification (Hewitt 1988). Although geographically restricted, pueriparous *S. s. bernardezi* is not isolated, and limit in its eastern part with a mixed reproductive strategy subspecie (*S. s. fastuosa*) and in the western and southern limits with two larviparous subspecies (*S. s. gallaica* and *S. s. bejarae*) (Velo-Antón and Buckley 2015). Hybrids are found in every contact zone, and they have been too informative to unravel the evolutionary history and dynamics of those subspecies (García-París et al. 2003). Therefore, this natural display offers an almost unique opportunity for further testing several hypotheses about how developmental programs between reproductive strategies diverge, the phenotypic implications of such changes, and the mechanisms involved (Voss and Shaffer 1996).

- **Ontogenetic sequences.** As previously discussed, considering how head morphology change from early ontogenetic stages to adulthood would be essential for a fully understanding of both internal (i.e. developmental and genetic) and external (e.g. ecological niche, functional constraints) factors shape morphological diversity (Adams and Nistri 2010; Cvijanović et al. 2014). The study of larval forms in pueriparous groups is complicated; nonetheless, one first approach will include the comparison of ontogenetic sequences, from newborns in pueriparous and metamorphs in larviparous, towards adults.
- **Physiological consequences of viviparity.** Viviparity provides higher independence from water for successful reproduction, as they do not depend upon suitable water masses for larval phase development. Although it may translate into fewer constraints about the habitats that it can potentially occupy, it does not necessarily imply a complete independence from humidity and water. For instance, direct development species are highly dependent on levels of humidity as their terrestrial eggs and juveniles are highly exposed to desiccation in the terrestrial environment (da Silva et al. 2012). Therefore, an eco-physiological comparative approach between modes of reproduction (e.g. in evaporative water loss rate and skin resistance) would shed some light on the potential physiological implications of a shifts in reproductive strategies that might lead to ecological differentiation.
- **Broad-scale study including all viviparous species within *Salamandra*.** Not only the species *S. salamandra*, but also all the genus constitutes an exceptional model to explore different evolutionary questions in a macroevolutionary scale. In that sense, further research combining information from all species within *Salamandra*, both larviparous and pueriparous ones, would help to identify and better understand the mechanisms and consequences of the acquisition of new reproductive strategies. For instance, it might help to identify shared traits (e.g. morphological, physiological, behavioral, reproductive) among pueriparous species that result from the acquisition of intraoviductal development (Wake 2015). In addition, it would help to clarify the validity of head morphology as a phylogenetic morphological trait and how intraspecific variation translates in morphological variation among taxa (Hanken and Hall 1993; Mabee et al. 2000).

REFERENCES

Adams, D. C. 2004. Character displacement via aggressive interference in Appalachian salamanders. Ecology 85:2664–2670.

———. 2010. Parallel evolution of character displacement driven by competitive selection in terrestrial salamanders. BMC Evolutionary Biology 10:72.

Adams, D. C., and A. Nistri. 2010. Ontogenetic convergence and evolution of foot morphology in European cave salamanders (Family: Plethodontidae). BMC Evolutionary Biology 10:216.

Alberch, P. 1980. Ontogenesis and morphological diversification. Integrative and Comparative Biology 20:653–667.

———. 1982. The generative and regulatory roles of development in evolution. Environmental adaptation and evolution 19–36.

———. 1987. Evolution of the developmental process: irreversibility and redundancy in amphibian metamorphosis. Pages 23–46 *in* R. Raff and E. Raff, eds. Development as an evolutionary process. Liss, New York, NY.

Alcobendas, M., and J. Castanet. 2000. Bone growth plasticity among populations of *Salamandra salamandra*: interactions between internal and external factors. Herpetologica 56:14–26.

April, J., R. H. Hanner, R. L. Mayden, and L. Bernatchez. 2013. Metabolic rate and climatic fluctuations shape continental wide pattern of genetic divergence and biodiversity in fishes. PLoS ONE 8:e70296.

Bass, S., and F. Gasser. 1994. Polytipism of *Salamandra salamandra* (L.) in north-western Iberia. Mertensiella 4:41–74.

Becker, C. G., R. D. Loyola, C. F. B. Haddad, and K. R. Zamudio. 2010. Integrating species life-history traits and patterns of deforestation in amphibian conservation planning. Diversity and Distributions 16:10–19.

Becker, C. G., Fonseca, C. R., Haddad, C. F. B., Batista, R. F., and P. I. Prado. 2007. Habitat split and the global decline of amphibians. Science 318:1775-1777.

Beldade, P., K. Koops, and P. M. Brakefield. 2002. Developmental constraints versus flexibility in morphological evolution. Nature 416:844.

Beukema, W., A. G. Nicieza, A. Lourenço, and G. Velo-Antón. 2016. Colour polymorphism in *Salamandra salamandra* (Amphibia: Urodela), revealed by a lack of genetic and environmental differentiation between distinct phenotypes. Journal of Zoological Systematics and Evolutionary Research 54:127–136.

Blankers, T., D. C. Adams, and J. J. Wiens. 2012. Ecological radiation with limited morphological diversification in salamanders. Journal of Evolutionary Biology 25:634–646.

Bolker, J. A. 1995. Model systems in developmental biology. BioEssays 17:451–455.

Bonett, R. M., and A. L. Blair. 2017. Evidence for complex life cycle constraints on salamander body form diversification. Proceedings of the National Academy of Sciences 114:9936–9941.

Bruce, R. C. R. C. 2003. Life histories. Pages 477–525 *in* B. Jamieson, ed. Reproductive biology and phylogeny of Urodela (Volume 1). Science Publishers.

Buckley, D., M. García-París, and M. H. Wake. 2007. Heterochrony, canibalism, and the evolution of viviparity in *Salamandra salamandra*. Evolution and Development 9:105–115.

Burney, C. W., and R. T. Brumfield. 2009. Ecology predicts levels of genetic differentiation in Neotropical birds. The American Naturalist 174:358–368.

Coleman, S. W., A. Harlin-Cognato, and A. G. Jones. 2009. Reproductive isolation, reproductive mode, and sexual selection: empirical tests of the Viviparity-Driven Conflict Hypothesis. The American Naturalist 173:291–303.

Collins, J. P. 2010. Amphibian decline and extinction: What we know and what we need to learn. Diseases of Aquatic Organisms 92:93–99.

Collins, J. P., and A. Storfer. 2003. Global amphibian declines: sorting the hypotheses. Diversity and distributions 9:89–98.

Cooper, W. J., and S. J. Steppan. 2010. Developmental constraint on the evolution of marsupial forelimb morphology. Australian Journal of Zoology 58:1–15.

Crean, A. J., K. Monro, and D. J. Marshall. 2011. Fitness consequences of larval traits persist across the metamorphic boundary. Evolution 65:3079–3089.

Cvijanović, M., A. Ivanović, M. L. Kalezić, and M. L. Zelditch. 2014. The ontogenetic origins of skull shape disparity in the *Triturus cristatus* group. Evolution & development 16:306–317.

da Silva, F. R., M. Almeida-Neto, V. H. M. do Prado, C. F. B. Haddad, and D. de Cerqueira Rossa-Feres. 2012. Humidity levels drive reproductive modes and phylogenetic diversity of amphibians in the Brazilian Atlantic Forest. Journal of Biogeography 39:1720–1732.

Deban, S. M., and S. B. Marks. 2002. Metamorphosis and evolution of feeding behaviour in salamanders of the family plethodontidae. Zoological Journal of the Linnean Society 134:375–400.

Dopazo, H., L. Boto, and P. Alberch. 1998. Mitochondrial DNA variability in viviparous and ovoviviparous populations of the urodele *Salamandra salamandra*. Journal of Evolutionary Biology 2:365–378.

Duellman, W. E., and L. Trueb. 1986. Biology of amphibians. New York. JHU press.

Duminil, J., S. Fineschi, A. Hampe, P. Jordano, D. Salvini, G. G. Vendramin, and R. J. Petit. 2007. Can population genetic structure be predicted from life-history traits? The American Naturalist 169:662–672.

Fitzpatrick, B. M. 2004. Rates of evolution of hybrid inviability in birds and mammals. Evolution 58:1865–1870.

Fluker, B. L., B. R. Kuhajda, and P. M. Harris. 2014. The influence of life-history strategy on genetic differentiation and lineage divergence in darters (Percidae: Etheostomatinae). Evolution 68:3199–3216.

Furness, A. I., K. R. Morrison, T. J. Orr, J. D. Arendt, and D. N. Reznick. 2015. Reproductive mode and the shifting arenas of evolutionary conflict. Annals of the New York Academy of Sciences 1360:75–100.

Fusco, G. 2001. How many processes are responsible for phenotypic evolution? Evolution and Development 3:279–286.

García-París, M., M. Alcobendas, D. Buckley, and D. B. Wake. 2003. Dispersal of viviparity across contact zoes in iberian populatios of fire salamanders (*Salamandra*) inferred from discordance of genetic and morphological traits. Evolution 57:129–143.

Gomez-Mestre, I., R. A. Pyron, and J. J. Wiens. 2012. Phylogenetic analyses reveal unexpected patterns in the evolution of reproductive modes in frogs. Evolution 66:3687–3700.

Hallgrímsson, B., W. Mio, R. S. Marcucio, and R. Spritz. 2014. Let's Face It—Complex Traits Are Just Not That Simple. PLoS Genetics 10:e1004724.

Hanken, J. 1989. Development and evolution in amphibians. American Scientist 77:336–343.

———. 1992. Life history and morphological evolution. Journal of Evolutionary Biology 5:549–557.

Hanken, J., and B. K. Hall. 1993. Mechanisms of skull diversity and evolution. Pages 1–36 *in* J. Hanken and B. K. Hall, eds. The skull (Volume 3). University of Chicago Press, Illinois, Chicago.

Hero, J. M., Williams, S. E., and W. E. Magnusson. 2005. Ecological traits of declining amphibians in upland areas of eastern Australia. Journal of Zoology 267:221–232.

Herrel, A., J. C. O'Reilly, A.-C. Fabre, C. Bardua, A. Lowie, R. Boistel, and S. N. Gorb. 2019. Feeding in Amphibians: Evolutionary Transformations and Phenotypic Diversity as Drivers of Feeding System Diversity. Pages 431–467 *in* V. Bels and I. Whishaw, eds. Feeding in Vertebrates. Springer, Cham.

Hewitt, G. M. 1988. Hybrid zones-natural laboratories for evolutionary studies. Trends in ecology & evolution 3:158–167.

Holtmeier, C. L. 2001. Heterochrony, maternal effects, and phenotypic variation among sympatric pupfishes. Evolution 55:330–338.

Ivanović, A., M. Cvijanović, and M. L. Kalezić. 2011. Ontogeny of body form and metamorphosis: Insights from the crested newts. Journal of Zoology 283:153–161.

Jaekel, M., and D. B. Wake. 2007. Developmental processes underlying the evolution of a derived foot morphology in salamanders. Proceedings of the National Academy of Sciences 104:20437–20442.

Jenner, R. A., and M. A. Wills. 2007. The choice of model organisms in evo–devo. Nature Reviews Genetics 8:311.

Jernvall, J. 2000. Linking development with generation of novelty in mammalian teeth. Proceedings of the National Academy of Sciences 97:2641–2645.

Jiménez-Robles, O., J. M. Guayasamin, S. R. Ron, and I. De la Riva. 2017. Reproductive traits associated with species turnover of amphibians in Amazonia and its Andean slopes. Ecology and Evolution 7:2489–2500.

Kelly, E. M., and K. E. Sears. 2011. Limb specialization in living marsupial and eutherian mammals: constraints on mammalian limb evolution. Journal of Mammalogy 92:1038–1049.

Liedtke, H. C., H. Müller, J. Hafner, J. Penner, D. J. Gower, T. Mazuch, M.-O. Rödel, et al. 2017. Terrestrial reproduction as an adaptation to steep terrain in African toads. Proceedings of the Royal Society B: Biological Sciences 284.

Lion, M. B., G. G. Mazzochini, A. A. Garda, T. M. Lee, D. Bickford, G. C. Costa, and C. R. Fonseca. 2019. Global patterns of terrestriality in amphibian reproduction. Global Ecology and Biogeography 28:744–756.

Lips, K. R., Brem, F., Brenes, R., Reeve, J. D., Alford, R. A., Voyles, J., C. Carey et al. 2006. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. Proceedings of the National Academy of Sciences 103: 3165-3170.

Liu, J. X., and J. C. Avise. 2011. High degree of multiple paternity in the viviparous Shiner Perch, *Cymatogaster aggregata*, a fish with long-term female sperm storage. Marine Biology 158:893–901.

Lourenço, A., D. Álvarez, I. J. Wang, and G. Velo-Antón. 2017. Trapped within the city: integrating demography, time since isolation and population-specific traits to assess the genetic effects of urbanization. Molecular Ecology 26:1498–1514.

Lourenço, A., B. Antunes, I. J. Wang, and G. Velo-Antón. 2018. Fine-scale genetic structure in a salamander with two reproductive modes: Does reproductive mode affect dispersal? Evolutionary ecology 32:699–732.

Loyola, R. D., C. G. Becker, U. Kubota, C. F. B. Haddad, C. R. Fonseca, and T. M. Lewinsohn. 2008. Hung out to dry: Choice of priority ecoregions for conserving threatened neotropical anurans depends on life-history traits. PLoS ONE 3:10–13.

Mabee, P. M., K. L. Olmstead, and C. C. Cubbage. 2000. An experimental study of intraspecfic variation, developmental timing, and heterochrony in fishes. Evolution 54:2091–2106.

Mank, J. E., and J. C. Avise. 2006. Supertree analyses of the roles of viviparity and habitat in the evolution of atherinomorph fishes. Journal of evolutionary biology 19:734–740.

Martel, A., M. Blooi, C. Adriaensen, P. Van Rooij, W. Beukema, M. C. Fisher, R. A. Farrer, et al. 2014. Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. Science 346:630–631.

Martel, A., A. Spitzen-van der Sluijs, W. Bert, R. Ducatelle, M. C. Fisher, A. Woeltjes, W. Bosman, et al. 2013. *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. Proceedings of the National Academy of Sciences 110:15325–15329.

Measey, G. J., P. Galbusera, P. Breyne, and E. Matthysen. 2007. Gene flow in a direct-developing, leaf litter frog between isolated mountains in the Taita Hills, Kenya. Conservation Genetics 8:1177–1188.

Moran, N. A. 1994. Adaptation and constraint in the complex life cycles of animals. Annual Review of Ecology and Systematics 25:573–600.

Müller, H., H. C. Liedtke, M. Menegon, J. Beck, L. Ballesteros-Mejia, P. Nagel, and S. P. Loader. 2013. Forests as promoters of terrestrial life-history strategies in East African amphibians. Biology letters 9:20121146.

Parichy, D. M. 2006. Evolution of danio pigment pattern development. Heredity 97:200–210.

Paz, A., R. Ibáñez, K. R. Lips, and A. J. Crawford. 2015. Testing the role of ecology and life history in structuring genetic variation across a landscape: A trait-based phylogeographic approach. Molecular Ecology 24:3723–3737.

Porter, A. H., and N. A. Johnson. 2002. Speciation despite gene flow when developmental pathways evolve. Evolution 56:2103–2111.

Raff, R. A. 1987. Constraint, flexibility, and phylogenetic history in the evolution of direct development in sea urchins. Developmental biology 119:6–19.

Richardson, M. K., and A. D. Chipman. 2003. developmental constraints in a comparative framework: a test case using variations in phalanx number during amniote evolution. Journal of Experimental Zoology Part B: Molecular and Developmental Evolution 296:8–22.

Rose, C. S. 2003. The developmental morphology of salamander skulls. Amphibian biology 5:1684–1781.

Roth, G., and D. B. Wake. 1985. Trends in the functional morphology and sensorimotor control of feeding behavior in salamanders: An example. Of the role of internal dynamics in evolution 192:175–191.

Schrader, M., and J. Travis. 2008. Testing the Viviparity-Driven-Conflict Hypothesis: parent-offspring conflict and the evolution of reproductive isolation in a poeciliid fish. The American Naturalist 172:806–817.

———. 2009. Do embryos influence maternal investment? Evaluating maternal-fetal coadaptation and the potential for parent-offspring conflict in a placental fish. Evolution 63:2805–2815.

Sears, K. E. 2014. Quantifying the impact of development on phenotypic variation and evolution. Journal of Experimental Biology. Part B: Molecular and Developmental Evolution 322B:643–53.

Sherratt, E., D. J. Gower, C. P. Klingenberg, and M. Wilkinson. 2014. Evolution of Cranial Shape in Caecilians (Amphibia: Gymnophiona). Evolutionary Biology 41:528–545.

Sherratt, E., M. Vidal-García, M. Anstis, and J. S. Keogh. 2017. Adult frogs and tadpoles have different macroevolutionary patterns across the Australian continent. Nature Ecology and Evolution 1:1385–1391.

Smith, F. J., C. J. Percival, N. M. Young, D. Hu, R. A. Schneider, R. S. Marcucio, and B. Hallgrímsson. 2015. Divergence of craniofacial developmental trajectories among avian embryos. Developmental Dynamics 244:1158–1167.

Smith, K. K. 2002. Sequence heterochrony and the evolution of development. Journal of morphology 252:82–97.

Spitzen-van der Sluijs, A., J. Asselberghs, S. Lötters, E. Goverse, B. R. Schmidt, W. Beukema, J. Sabino-Pinto, et al. 2016. Expanding distribution of lethal amphibian fungus *Batrachochytrium salamandrivorans* in Europe. Emerging Infectious Diseases 22:1286–1288.

Stegen, G., F. Pasmans, B. R. Schmidt, L. O. Rouffaer, S. Van Praet, M. Schaub, S. Canessa, et al. 2017. Drivers of salamander extirpation mediated by *Batrachochytrium salamandrivorans*. Nature 544:353.

Stuart, S. N., J. S. Chanson, N. A. Cox, B. E. Young, A. S. L. Rodrigues, D. L. Fischman, and R. W. Waller. 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306:1783–1786.

Tilley, S. G. 2016. Patterns of Genetic Differentiation in Woodland and Dusky Salamanders. Copeia 104:8–20.

Van Bocxlaer, I., S. P. Loader, K. Roelants, S. D. Biju, M. Menegon, and F. Bossuyt. 2010. Gradual adaptation toward a range-expansion phenotype initiated the global radiation of toads. Science 327:679–682.

Velo-Antón, G., and D. Buckley. 2015. Salamandra común – *Salamandra salamandra* (Linnaeus, 1758). Enciclopedia Virtual de los Vertebrados Españoles. Museo Nacional de Ciencias Naturales, Madrid.

Velo-Antón, G., M. García-París, P. Galán, and A. Cordero-Rivera. 2007. The evolution of viviparity in holocene islands: ecological adaptation versus phylogenetic descent along the transition from aquatic to terrestrial environments. Journal of Zoological Systematics and Evolutionary Research 45:345–352.

Vences, M., F. Andreone, F. Glaw, J. Kosuch, A. Meyer, H. C. Schaefer, and M. Veith. 2002. Exploring the potential of life-history key innovation: Brook breeding in the radiation of the Malagasy treefrog genus *Boophis*. Molecular Ecology 11:1453–1463.

Voss, S. R., and H. B. Shaffer. 1996. What insights into the developmental traits of urodeles does the study of interspecific hybrids provide? International Journal of Developmental Biology 40:885–893.

Vučić, T., M. Sibinović, T. D. Vukov, N. T. Kolarov, M. Cvijanović, and A. Ivanović. 2019. Testing the evolutionary constraints of metamorphosis: the ontogeny of head shape in *Triturus* newts. Evolution 73:1253–1264 .

Wagner, C. E., L. J. Harmon, and O. Seehausen. 2012. Ecological opportunity and sexual selection together predict adaptive radiation. Nature 487:366–369.

Wainwright, P. C., and S. M. Reilly. 1994. Ecological morphology: integrative organismal biology. University of Chicago Press, Chicago.

Wake, D. B. 1982. Functional and developmental constraints and opportunities in the evolution of feeding systems in urodeles. Environmental adaptation and evolution 51–66.

———. 2009. What salamanders have taught us about evolution. Annual Review of Ecology, Evolution, and Systematics 40:333–352.

Wake, D. B., and J. Hanken. 2004. Direct development in the lungless salamanders: what are the consequences for developmental biology, evolution and phylogenesis? International Journal of Developmental Biology 40:859–869.

Wake, M. H. 2003. Reproductive modes, ontogenies , and the evolution of body form. Animal biology 53:209–223.

———. 2015. How do homoplasies arise? Origin and maintenance of reproductive modes in amphibians. Page 375 *in* K. P. Dial, N. Shubin, and E. L. Brainerd, eds. Great Transformations in Vertebrate Evolution. University of Chicago Press.

Wang, I. J. 2013. Examining the full effects of landscape heterogeneity on spatial genetic variation: a multiple matrix regression approach for quantifying geographic and ecological isolation. Evolution 67:3403–3411.

Wells, K. D. 2007. The Ecology and Behavior of Amphibians. The University of Chicago Press, Chicago.

Wollenberg Valero, K. C. 2015. Evidence for an intrinsic factor promoting landscape genetic divergence in Madagascan leaf-litter frogs. Frontiers in genetics 6:155.

Zeh, D. W., and J. A. Zeh. 2000. Reproductive mode and speciation: The viviparity-driven conflict hypothesis. BioEssays 22:938–946.

Zeh, J. A., and D. W. Zeh. 2008. Viviparity-driven conflict. Annals of the New York Academy of Sciences 1133:126–148.

Zimkus, B. M., L. Lawson, S. P. Loader, and J. Hanken. 2012. Terrestrialization, miniaturization and rates of diversification in african puddle frogs (anura: Phrynobatrachidae). PLoS ONE 7:15–21.

Conclusions

Conclusions

1) The landmark-based geometric morphometrics method described here constitutes a powerful tool for the exploration and description of dorsal head shape in urodeles. It has been proven to be highly accurate and its non-invasive character broadens its potential to be implemented in alive individuals from natural populations, being able to detect morphological differentiation patterns at fine-scale.

2) Head morphological differentiation in *Salamandra salamandra* is not related to the ontogenetic modifications associated with the acquisition of a viviparous reproductive strategy (i.e. developmental acceleration, precocious formation of feeding structures and active intrauterine feeding behavior), at least in the adult stage. Conversely, it seems to result from different historical and evolutionary processes acting differentially in each subspecies displaying viviparity.

3) Head morphological adult features constitute effective diagnostic traits for the description of subspecies within *Salamandra salamandra*. Therefore, quantitative description of head morphology should be integrated together with other sources of information for a comprehensive reevaluation of the taxonomy within this species.

4) The polymorphic viviparous subspecies *Salamandra salamandra bernardezi* is confirmed to present a structuration of genetic and phenotypic diversity, which present a high geographic concordance. While neutral genetic structure follows an isolation by distance pattern, different evolutionary mechanisms may generate spatial concordance of genetic and phenotypic variation.

5) Different colorations within the subspecies *Salamandra salamandra bernardezi* display significant differences in head morphology and body size. Those distinguishing features should be considered in the exploration of the evolutionary forces underlying intraspecific diversity and polymorphism within this subspecies.

6) Populations of *Salamandra salamandra* from both independent viviparous origins present a strong incidence of multiple paternity despite the reduction in brood sizes associated with the acquisition of a viviparous strategy. Apparently, multiple paternity is evolutionary favored for the insurance of fertilization success and the reduction of mating failures, although whether it acts as a genetic compensation mechanism for increasing genetic diversity should also be considered.

7) The high incidence of multiple paternity as well as the high number of mean fathers siring a clutch in the viviparous insular populations from the subspecies *Salamandra salamandra gallaica* point to the special relevance of the benefits derived from a polyandrous system in an isolated population.

8) *Salamandra salamandra* conforms an extremely diverse system with a high potential for the exploration of a number of ecological and evolutionary questions on the origin of new reproductive strategies. The special configuration of the pueriparous/larviparous system allows for comparative studies between and within modes of reproduction, the description of patterns of variation, and the identification of the processes underlying them.

9) Viviparity and its associated modifications in development and life-history traits regarding the ancestral larviparous mode of reproduction seem to have unequal consequences at each studied level. While developmental modifications do not impact intraspecific adult morphological diversity, viviparous mode of reproduction seem to be highly related to some reproductive traits, such as multiple paternity patterns.

10) The results of this thesis support the need of integrative approaches that combine information from several levels to reach a solid understanding of the consequences of the evolution of new reproductive strategies.

Resumen y conclusiones

Resumen y conclusiones

La gran diversidad de modos reproductivos que podemos encontrar en la naturaleza son el resultado de la combinación de diversos rasgos reproductivos, como son el lugar en el que se produce la ovoposición y desarrollo, el número de descendientes y tamaño de la puesta, el estadio de desarrollo en el que se produce el nacimiento/eclosión o la cantidad y tipo de cuidado parental. De esta manera, podemos encontrar desde organismos en los que todo el proceso reproductivo de un elevado número de descendientes, desde la fecundación hasta la eclosión, tiene lugar de forma libre, hasta organismos en los que un pequeño número de descendientes son retenidos dentro del cuerpo de uno de los progenitores (generalmente la madre) hasta que se produce el nacimiento de individuos en fases avanzadas del desarrollo.

Dentro del gran abanico de estrategias reproductivas que podemos encontrar en la naturaleza, el viviparismo, entendido como la retención de huevos y embriones en el tracto reproductivo de la madre a lo largo de un periodo de tiempo durante el cual se proporcionan nutrientes adicionales a los contenidos en el huevo, y que concluye con la liberación (nacimiento) de individuos juveniles en estados avanzados de desarrollo, ha evolucionado independientemente en todos los taxones de vertebrados, exceptuando las aves y ciclóstomos, y ha sido objeto de múltiples estudios y disciplinas. La adquisición de un modo de reproducción vivíparo puede presentar una serie de potenciales ventajas adaptativas, como una mayor protección de la descendencia frente a factores bióticos y abióticos del medio externo y un mayor grado de desarrollo de los neonatos, lo que puede implicar una mayor eficacia biológica. Sin embargo, lleva también asociados una serie de costes en términos energéticos, de desempeño locomotor y de compromiso con otros rasgos de historia de vida, como la fecundidad (tanto en número de descendientes por puesta como en número de eventos reproductivos a lo largo de la vida), esfuerzo reproductor, cuidado parental y tamaño corporal del progenitor gestante. Por tanto, el viviparismo se verá favorecido evolutivamente cuando los beneficios superen los costes asociados.

Por otro lado, la evolución de un modo reproductor vivíparo lleva asociados una serie de cambios a diversos niveles, tanto de los individuos adultos como de la descendencia. Estos cambios incluyen modificaciones en el comportamiento, secuencias de desarrollo, fisiología y morfología para permitir el correcto desarrollo de los embriones dentro del oviducto, así como para facilitar la provisión de nutrientes por diversas vías. No obstante, el viviparismo ha evolucionado independientemente en los diferentes grupos, de manera que los mecanismos que han permitido su adquisición son extremadamente variables. En los grupos vivíparos, la mayor protección de las fases iniciales del desarrollo reduce las limitaciones impuestas por el ambiente para la reproducción y desarrollo inicial (e.g. ambientes acuáticos para el desarrollo de huevos/larvas de vida acuática libre), lo que puede favorecer la colonización de nuevos hábitats, el aislamiento entre grupos y, en última instancia, la diversificación o especiación de taxones. Este patrón es, sin embargo, variable en los diferentes grupos.

En el caso concreto de los anfibios, además del ciclo de vida bifásico 'clásico' consistente en huevos y larvas acuáticas de vida libre que tras un periodo de crecimiento metamorfosean en juveniles terrestres, éstos han desarrollado una gran variedad de historias de vida, entre ellas el viviparismo, que aparece en los tres órdenes. En el caso del orden Urodela, el viviparismo ha evolucionado únicamente y de forma independiente en dos géneros de una misma familia: *Lyciasalamandra* y *Salamandra.* Mientras que todas las especies incluidas en el primer género son vivíparas, dentro del género *Salamandra* encontramos dos especies que presentan polimorfismo reproductivo: *S. algira* y *S. salamandra*, ya que aunque a lo largo de la mayor parte de su área de distribución ambas especies son larvíparas (liberación de larvas de vida acuática libre), algunas poblaciones y subespecies presentan un modo de reproducción vivíparo o pueríparo (liberación de juveniles terrestres). En el caso de *S. algira* únicamente se han encontrado poblaciones vivíparas en algunas poblaciones de una subespecie, sin embargo, dentro de *S. salamandra* se han identificados dos núcleos en los que el viviparismo ha evolucionado de manera independiente. El primero se encuentra localizado en la Cordillera Cantábrica, al norte de la Península Ibérica, e incluye todas las poblaciones de la subespecie *S. s. bernardezi.* La subespecie colindante, *S. s. fastuosa,* presenta un modo reproductor mixto como resultado de diversos procesos de introgresión desde el área de distribución de *S. s. bernardezi.* El segundo origen del viviparismo aparece en dos poblaciones insulares en el noroeste de la Península Ibérica. Dichas poblaciones pertenecen a la subespecie *S. s. gallaica* la cual presenta a lo largo del resto de su rango de distribución un modo reproductor larvíparo.

Mientras que los mecanismos subyacentes a la evolución del viviparismo en *S. algira* son poco conocidos, la adquisición del viviparismo en *S. salamandra* ha tenido lugar mediante una serie de modificaciones heterocrónicas en el desarrollo embrionario respecto a las poblaciones larvíparas, entre las que encontramos: una aceleración generalizada del desarrollo (incluida el momento de metamorfosis), un crecimiento precoz de la parte anterior del cuerpo, así como de las estructuras relacionadas con la alimentación, y una eclosión intrauterina temprana. Todas estas modificaciones permiten una alimentación intrauterina temprana, mediante oofagia, sobre huevos abortivos, o adelfofagia, sobre embriones menos desarrollados. Este diseño natural que presenta la salamandra común ofrece la oportunidad para realizar estudios comparativos a nivel intraespecífico, lo que puede proporcionar una valiosa información a la que no se puede acceder cuando se trabaja a niveles taxonómicos superiores. De hecho no solo desde un punto de vista reproductivo, sino que *S. salamandra* es una especie altamente variable en otros aspectos, con una gran diversidad ecológica y morfológica (en forma, coloración y tamaño). Por todo ello, la salamandra común se presenta como un sistema de estudio ideal para explorar las consecuencias de la adquisición de un nuevo modo reproductivo a diferentes niveles de la organización biológica, así como profundizar en la comprensión acerca del origen y evolución de la complejidad biológica que podemos observar en la naturaleza.

El objetivo general de esta tesis es la investigación de las potenciales consecuencias morfológicas y reproductivas de la adquisición de un modo reproductor vivíparo, utilizado como sistema de estudio el polimorfismo reproductivo y la diversidad que encontramos dentro de la especie *S. salamandra.* Para ello, se abordarán diversos objetivos específicos a lo largo de cuatro capítulos:

1. Desarrollar un método no invasivo de morfometría geométrica para la exploración de la morfología dorsal de la cabeza en urodelos. Esta metodología nos permitirá, por un lado, explorar las diferencias morfológicas entre individuos procedentes de poblaciones naturales y testar diversas hipótesis relativas a la diferenciación morfológica (**CAPÍTULO 1**).
2. Determinar si los cambios en las secuencias ontogenéticas asociados con la adquisición de un modo reproductor vivíparo están relacionados con las diferencias en la morfología de la cabeza entre subespecies, o si bien, ésta es el resultado de historias evolutivas diferenciadas (**CAPÍTULO 2**).
3. Determinar la validez y utilidad taxonómica de las diferencias morfológicas de la cabeza para la definición y diferenciación de subespecies dentro de *S. salamandra* (**CAPÍTULO 2**).
4. Definir las diferencias morfológicas entre los polimorfismos de coloración que se encuentran a lo largo del área de distribución de la subespecie pueríapara *S. s. bernardezi* (**CAPÍTULO 3**).
5. Determinar los procesos evolutivos subyacentes a la estructuración genética y de la diversidad morfológica dentro de esta subespecie (**CAPÍTULO 3**).
6. Identificar los patrones de paternidad que presentan ambos núcleos vivíparos en las subespecies *S. s. bernardezi* y *S. s. gallaica* (**CAPÍTULO 4**).
7. Comparación de los patrones de paternidad entre núcleos vivíparos, modos reproductivos dentro de *S. salamandra* y determinar los posibles beneficios derivados de la presencia de múltiples padres en una única puesta (**CAPÍTULO 4**).

En el **PRIMER CAPÍTULO** de la presente tesis se ha descrito un método no invasivo de morfometría geométrica que permite explorar la morfología de la vista dorsal de la cabeza de urodelos utilizando como sistema de estudio dos poblaciones naturales de la subespecie *S. s. gallaica*. La cabeza en los urodelos desempeña un gran número de funciones ecológicas y sociales, por lo que representa un objetivo potencial de los procesos evolutivos. De hecho, la vista dorso-ventral de la cabeza en urodelos, debido a su forma ancha y aplanada, captura la mayor parte de la variación ontogenética y evolutiva de la cabeza. La metodología descrita ha demostrado ser altamente precisa y de gran utilidad a la hora de detectar diferencias incluso a escalas muy reducidas, como por ejemplo, detectando dimorfismo sexual dentro de cada población. Además, su aplicación sobre individuos vivos amplía el abanico de situaciones en las que puede ser implementado, reduciendo las restricciones en el tamaño de muestra y accesibilidad impuestas cuando trabajamos con muestras óseas o con especímenes de museo. Esta metodología descrita se aplicará en el **SEGUNDO** y **TERCER CAPÍTULO** para testar diversas hipótesis sobre los procesos que subyacen la diversificación fenotípica.

Las diferencias en las secuencias de desarrollo entre los dos modos de reproducción que encontramos en salamandra se centran principalmente en la formación temprana de las estructuras cefálicas que permiten una alimentación exógena intrauterina precoz, es decir, la boca y las mandíbulas, además del aparato digestivo. La forma de la cabeza además, se ha utilizado como rasgo característico para la descripción de las diversas subespecies, sin embargo, nunca se ha caracterizado cuantitativamente aplicando técnicas morfométricas. Por todo ello, en el **SEGUNDO CAPÍTULO** se explora la hipótesis de que los cambios en el desarrollo temprano en las poblaciones pueríparas de ambas subespecies tienen como resultado una convergencia en la forma de la cabeza en los individuos adultos. Para ello, aplicando la metodología descrita en el **PRIMER CAPÍTULO**, se estudió y comparó la morfología entre poblaciones procedentes de dos subespecies: *S. s. bernardezi* y *S. s. gallaica*, incluyendo tanto poblaciones pueríparas como larvíparas de esta última. La diferenciación morfológica entre las poblaciones de ambas subespecies, independientemente del modo reproductor, así como la ausencia de diferencias significativas entre modos reproductivos dentro de la subespecie *S. s. gallaica* apuntan a que las diferencias en la morfología cefálica son el resultado de la historia evolutiva independiente entre subespecies, rechazando la hipótesis inicial de convergencia morfológica derivada de similitudes en el desarrollo. Aunque se precisan estudios adicionales ya que no podemos descartar que esta convergencia se produzca en estadios ontogenéticos más tempranos y que a lo largo del crecimiento se diluya, este trabajo destaca la importancia que tienen los estudios comparativos que integran historias evolutivas y trayectorias ontogenéticas en la exploración de las diferentes impulsores la diversidad morfológica observada.

De esta manera, la forma de la cabeza aparece como un rasgo subespecífico, que puede ser utilizado como rasgo diagnóstico a la hora de la determinación de subespecies, aunque también existe una gran diversidad dentro de cada una de ellas. Los últimos estudios moleculares y morfológicos apuntan a la necesidad de una re-evaluación taxonómica integrando datos moleculares, ecológicos y morfológicos de las subespecies descritas dentro de *S. salamandra*, para lo cual la morfología de la cabeza debería ser un rasgo a considerar. Por ello, en el **TERCER CAPÍTULO** se ha abordado el estudio de la diversidad morfológica que existe dentro de la subespecie puerípara *S. s. bernardezi*, que es la subespecie de la Península Ibérica que presenta una mayor estructuración y diferenciación de la diversidad a lo largo de su rango de distribución. Esta subespecie presenta en la parte este de su distribución un polimorfismo de coloración, con individuos que presentan el fenotipo típicos de esta subespecie (fenotipo rayado), y otros que carecen del patrón rayado y presentan una coloración que varía desde el amarillo hasta el marrón-oliváceo (fenotipo no rayado). Pese a la existencia de estos morfotipos aproximadamente discretos, otros rasgos morfológicos no han sido abordados. En este capítulo demostramos la existencia de claras diferencias morfológicas entre los diferentes fenotipos en tamaño corporal (SVL), y forma y tamaño de la cabeza.

Además, se ha encontrado que existe una importante concordancia en la estructuración geográfica de la diversidad genética y morfológica (distribución geográfica de las coloraciones). Las coloraciones no rayadas aparecen únicamente en uno de los dos clusters genéticos identificados, mientras que las coloraciones típicas rayadas aparecen en ambos clusters. Estos patrones observados apoyan la existencia de diferentes mecanismos que favorecen la concordancia geográfica de la diversidad genética y morfológica, como una selección diferencial sobre cada fenotipo, el origen alopátrico de los fenotipos no rayados, o bien, una combinación de ambos. Por otro lado se estudió la posible correlación entre los patrones de diferenciación de la diversidad genética neutra, mediante el uso de marcadores moleculares microsatélites, y de la morfología de la cabeza. De existir, esta correlación apoyaría la hipótesis de que la diferenciación en la forma de la cabeza entre poblaciones es el resultado de procesos neutros, como deriva génica y aislamiento por distancia. Sin embargo, la diferenciación morfológica no presenta un patrón de aislamiento por distancia, ni una correlación con diversidad genética neutra, la cual sí sigue un patrón de aislamiento por distancia, apuntando la existencia de otros procesos evolutivos que determinan la diversidad morfológica dentro de esta subespecie. Esta estructuración de la diversidad genética y morfológica dentro a una escala tan pequeña apunta a la existencia de una baja conectividad entre poblaciones, aunque la diferenciación entre poblaciones también podría estar relacionada con la existencia de un modo de reproducción pueríparo ya que la independencia de los ecosistemas acuáticos facilitaría la ocupación de nuevos y más variados hábitats, favoreciendo la diferenciación interpoblacional.

Además de las potenciales consecuencias morfológicas, la adquisición de un modo reproductor pueríparo presenta una serie de consecuencias en otros aspectos y rasgos de historia de vida, siendo uno de los más destacables la reducción que se produce en el número de individuos que nacen en cada puesta, es decir, en la fecundidad. Mientras que las hembras larvíaparas liberan ente 20 y 90 larvas acuáticas de vida libre, en las poblaciones pueríparas (*S. s. bernardezi* y las poblaciones insulares de *S. s. gallaica*) las hembras liberan generalmente entre 1 y 35 juveniles terrestres. Pese a las posibles ventajas derivadas del viviparismo como es la eliminación de una fase con altas tasas de mortalidad, esta reducción en la fecundidad puede tener también consecuencias negativas, como una reducción de la diversidad genética de la especie. Se han observado patrones de múltiple paternidad previamente en poblaciones larvíparas de *S. salamandra*, pero nunca han sido exploradas en las poblaciones pueríparas, donde, como consecuencia del menor número de descendientes por puesta se espera una menor incidencia de múltiple paternidad. Además, los eventos de canibalismo que se producen durante el desarrollo intrauterino de los embriones pueríparos pueden modificar el número final de padres que engendran una determinada puesta. Para resolver estas dos cuestiones, en el **CAPÍTULO CUATRO** se estudió si en las poblaciones pueríparas también existían eventos de múltiple paternidad y se trataron de determinar y explicar los patrones observados. Contrario a lo esperado, a pesar de la reducción en la fecundidad, las poblaciones vivíparas mantienen, e incluso aumentan tanto la frecuencia como el número de padres implicados en la paternidad de una determinada puesta. La poliandria y paternidad múltiple se cuentan entre los mecanismos favorecidos evolutivamente para contrarrestar los posibles efectos adversos de la reducción en fecundidad. Es especialmente destacable el patrón observado en las poblaciones insulares, donde se ha detectado el número más elevado de padres en una única puesta de esta especie, apuntando a la selección de la paternidad múltiple como mecanismo para garantizar el éxito reproductivo en situaciones de altos niveles de endogamia, donde el riesgo de incompatibilidades y fracasos reproductivos es mayor.

Entender los procesos subyacentes así como las consecuencias derivadas de la evolución de nuevos modos reproductivos a diversos niveles requiere de estudios que integren en la medida de lo posible información procedente de diversas fuentes y disciplinas. Además, aprovechar la información que se puede extraer de los múltiples sistemas que encontramos en la naturaleza permitirá importantes avances en la comprensión de los procesos y patrones que podemos observar. En este sentido, el diseño natural que presenta la especie *S. salamandra* la convierte en un sistema prácticamente único para abordar múltiples preguntas acerca de las implicaciones de la evolución del viviparismo a un nivel intraespecífico.

CONCLUSIONES

1) El método de morfometría geométrica basado en landmarks aquí descrito constituye una herramienta con un gran potencial para estudiar y describir la forma dorsal de la cabeza en urodelos. Se ha demostrado como un método altamente preciso y su carácter no invasivo amplía su rango de aplicación a organismos vivos procedentes de poblaciones naturales, siendo capaz de detectar patrones de diferenciación morfológica a escala fina.

2) La diferenciación morfológica de la cabeza en *Salamandra salamandra* no está relacionada con las modificaciones ontogenéticas propias de la adquisición de un modo de reproducción vivíparo (i.e. aceleración del desarrollo, formación precoz de las estructuras de alimentación y una alimentación intrauterina activa), al menos en la fase adulta. Por el contrario, ésta parece resultar de diversos procesos históricos y evolutivos que actúan de forma diferenciada en cada una de las subespecies que presenta un modo de reproducción vivíparo.

3) Las características morfológicas de la cabeza en adultos constituyen un rasgo diagnóstico eficaz para la descripción de subespecies dentro de *Salamandra salamandra.* Por ello, es necesario integrar la descripción cuantitativa de la morfología de la cabeza con otras fuentes de información para llevar a cabo una reevaluación completa de la taxonomía de esta especie.

4) Se ha confirmado una estructuración de la diversidad fenotípica y genética dentro de la subespecie polimórfica *Salamandra salamandra bernardezi,* así como una gran concordancia geográfica entre ambas. Mientras que la estructura genética neutra sigue un patrón de aislamiento por distancia, diversos mecanismos evolutivos generan la concordancia espacial entre la variación genética y fenotípica.

5) Las diferentes coloraciones observadas en la subespecie *Salamandra salamandra bernardezi* difieren significativamente en la morfología de la cabeza y en el

tamaño corporal. Estas características distintivas deben ser tenidas en cuenta en la exploración de las fuerzas evolutivas subyacentes a la diversidad intraespecífica y al polimorfismo de esta subespecie.

6) Las poblaciones de *Salamandra salamandra* de los dos orígenes independientes vivíparos presentan una gran incidencia de paternidad múltiple pese a la reducción del tamaño de puesta asociado a la adquisición de un modo de reproducción vivíparo. Aparentemente, la paternidad múltiple se ha favorecido evolutivamente para asegurar la fertilización efectiva y la reducción del riesgo de apareamientos fallidos aunque la posibilidad de que sirva como un mecanismo compensatorio para aumentar la diversidad genética también se debe considerada.

7) La gran incidencia de paternidad múltiple así como el elevado número de padres en cada puesta encontrado en las poblaciones insulares vivíparas de la subespecie *Salamandra salamandra gallaica* indican a la especial relevancia de los beneficios derivados de un sistema poliándrico en una población aislada.

8) *Salamandra salamandra* constituye un sistema extremadamente diverso con un elevado potencial para abordar diversas cuestiones ecológicas y evolutivas acerca del origen de una nueva estrategia reproductora. La singular configuración que presenta el sistema pueríparo/larvíparo permite estudios comparativos entre y dentro de modos de reproducción, la descripción de patrones de variación y la identificación de los procesos subyacentes.

9) El modo de reproducción vivíparo y las diferencias en el desarrollo y en los rasgos de historia de vida que presenta respecto al modo de reproducción ancestral larvíparo tiene consecuencias desiguales en cada nivel estudiado. Mientras que las modificaciones en el desarrollo no tienen efectos sobre la diversidad morfológica intraespecífica de los adultos, el modo de reproducción vivíparo está altamente relacionado con otros rasgos reproductivos, como la presencia de paternidad múltiple.

10) Los resultados de esta tesis apoyan la necesidad de aproximaciones que integren información procedente de diversos niveles para obtener una compresión sólida y completa de las consecuencias derivadas de la evolución de nuevos modos de reproducción.