

**Life history strategies and sexual dimorphism of the  
Amphibia with special emphasis on the Urodela (Newts  
and Salamanders) and the Gymnophiona (Caecilians)**

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For my mother Bärbel Reinhard, née Lindenlaub (1957 - 2001)



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## SUMMARY

The life history strategies of amphibians are extremely diverse and they demonstrate a greater diversity of reproductive modes than other vertebrates. Many evolutionary transitions between oviparity and viviparity exist, whereas some amphibians exhibit shifts towards terrestriality next to the phylogenetically basal biphasic lifecycle, which includes an aquatic larva and is dependent from aquatic habitats. Reproductive modes such as direct development or viviparity circumvent the necessity of aquatic reproduction. This in turn promotes the development of parental care strategies, which maximize the fitness of the offspring. Viviparous species especially have a lower fecundity (due to reduced clutch and ovum sizes) in contrast to oviparous species, but they often redirect their investment by providing various modes of parental care. Parental care exists in different varieties throughout all amphibian orders and especially caecilian amphibians have evolved highly unusual strategies, like maternal dermatotrophy as a post-hatching or post-birth nutrient provision for altricial young.

Another important factor, which helps to understand amphibian mating systems, is the evolution of sexual size dimorphism (SSD). SSDs arise through the different reproductive roles of the sexes of the same species and are favoured by natural selection (fecundity theory), sexual selection (especially concerning male rivalry) or ecological and behavioural factors (*e.g.*, niche divergence, courtship behaviour, parental care behaviour). All those factors are more or less mutually dependent and need to be studied in connection.

This dissertation deals with the reproductive biology of newts and salamanders (Urodela) and with caecilian amphibians (Gymnophiona) and focuses on the evolution of their life history strategies, especially parental investment and SSD. The database for the studies is formed by direct investigation of unknown reproductive behaviours of selected species, which were bred in laboratory populations and completed by collection-based research using large samples of specimen. The results contribute to the general understanding of the evolution of amphibian reproduction and could clarify some long debated enigmas.

**First of all**, a review of amphibian reproduction is given, providing a general summary of the relevant reproductive morphologies of the three amphibian orders: Urodela, Anura and Gymnophiona.

**Secondly**, large sample series of two Mediterranean, terrestrial salamanders (*Salamandra algira* and *Mertensiella caucasica*), housed at natural history collections, were examined for sexual dimorphism and additionally controlled for age using skeletochronological methods. The sexes of both species were monomorphic for body size, but revealed a significant, male-biased limb dimorphism in connection with courtship and behavioural strategies. Arm size and shape differences remained dimorphic during ontogeny in both species. Compared to other salamandrids, the phylogenetically basal 'True salamanders' generally lack a body size dimorphism, but exhibit size and shape differences of other characters.

**Thirdly**, relations of female and male fecundity traits (*e.g.*, egg volume or number of testes lobes respectively) with age were analysed for *Salamandra algira*. The study provides baseline data for this species and it was shown that female and male fecundity traits are positively influenced by age. A delayed sexual maturity is suggested, as the examined population originates from a higher elevation region.

**Fourthly**, the driving factors underlying the manifestation of sexual dimorphism could be ideally demonstrated, whilst studying the SSD patterns of a phylogenetically derived newt in contrast to the basal terrestrial salamanders of the Salamandridae that were regarded previously. The fourth chapter deals with the study of SSD of the Marbled Newt *Triturus marmoratus*. More than the half of 27 traits analysed were sexually dimorphic and the general SSD is female-biased. An important, sexually dimorphic trait is the distance between the extremities, which indicates the space for eggs in females and is thus strongly female-biased. The set of sexually dimorphic characters found in *T. marmoratus* is likely a common pattern among the other oviparous, aquatic-breeding members of the entire European Newt complex.

**Fifthly**, sexual dimorphism is also present in the aquatic, paedomorphic salamander species *Siren intermedia* (Urodela: Sirenidae), but in contrast it is male-biased. This can be attributed to aggressive behaviour of the males during the breeding season and to a for urodeles rarely reported form of paternal care. This

study provides the first observation of the mating behaviour and provides further proof for the long-debated existence of external fertilization, as well as the first report of male parental care. The mode of fertilization and the possible existence of parental care has been an element of uncertainty for a long time. Resolving this question also strengthened the currently preferred phylogenetic hypotheses from a behavioural point of view.

**Sixthly**, caecilian amphibians are also known for their extraordinary diversity of parental care strategies and for a high percentage of viviparous species (approx. 30 %) in contrast to anuran and urodelan amphibians. They retain their eggs inside their oviducts and have evolved a specialised foetal dentition, which ensures intra-oviductal feeding that provides the young during a longer gestation with enough nutrients to be born precocial and independent of further care. The maternal investment of the aquatic, viviparous *Typhlonectes natans* is very high. The examined newborn young averaged a size of 37.2 % and a body mass of 34.4 % of their mothers. Newborn siblings also varied in birth mass and body volume, but how far intrauterine competition functions needs to be addressed in future studies.

**Finally**, the breeding of the terrestrial, viviparous caecilian *Geotrypetes seraphini* showed the opposite possibility of the developmental stage of newborn caecilians in contrast to *T. natans*. The young of *G. seraphini* are born altricial and are dependent on extended parental care. This includes the guarding of the young by the female and their nourishment via maternal dermatotrophy. This study demonstrates the developmental process after birth including dentitional metamorphosis. The foetal-like dentition of the young has been considered a preadaptation to intraoviductal feeding, when it was previously reported in oviparous species.

The eggs of *G. seraphini*, which are retained in the oviduct, are mesolecithal in comparison to oviparous species having macrolecithal eggs and other viviparous species having microlecithal eggs (such as *T. natans*). Thus, an intermediate novel reproductive mode indicating a transition between oviparity and viviparity for *G. seraphini* is described. Additionally, a communal nesting implying social behaviour has been recorded in the laboratory population.

**Generally**, the results presented in my dissertation provide important insights into amphibian life history strategies. Previously unrecognised and unreported behaviours

could be identified and the new data can contribute to future comparative studies of vertebrate reproduction and life history evolution. Because all aspects of reproduction are tightly interlinked and can influence each other, future studies should regard all life history traits possible, including age, habitat, behavioural strategies, the mode of reproduction and possible effects of parental care.

## ZUSAMMENFASSUNG

Amphibien zeigen bezüglich ihrer Lebensgeschichten (life history) eine Vielzahl verschiedener Strategien und die Diversität ihrer Reproduktionsmodi ist deutlich größer, als bei anderen Vertebraten. Es kommen sowohl Oviparie als auch Viviparie mit verschiedenen Zwischenstufen vor, wobei ein genereller Trend zur terrestrischen Lebensweise besteht, der die betreffenden Arten von aquatischen Habitaten unabhängig macht, wie es bei dem ursprünglichen, biphasischen Lebenszyklus mit aquatischer Larve nicht möglich ist. Dies begünstigt zum Beispiel Direktentwicklung oder Viviparie, was wiederum die Entwicklung von Brutpflegestrategien, die die Fitness der Nachkommenschaft erhöhen, fördert. Vivipare Arten haben durch reduzierte Eizahlen, beziehungsweise -größen, eine geringere Fekundität als die meisten oviparen Arten, können jedoch ihr Investment umleiten, zum Beispiel in extensive Brutpflege, wodurch sie dem Fekunditätsverlust entgegenwirken können. Brutpflegemechanismen gibt es in verschiedenen Formen in allen drei Amphibienordnungen, wobei die Schleichenlurche (Blindwühlen/Gymnophiona) sehr spezielle Strategien, wie Maternale Dermatophagie ("Hautfressen"), als Nährstoffversorgung für "Nesthocker"-Junge nach dem Schlupf oder der Geburt entwickelt haben.

Ein weiterer sehr wichtiger Faktor zum Verständnis der Paarungssysteme der Amphibien ist die Evolution von Sexualdimorphismen (SSD). SSDs entstehen durch die abweichenden Reproduktionsrollen der Geschlechter einer Art und werden durch Natürliche Selektion (Fekunditäts-Theorie), Sexuelle Selektion (vor allem bei Arten mit aggressiven männlichen Interaktionen, Rivalität, Territorialität) oder durch ökologische Faktoren sowie Verhaltensweisen (zum Beispiel unterschiedliche Ressourcennutzung bei der Nahrungssuche, Paarungsverhalten, Brutpflegeverhalten) beeinflusst. Alle genannten Faktoren sind mehr oder weniger stark miteinander verknüpft und müssen deshalb stets im Zusammenhang betrachtet werden.

Diese Dissertation beschäftigt sich mit der Reproduktionsbiologie von Schwanzlurchen (Urodela) und Blindwühlen (Gymnophiona) und zielt hierbei auf die Life-history-Strategien, vor allem auf verschiedene Formen von elterlichem Investment sowie die Entstehung von Sexualdimorphismen, ab. Ein Hauptbestandteil bildete die direkte Untersuchung unbekannter Reproduktionsverhaltensweisen

ausgesuchter Arten, welche in Laborpopulationen gehalten wurden sowie sammlungsbasierte Arbeit an größeren Serien von Museumsexemplaren. Die Ergebnisse tragen zum generellen Verständnis der Evolution der Reproduktion von Amphibien bei und konnten einige bereits seit langer Zeit in der Diskussion stehende Fragen klären.

**An erster Stelle** gibt ein Review einen Überblick über die Reproduktion der Amphibien aller rezenten Ordnungen (Schwanz-, Frosch- und Schleichenlurche).

**Zweitens** wurden größere Serien von Museumsexemplaren von zwei mediterranen, terrestrischen Salamanderarten (*Salamandra algira* und *Mertensiella caucasica*) auf das Vorhandensein von Sexualdimorphismen untersucht. Um die gewonnenen Daten in Relation zum individuellen Alter der Tiere zu testen, wurden skeletochronologische Methoden eingesetzt. Beide Arten zeigten keine generellen Größendimorphismen, jedoch signifikante, männlich-verlagerte Dimorphismen der Extremitäten in Zusammenhang mit ihrem Verhalten (Paarung, Rivalität). Diese Unterschiede bestehen bei beiden Arten auch während der Ontogenese. Durch den Vergleich mit anderen Salamandriden fällt ein generelles Fehlen von Größendimorphismen innerhalb der basalen Echten Salamander auf, wohingegen Dimorphismen anderer Körpermerkmale auftreten können.

**An dritter Stelle** wurde die bereits betrachtete Art *S. algira* weiterhin in Bezug auf Fekunditätsmerkmale sowohl der Weibchen als auch der Männchen im Altersverlauf untersucht (zum Beispiel Eivolumen, Anzahl der Hodenlappen). Diese Studie liefert grundlegende Daten für diese Art und es zeigte sich, dass die männliche und weibliche Fekundität positiv mit dem Alter korrelieren. Es erscheinen außerdem Hinweise auf einen späteren Eintritt in die Geschlechtsreife, was auf den Fundort der Tiere zurückgeführt werden kann, da sich dieser in höher gelegenen Regionen befindet.

**Im vierten Kapitel** verändert sich die Lebensweise der untersuchten Arten von terrestrisch zu aquatisch, wodurch sich sehr gut zeigen lässt, welche zusätzlichen Faktoren eine aquatische, phylogenetisch abgeleitete Art in der Ausprägung ihres Sexualdimorphismus beeinflusst, im Gegensatz zu den bisher betrachteten terrestrischen, phylogenetisch basalen Salamandern. Zur Verfügung stand eine größere Serie des Molches *Triturus marmoratus* aus einer naturhistorischen

Sammlung. Mehr als die Hälfte der 27 untersuchten Charaktere waren sexuell dimorph und der generelle Größendimorphismus verschob sich zu größeren Weibchen. Ein sehr wichtiges dimorphes Merkmal ist der Abstand zwischen den Extremitäten, der direkt für den möglichen Platz für Eier steht und somit in seiner Größe stark weiblich-verlagert ist. Es wird angenommen, dass dieses Merkmal innerhalb des ganzen Europäischen Molch-Komplexes sexuell dimorph ist.

**Desweiteren** zeigen sich auch Sexualdimorphismen in der aquatischen, pädomorphen Salamanderart *Siren intermedia* aus der Familie der Sirenidae, jedoch diesmal mit starker männlicher Ausprägung. Der Grund hierfür besteht im aggressiven Verhalten der Männchen während der Paarungszeit und in einer für Urodelen nur sehr selten berichteten Form der Brutpflege. Diese Studie liefert die erste Beobachtung des Paarungsverhaltens und weitere Beweise für die lang debattierte Existenz von externer Befruchtung sowie den Erstbericht von männlicher Brutpflege. Die Form der Befruchtung und die Möglichkeit der Existenz von Brutpflege dieser Art stellten lange Zeit einen Unsicherheitsfaktor dar. Die Auflösung dieses Rätsels stärkt die aktuelle phylogenetische Hypothese der Verwandtschaftsbeziehungen der Schwanzlurche aus einer ethologischen Sichtweise.

**Sechstens** sind Schleichenlurche für ihre außerordentliche Diversität der Brutpflegestrategien bekannt und weisen ebenso eine hohe Anzahl viviparer Arten im Vergleich zu Urodelen und Anuren auf. Diese Arten halten ihre Eier innerhalb der Ovidukte zurück und entwickelten eine spezielle fetale Bezahnung, die eine Ernährung innerhalb des Ovidukts mittels Abschaben des Epithels möglich macht. Auf diese Weise erhalten die heranwachsenden Jungen genügend Nährstoffe, um größer und unabhängig von weiterer Pflege geboren zu werden. Das mütterliche Investment ist hierbei sehr hoch, was sich auch in den, in diesem Kapitel untersuchten, neugeborenen Jungtieren der aquatischen Schwimmwühle *Typhlonectes natans* zeigt, die eine durchschnittliche Größe von 37.2 % und ein durchschnittliches Gewicht von 34.4 % der Mutter erreichten. Die Neugeborenen zeigten ebenso eine erhöhte Variation ihres Geburtsgewichtes sowie ihres Körpervolumens. Wie weit dies jedoch auf intrauterine Konkurrenz zurückzuführen ist, bleibt weiterhin zu klären.



**Letztlich** zeigte die Zucht der terrestrischen, viviparen Gymnophione *Geotrypetes seraphini* das mögliche Gegenteil des Entwicklungsgrades bei der Geburt von Blindwühlen im Vergleich zu *T. natans*. Die Jungtiere von *G. seraphini* werden als "Nesthocker" geboren und sind von weiterer Pflege abhängig. Dies wird durch das Bewachen der Jungen vom Muttertier sowie durch Fütterung mittels Maternaler Dermatophagie bewerkstelligt. Diese Studie zeigt den Entwicklungsprozess nach der Geburt, die Zahnmetamorphose sowie die erhaltene fetale Bezahnung der Jungen, welche als nötige Vorstufe der intrauterinen Ernährung durch eine ähnliche fetale Bezahnung angesehen wurde, als diese bereits bei oviparen Arten entdeckt wurde. Im Zusammenhang mit der Entdeckung von mesolecithalen Eiern (ovipare Arten legen meist makrolecithale Eier, während vivipare Arten oft eine Reduktion zu mikrolecithalen Eiern erfahren haben) beschreibt *G. seraphini* einen neuen, bisher unbekannten Reproduktionsmodus innerhalb der Blindwühlen. Außerdem besteht eine Form von kommunalem Nisten durch ausgeprägtes soziales Verhalten.

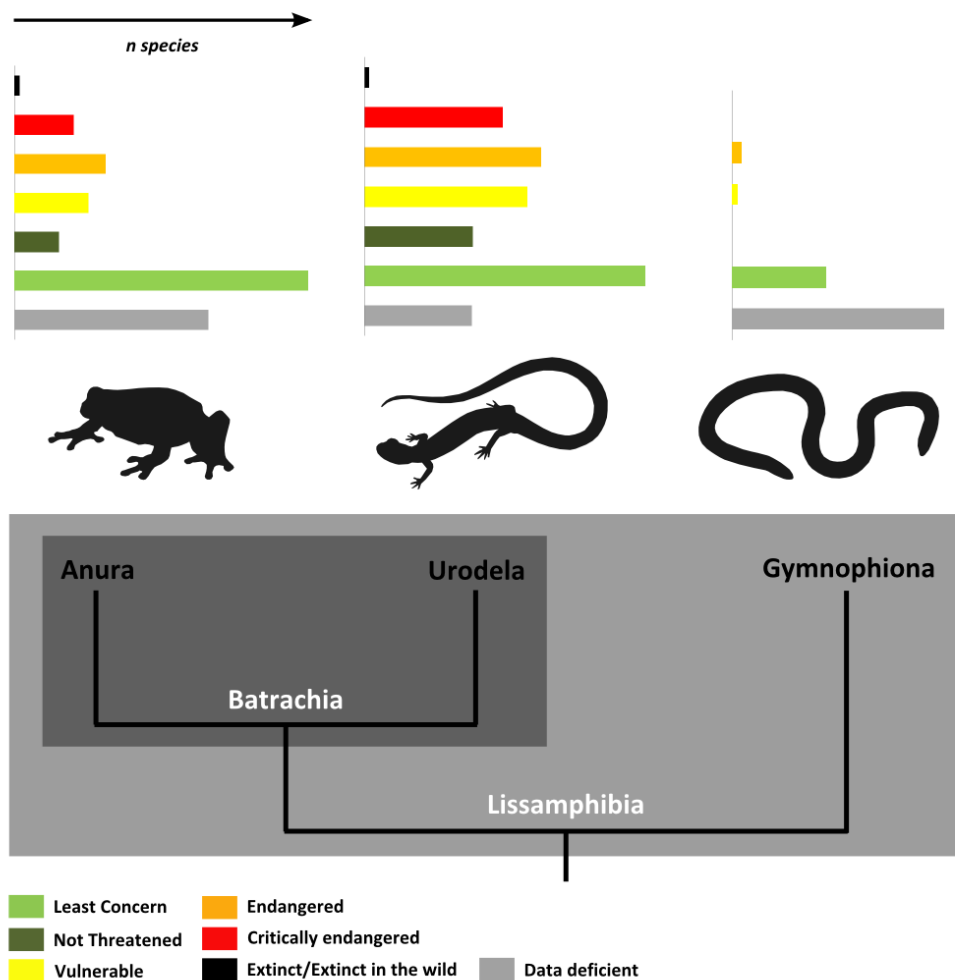
**Generell** stellen die Ergebnisse dieser Dissertation wichtige weitere Einblicke in die Lebensstrategien der Amphibien dar und bisher unbemerkte oder unbekannte Verhaltensweisen konnten beschrieben werden, wodurch ein Beitrag für zukünftige vergleichende Studien der Reproduktion von Vertebraten und Lebensstrategien geleistet werden konnte. Ein wichtiger Faktor, der sich zeigte, besteht darin, dass alle Aspekte der Reproduktion sehr eng miteinander verknüpft sind und sich gegenseitig beeinflussen, weshalb zukünftige Studien sowohl stets große Stichprobenzahlen als auch alle möglichen Lebensstrategie-Merkmale, wie Alter, Habitat, Verhalten, Reproduktionsmodus und Brutpflege, betrachten sollten.

## INTRODUCTION

Modern science proved amphibians to be a highly successful group of tetrapod vertebrates. They have a world-wide distribution (except in the polar regions and the open oceans) and more than 7200 species have been described - a number that continues to increase (Wells 2007, see AmphibiaWeb 2014 for an up-to-date species count). Amphibians inhabit nearly every ecozone, ranging from rainforests and deserts to the Siberian tundra (the salamanders *Salamandrella keyserlingii* and *S. schrenkii* are able to survive temperatures plunging as low as -35°C [Berman et al. 2010]) and they can be fully aquatic or even fully terrestrial with many intermediate stages possible (see Wells 2007).

Living amphibians are comprised of three orders - the Anura (frogs and toads, 6431 species), the Urodela (salamanders and newts, 665 species) and the Gymnophiona (caecilians, 200 species) (see AmphibiaWeb 2014 for an up-to-date species count and Figure 1 for phylogenetic relationships). In contrast to the ongoing description of new species, amphibians are declining at the very same moment, because they are suffering from habitat loss, alteration and fragmentation (Gower & Wilkinson 2005, Gallant et al. 2007), as well as from infectious diseases, *e.g.*, from chytridiomycosis caused by *Batrachochytrium dendrobatidis* (*e.g.*, Olson et al. 2013, Gower et al. 2013) or by *Batrachochytrium salamandrivorans* (Martel et al. 2013). About 41 % of amphibian species are recognized as threatened according to the IUCN Red List (IUCN 2013; see an overview of amphibian Red List categories and their pattern of distribution in Figure 1). Moreover, many species are listed as "data deficient", what applies especially to caecilians (Gower & Wilkinson 2005). These tropical, snake-like, limbless amphibians live a highly secretive soil-dwelling life, with only a few species being secondarily aquatic (Gower & Wilkinson 2005). They are an ancient group of lissamphibians and split off from their batrachian sister clade about 325 Mya (see Hedges et al. 2006 - [www.timetree.org](http://www.timetree.org)). Furthermore, caecilians are characterized by a unique morphology, including compact skulls, reduced eyes, an additional sensory organ (the tentacle) and the absence of both a shoulder- and a hip-girdle (*e.g.*, Duellman & Trueb 1994, Wells 2007). In comparison, salamanders and newts are rather lizard-shaped with well-developed limbs and tail. Both terrestrial and aquatic or semi-aquatic forms occurs, and they often have the typical

biphasic life-cycle that is characteristic for amphibians (e.g., Duellman & Trueb 1994, Wells 2007).



**Figure 1.** Phylogenetic relationships of the amphibian orders (after Zhang & Wake 2009) and distribution of IUCN Red List categories of extinction risks (after IUCN 2013).

### *Life history and reproductive strategies*

Amphibian life histories are extremely diverse and especially the diversity of their reproductive modes is greater than in other vertebrates (Duellman & Trueb 1994).

A female Cape river frog, *Amietia fuscigula*, lays up to 15.000 eggs (Wager 1965, Duellman & Trueb 1994). By contrast, a female Cascade torrent salamander *Rhyacotriton cascadae* lays only as few as eight eggs (Good & Wake 1992) and females of the caecilian *Boulengerula boulengeri* will even guard a clutch of only four eggs (A. Kupfer, pers. comm.). These three species vividly demonstrate that

organisms can differ in the amount of offspring they produce. But furthermore, life history evolution as a whole shapes how individuals develop, how much time they take to grow and to mature, how they reproduce, as well as their ultimate life-span (Fabian & Flatt 2012). The assessment of life history traits is important for understanding adaptation and how evolutionary forces shape reproduction and survival in certain environments to reach a maximum fitness within the given evolutionary constraints (Fabian & Flatt 2012). The most important constraints are so-called life history "trade-offs" (Stearns 1992, Fabian & Flatt 2012) that arise, if one fitness increasing change in one trait is linked to another change that decreases the fitness of the other trait (Stearns 1989, Stearns 1992). For example, egg volume is fitness-increasing, as large eggs lengthen the embryonic development inside the egg, which leads to large, developmentally advanced hatchlings (Nussbaum 1987). However, a larger egg volume is only possible, if another, originally fitness increasing trait - clutch size - is reduced.

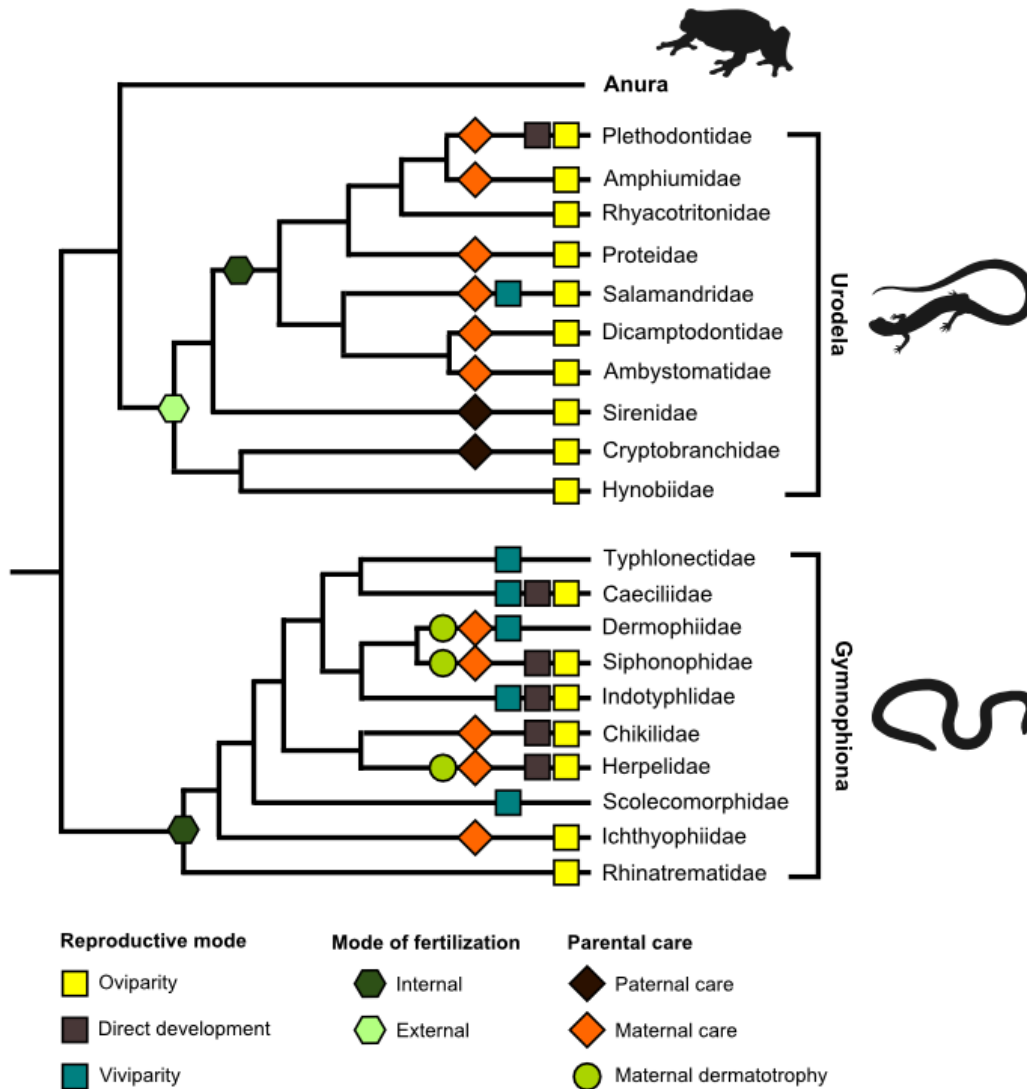
The mode of fertilisation is fundamental in the evolution of reproductive strategies (Townsend et al. 1981) and can be external (the ancestral condition), as well as internal within all three amphibian orders (Wells 2007). It must be emphasised that all caecilians have internal fertilization and direct sperm transfer (*e.g.*, Kupfer et al. 2006a, Kühnel et al. 2011), because they possess a unique reproductive morphology. Male caecilians have an intromittent organ (phallodeum), as the posterior part of the cloaca is evertible (Wake 1977a). In contrast to that, only a few anurans practice internal fertilization (*e.g.*, *Eleutherodactylus coqui* [Townsend et al. 1981], *Nectophrynoides malcolmi* [Wake 1980], *Ascaphus truei* [Noble & Putnam 1931]), but most of the urodeles do (except the most basal families Hynobiidae, Cryptobranchidae and Sirenidae) (see Sever 2003a, Wells 2007, Reinhard et al. 2013/chapter 5). Most newts and salamanders are known for another major innovation, because they have cloacal glands, which produce a spermatophore that contains the sperm and ensures a reliable sperm transfer from the male to the female (Sever 1994, Bruce 2003, Sever 2003b, Wells 2007; for phylogenetic distribution of reproductive modes see Figure 2). Internal fertilization additionally allows a decoupling of times for mating and oviposition or egg brooding (Bruce 2003).

Although the typical amphibian biphasic life cycle involves aquatic eggs and a lot of species reproduce in different types of water bodies, there is a clear trend towards

terrestriality and thus reproductive independence from aquatic habitats (Wells 2007). This is especially applicable for species with direct development, whose eggs are laid in moist spots on land, skip the aquatic larval stage and hatch into terrestrial juveniles (Stebbins & Cohen 1997, Bruce 2003) or viviparous species, that are independent from the water for reproduction through retention of developing embryos in the female oviduct until the end of gestation and delivery of fully terrestrial juveniles (Buckley 2012) (apart from some lecithotrophic viviparous species such as the Fire Salamander *Salamandra salamandra*, whose larvae hatch before or at birth and are dependent on further development in the water [e.g., Alcobendas et al. 2004, Thiesmeier 2004]). Only species with internal fertilization are capable of giving birth to living young (Wake 1993, Blackburn 1999) and viviparity (live bearing) has evolved once in salamanders (Wake 1993), twice among anurans (Wake and Dickie 1998) and several times within caecilians (see Gower et al. 2008).

### *Parental Care*

Most amphibian species abandon their eggs once they are laid and provide no further care for them, neither before nor after hatching (Stebbins & Cohen 1997). On the other hand, amphibians are also known to exhibit an extraordinary diversity of parental care strategies (see reviews in Duellman & Trueb 1994, Stebbins & Cohen 1997, Wells 2007). These caring behaviours can range from egg-guarding to transport of eggs or larvae and even feeding of the young (Forester 1979, Duellman & Trueb 1994, Stebbins & Cohen 1997, Wells 2007; for the phylogenetic distribution of the occurrence of parental care in salamanders and caecilians see Figure 2). Parental care has considerable benefits for the offspring, as it is highly fitness-increasing. However, the associated parents have to cope with reduced investment into further offspring or even reduced survival (Trivers 1972, Duellman & Trueb 1994, Gonzalez-Voyer & Kolm 2010).



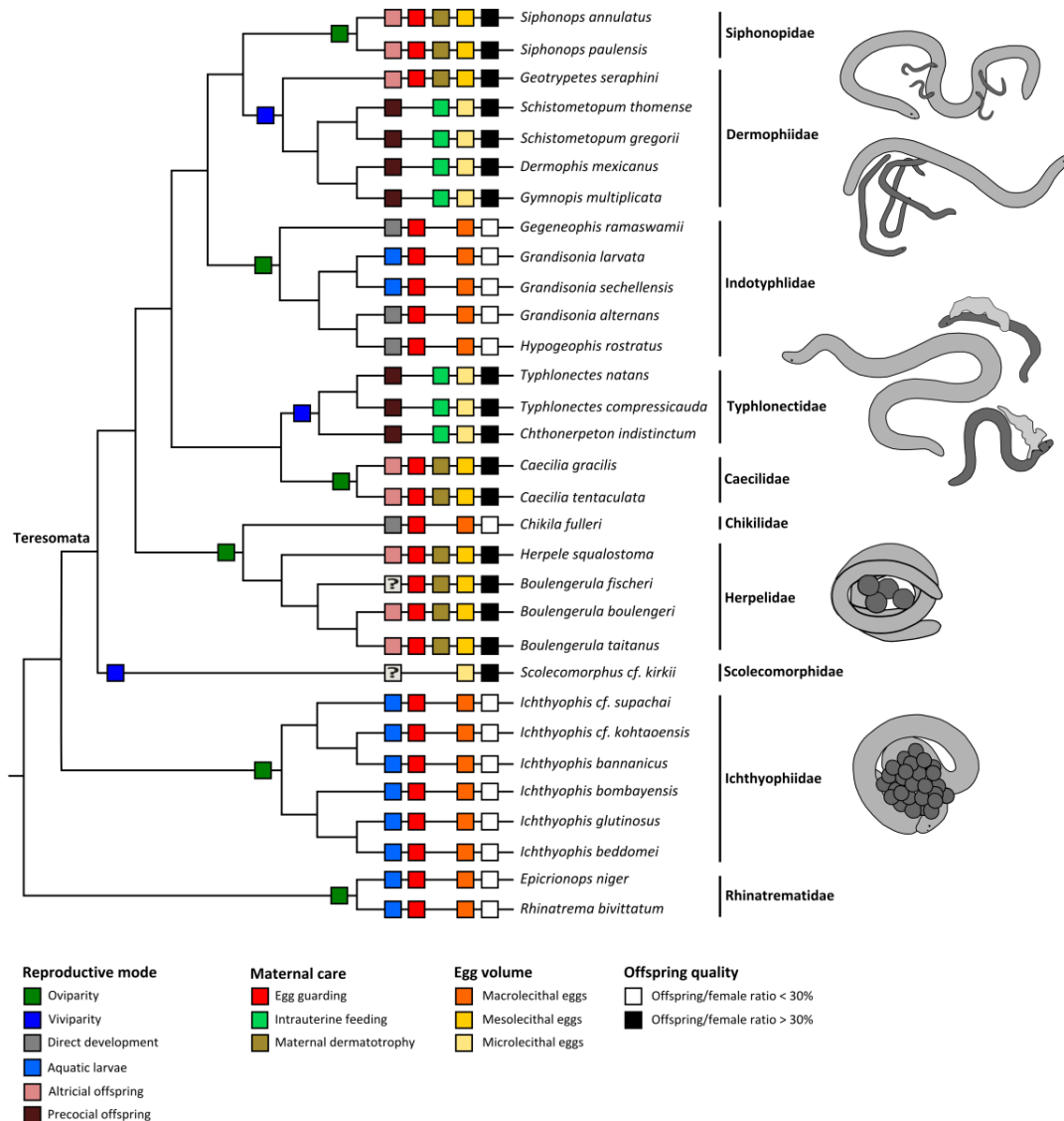
**Figure 2.** Phylogenetic relationships of urodeles (modified after Pyron & Wiens 2011, Reinhard et al. 2013) and caecilians (modified after Kamei et al. 2012) with distribution of reproductive modes and presence of parental care. Some reports of parental care are rather contradictory, *e.g.*, for the salamander family Hynobiidae, in which males are believed to guard the eggs, the behaviour is only of a very limited duration and rather resembles a scramble competition situation between the males sitting on the egg sacs. Here, fertilization is external and males must compete for a successful fertilization of eggs (*e.g.*, Hasumi 1994).

In most cases, female amphibians are associated with parental care, especially if fertilization is internal, whereas species with external fertilization are more likely to exhibit paternal care (Ridley 1978, Gross & Shine 1981).

One important and common caring behaviour that has evolved independently in all three amphibian orders is the attendance and guarding of egg clutches by one parent

(Duellman & Trueb 1994), which heightens egg and thus offspring survival through protection against predators, reduction of desiccation (in terrestrial nest sites), protection from pathogens (through contact with skin secretions of the attending parent or even removal of infected eggs), active oxygen supply by creation of water currents and so forth (*e.g.*, Stebbins & Cohen 1997, Nussbaum 2003). Guarding of eggs and hatchlings is the only form of parental care, which is exhibited by salamanders (Wells 2007). As an example, females of the salamander *Desmognathus ochrophaeus* guard their egg clutches and actively defend them against attacks from predatory intruders, purposefully remove infected eggs by oophagy and oscillate the eggs by gular pulsations (Forester 1979). Thus, Forester (1979) was able to show via field survivorship experiments that the mortality of the clutches was reduced from 100 % to 47 %, if the female was present and actively performing parental care. Although data on the reproductive biology of caecilians are more frugal than for urodeles and anurans, some species are known to guard their egg clutches in a similar way, *e.g.*, *Ichthyophis* ssp. (Bhatta 1999, Kupfer et al. 2004, Daniels 2005, Bei et al. 2012). The captive breeding of *Ichthyophis kohtaoensis* showed that the guarding and the care of the female was vital for the survival of the clutch, whilst another clutch, which was abandoned by the female, was quickly infected with fungus (Kramer et al. 2001). It is even assumed, that all oviparous caecilians practice egg-guarding (Gower et al. 2008) and thus enhance offspring survival. Species with direct development have particularly been reported to guard their eggs (*e.g.*, Brauer 1897, Kupfer et al. 2008, Wells 2007).

Viviparous caecilians, which retain developing embryos in their oviducts and give birth to live young, provide nutrients for their offspring after all their yolk is resorbed and they hatch inside the oviducts (Wake 1977b). Hypertrophied tissue of the maternal oviduct is scraped off with a specialised foetal dentition that differs from the adult teeth (Parker 1956, Wake 1977b, Kupfer et al. 2006b). The foetal dentition is resorbed before birth or shortly afterwards (Wake 1977b) in species with precocial young, like the Rio Cauca Caecilian *Typhlonectes natans*, which can bear eight young with total lengths of up to 43 % of the mothers total length (see chapter 6).



**Figure 3.** Reproductive biology of caecilians: portrayed are the modes of reproduction, maternal care and maternal investment with their phylogenetic distributions.

However, some oviparous and viviparous species have puzzled herpetologists for years, as they retain the foetal dentition for longer periods after hatching or birth (Parker 1956, Parker & Dunn 1964, Wake 1977b). Kupfer et al. (2006b) found the solution for this enigma and described a remarkably new form of parental care in caecilians. The females of the oviparous, direct-developing species *Boulengerula taitanus* provide nutrients for their freshly hatched, altricial and thus helpless young - their skin becomes thicker and rich in lipids as well as proteins and finally serves as food for their offspring, as it is peeled off by the use of the retained foetal dentition



(dermatophagy) (Kupfer et al. 2006b, Kupfer et al. 2008). By today, other oviparous, direct-developing species have been shown to perform the same remarkable parental care: the Ringed Caecilian *Siphonops annulatus* (Wilkinson et al. 2008), the recently described *Microcaecilia dermatophaga* (Wilkinson et al. 2013) and (through indirect evidence) *Herpele squalostoma* (Kouete et al. 2012), but it is assumed that skin-feeding might prove to be common in caecilians (Wilkinson et al. 2008, Wilkinson et al. 2013). Maternal dermatotrophy is seen as an important intermediate stage between oviparity (provisioning of yolk: lecithotrophy) as the ancestral condition and viviparity (foetal feeding in the oviduct with use of specialised dentition: matrotrophy) as the derived condition (Kupfer et al. 2006b; see Figure 3 for details on caecilian reproductive biology).

Within urodeles, only two species are known to care for their offspring after hatching - the French Cave Salamander *Hydromantes strinatii* (Oneto et al. 2010) and the Lesser Siren *Siren intermedia* (Reinhard et al. 2013, see chapter 5), but this behaviour only includes attendance and no nutrient supply.

### *Sexual size dimorphism*

All parameters of life history strategies are more or less tightly interrelated. Life history traits, such as clutch size or volume, can directly influence the phenotype of the individuals. For example, female and clutch size or volume are often positively correlated, which is why larger females can produce more or larger eggs (fecundity theory) (e.g., Shine 1979, Fairbairn 2007, Kupfer 2007, Reinhard 2010, Reinhard & Kupfer 2013). An increased selection pressure for fecundity drives the evolution of sexual dimorphism and entails that most amphibian females are larger than their conspecific males (Shine 1979, Kupfer 2007). Male-biased sexual dimorphism is a much rarer condition among amphibians and is predominantly promoted through sexual selection with the highest possible reproductive success as an objective, for example advantages in male-male combat or female choice. Both sexes of a species tend to develop a size that optimizes their fitness, what leads to diverging body sizes or even different shapes (Fairbairn 2007, Kupfer 2007, Reinhard 2010, Reinhard & Kupfer 2013). In addition to fecundity and sexual selection, ecology and behaviour are important influences, which can lead to the manifestation of a sexual dimorphism (Shine 1989, Fairbairn 2007).

Sexual dimorphism is an important factor, which helps to understand life history strategies, mating systems and underlying selection pressures and therefore has encouraged a lot of scientists to study the mechanisms causing it (see Fairbairn et al. 2007). Of particular interest for such studies are taxa that are characterised by a variable reproductive biology and behavioural strategies - what makes amphibians an ideal model group. Nevertheless, knowledge about sexual dimorphism in amphibians is still incomplete in comparison to other vertebrates (Kupfer 2007). Within urodeles, salamandrids exhibit a variety of different sexual dimorphism: female-biased size dimorphism can be found in 56 % of the species and male-biased size dimorphism in at least 15 % of the species. Additionally, phylogenetically young salamandrids are often characterised by further shape dimorphism or secondary sexual traits in connection with courtship and mating modes (Reinhard 2010, Reinhard & Kupfer 2013).

The knowledge of sexual dimorphism in caecilians is sparse and their mostly uniform morphology, without obvious secondary sexual traits, makes intersexual comparisons even harder. Only the comparison and measurement of large series of caecilians can bring insights and reveal cryptic dimorphic characters (see Kupfer 2009). In this way, some size and shape dimorphism was identified (cloacal dimorphism in *Ichthyophis* cf. *kohtaoensis* as well as in *Typhlonectes* and *Chthonerpeton* or head size dimorphism, e.g., in *Scolecomorphus uluguruensis*, *Boulengerula boulengeri*, *Dermophis mexicanus* and *Gegeneophis ramaswamii* [for a review of SSD in caecilians see Kupfer 2009]).

### *Study aims*

This study deals with several different amphibian species ranging from Urodela to Gymnophiona and shall contribute to the understanding of amphibian mating systems and life history strategies.

(1) One central aspect is the captive care and breeding of species (salamanders and caecilians), whose reproductive biologies or modes of parental care have been characterised by contradicting or confusing findings, whereby essential questions have remained unanswered and shall be resolved with this study (chapters 5 - 7). A

review of the amphibian reproductive morphology provides further access to the topics (see chapter 1).

(2) Another aspect is the examination of sexual dimorphism of large series of salamander species. The influence of age is considered to some extent, as the indeterminate growth of amphibians and sexual bimaturity might influence sexual dimorphism (chapters 2 - 4).

This doctoral dissertation is a cumulative thesis. The individual chapters, which are unified under the main topic of amphibian life history and sexual dimorphism, were individually prepared for publication in international peer-reviewed and ISI evaluated journals. As a consequence, certain aspects of the general introduction may appear again in the following chapters.

## OVERVIEW OF MANUSCRIPTS

### Chapter 1

Susanne Kühnel, **Sandy Reinhard**, Alexander Kupfer

#### **Evolutionary reproductive morphology of amphibians: an overview**

*Bonn zoological Bulletin* 57: 119-126

This chapter provides a brief review of the reproduction of all three major lineages, *i.e.*, anurans, urodeles and caecilians and their morphological adaptations. A special focus lies on internal fertilization and the cloaca in general.

SR wrote the urodele part and drew Figure 1. SK drafted other parts of the manuscript and AK gave additional input to all sections. All authors read and edited the final manuscript.

### Chapter 2

**Sandy Reinhard**, Sandra Renner, Alexander Kupfer

#### **Sexual dimorphism and age of Mediterranean salamanders**

*Zoology* 118: 19-26

Sexual dimorphism (SD) describes differences of size and shape between the sexes of the same species. SD is usually seen as a result of natural and sexual selection, as well as ecological factors shaping the phenotypes of males and females as an adaptation to diverging reproductive roles. Both species of Mediterranean salamanders (*Salamandra algira* and *Mertensiella caucasica*) were monomorphic in body size, but showed a significant male-biased dimorphism in limb length, likely related to the particular mating system. A review on SD indicated monomorphy of body size as a common lineage specific pattern among the 'True Salamander' clade, but also the presence of other sexually dimorphic traits.

SReinhard and SRenner visited natural history museum collections, where SReinhard carried out the morphometrics and dissections of the specimens for sexing purposes with the assistance of SRenner. Skeletochronology was performed by SRenner. SReinhard and SRenner carried out the statistical analysis. SReinhard drafted the

manuscript including the parts based on data from SRenner's diploma thesis. All authors conceived the study, interpreted the data and contributed to the final manuscript. SReinhard prepared all figures or redesigned the figures that were based on data from SRenner's diploma thesis. SReinhard and AK edited the revised version of the manuscript for final submission.

### **Chapter 3**

**Sandy Reinhard**, Sandra Renner, Alexander Kupfer

#### **Age and fecundity in *Salamandra algira* (Urodela: Salamandridae)**

Accepted for publication in *Salamandra* (MS. Ref. Nr.: 0692)

We examined fecundity patterns in the terrestrial salamander *Salamandra algira* (Salamandridae) and tested if age, body size and fecundity of both sexes are connected and how these reproductive traits interact. Positive correlations of body size, age and fecundity traits, *i.e.*, egg number and number of testes lobes were revealed. This chapter is complementary to chapter 2.

Specimen and statistical analysis were carried out by SReinhard and SRenner. SRenner carried out the skeletochronological analysis. SReinhard drafted the manuscript and prepared the figures. All authors designed the study, interpreted the data and approved the final manuscript. SReinhard and AK edited the revised version of the manuscript for final submission.

## Chapter 4

**Sandy Reinhard**, Alexander Kupfer

### **Sexual dimorphism in a French population of the Marbled Newt *Triturus marmoratus* (Urodela: Salamandridae)**

Accepted for publication in *Salamandra* (MS. Ref. Nr.: 0718)

In contrast to the previous two chapters that dealt with the sexual dimorphism and reproduction of terrestrial salamanders, this chapter focuses on an aquatic newt species.

A complex set of dimorphism was identified in Marbled newts. The adaptive significance is completely different, due to variant lifestyle regimes in aquatic newts and terrestrial salamanders, as summarised in the paper.

SR obtained all data from specimens housed in a natural history museum collection. SR did all measurements and statistical tests. SR wrote and designed the first manuscript draft, AK reviewed and approved the final version of the manuscript.

## Chapter 5

**Sandy Reinhard**, Sebastian Voitel, Alexander Kupfer

### **External fertilisation and paternal care in the paedomorphic salamander *Siren intermedia* Barnes, 1826 (Urodela: Sirenidae)**

*Zoologischer Anzeiger* 253: 1-5

Whether fertilisation in the paedomorphic salamander *Siren intermedia* is external or internal has been debated for decades, but the reproductive biology of sirenids also includes information crucial for resolving the phylogeny of urodeles. Herein, observations of external fertilisation and an unexpected case of paternal care in *S. intermedia* are reported and discussed in the light of phylogeny.

SR and SV bred *Siren* in two independent breeding groups facilitating rare observations of reproductive behaviour. Courtship sequences were documented and interpreted by SR. SR and SV both documented paternal care. SR drafted the original manuscript, AK gave further input and edited the revised manuscript together with SR. All authors approved the final version of the manuscript.

## Chapter 6

**Sandy Reinhard**, Alexander Kupfer

### **Mating and maternal investment of the viviparous caecilian amphibian *Typhlonectes natans* (Amphibia: Gymnophiona: Typhlonectidae)**

To be submitted to *Herpetology Notes* and *Amphibia-Reptilia/Journal of Herpetology*

We examined aspects of the reproductive biology of the aquatic viviparous caecilian *Typhlonectes natans*. Mating sequences were described. Litter sizes ranged from three to eight and the offspring reached sizes of up to 43 % of the maternal total length. Despite this very high investment, annual breeding was observed. Although fecundity is reduced in viviparous caecilians, as clutch size is decreased in comparison to many oviparous species, lower fecundity in viviparous taxa is compensated via uterotrophy leading to increased offspring quality.

SR collected long-term data on maternal investment from a breeding colony of *Typhlonectes natans* housed at the Institute of Systematic Zoology and Evolutionary Biology (University of Jena). SR carried out analyses and SR drafted the manuscript, including all figures. AK gave further input and edited the manuscript together with SR.

The manuscript will be split up into two publications: a brief description of breeding and courtship will be submitted as a short note, probably to the open access journal *Herpetology Notes*. The extensive part of the manuscript that deals with maternal investment will be combined with additional data on other viviparous caecilians and submitted to *Amphibia-Reptilia* or *Journal of Herpetology*.

**Chapter 7****Sandy Reinhard**, Felix Beckmann, Alexander Kupfer**Life history of the West African caecilian *Geotrypetes seraphini* (Amphibia: Gymnophiona: Dermophiidae)**To be submitted to the *Biological Journal of the Linnean Society*

SR collected long-term data from a breeding colony of the terrestrial *Geotrypetes seraphini* housed at the Institute of Systematic Zoology and Evolutionary Biology (University of Jena). SR carried out all morphometric analyses and statistical tests. SR produced the original  $\mu$ CT-images of caecilian skulls at the DESY Hamburg, where FB assisted in creating SR $\mu$ CT scans. FB rendered initial 3D-reconstructions from the scanned image stacks. SR further processed the SR $\mu$ CT scans. Some scanning electron microscopy images were produced by a student assistant supervised by SR and AK. SR drafted the manuscript including all figures and AK co-drafted and -edited the manuscript.

The altriciality of newborn young in connection with viviparity and mesolecithal eggs in *G. seraphini* characterises a previously unknown mode of caecilian reproduction. Interestingly, we could also record communal nesting during the breeding period, likely indicating complex social interactions. The breeding colony of the West African caecilian *Geotrypetes seraphini* provided the key source for studying the reproductive biology of these otherwise inaccessible, fossorial vertebrates.

I have read the authors' contributions stated above and confirm their correctness.

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Prof. Dr. Lennart Olsson (supervisor)



## **CHAPTER 1**

### **Evolutionary reproductive morphology of amphibians: an overview**

Susanne Kühnel, Sandy Reinhard, Alexander Kupfer

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## Evolutionary reproductive morphology of amphibians: an overview

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**Abstract.** Reproduction is a crucial trait in the life history of any organism, and vertebrates, whether aquatic or terrestrial, have evolved an extraordinary diversity of reproductive strategies and morphologies. Among tetrapods, the diversity of reproductive modes is exceptionally high in amphibians, who also show multiple trends towards terrestrialsation and internal fertilisation. Herein we give a brief overview of the diversity of amphibian reproductive morphology, with a special emphasis on the cloaca, for all three major lineages, i.e., anurans, urodeles and caecilians.

**Key words.** Reproduction, genital morphology, Amphibia.

### INTRODUCTION

Reproduction is a crucial trait in the life history of any organism and scientists have been intrigued and challenged by this event, and the structures associated with it, ever since the days of Darwin (1871). Both aquatic and terrestrial vertebrates have evolved an extraordinary diversity of reproductive strategies and morphologies, including varieties of oviparity and viviparity (Meisenheimer 1921; Lombardi 1998). Among tetrapods, the diversity of reproductive modes is exceptionally high in amphibians. In this group, we also see multiple trends towards terrestrialsation and internal fertilisation (e.g. Haddad & Prado 2005). Reproductive modes such as viviparity have evolved independently in all three lineages of modern amphibians (e.g. Noble 1927; Wake & Dickie 1998; Wells 2007). Herein we interpret a reproductive mode as a combination of several reproductive traits, including oviposition site, clutch characteristics, stage and size of hatchling, and type of parental care (sensu Salthe 1969).

Internal fertilization is a precondition for viviparity (Wourms & Lombardi 1992; Böhme & Ziegler 2008). It is associated with different strategies of sperm transfer, which have evolved within all three amphibian orders, ranging from cloacal apposition in anurans to a true copula via a male intromittent organ in caecilians (Sever et al. 2001; Kupfer et al. 2006). Like most other tetrapods, amphibians have a cloaca, a chamber that receives products from the kidneys, the intestine and the gonads, and opens to the outside through a cloacal opening or vent (Kardong 2006).

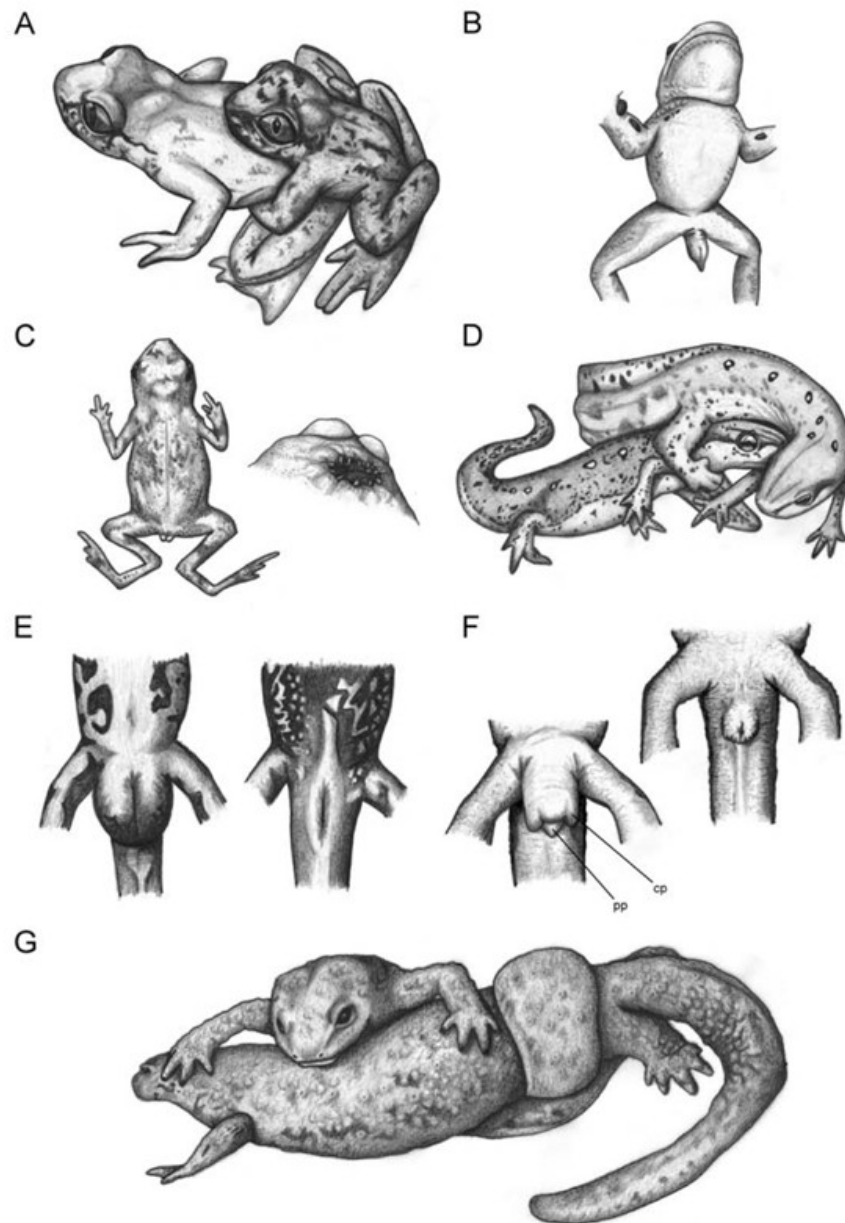
Below we review the diversity of amphibian cloacal morphologies involved in ensuring a secure direct sperm transfer and internal fertilization among anurans, urodeles and caecilians.

### REPRODUCTIVE MORPHOLOGY OF AMPHIBIANS

#### Anura

The majority of anurans, currently including almost 6000 species (AmphibiaWeb 2010), practice external fertilization, and thus have no special male cloacal arrangements facilitating direct sperm transfer (recently summarised by Wells 2007). During copulation, males grasp females firmly with their forearms (termed amplexus). In most cases, sperm is directly released on the eggs protruding from the female cloaca, but in some cases fertilisation takes place without amplexus (e.g. Crump 1974; Kunte 2004). Internal fertilisation is rare among anurans, and mostly connected to viviparity or other complex parental care mechanisms (e.g. Wake 1993; Beck 1998).

Exceptionally, the phylogenetically basal tailed frogs *Ascaphus truei* and *A. montanus* are the only anurans known to have evolved a true intromittent organ in males (see Figs 1A–B). During courtship they practise a combination of amplexus and copulation called “copulexus” (see Sever et al. 2001; Stephenson & Verrell 2003). The so-called



**Fig. 1.** Reproductive morphology of anurans and salamanders. (A) Inguinal amplexus ("copulexus") of *Ascaphus truei*. (B) male *Ascaphus truei*. The "tail", a cloacal extension, can be inserted into the cloaca of the female during amplexus, ventrolateral view. (C) male *Mertensophryne micranotis* (Anura: Bufonidae), left, dorsal view and its cloaca, right, caudal view (after Grandison 1980). (D) cephalic amplexus of *Notophthalmus viridescens* (Urodela: Salamandridae). The male grasps the females's neck whilst fanning pheromones towards her nostrils. (E) cloacal region of lentic breeding *Cynops pyrrhogaster* (Urodela: Salamandridae). The male's cloaca (left) is heavily swollen compared to that of the female (right). (F) cloacal region of lotic breeding *Euproctus montanus* (Urodela: Salamandridae, after Brizzi et al. 1995). Males (left) possess a cloacal protuberance (cp) which bears a protusible pseudopenis (pp), whereas the female cloaca is slightly conical shaped and its opening is located ventrally (right). (G) amplexing pair of *Calotriton arnoldi* (Urodela: Salamandridae). The male grasps the female's trunk with his tail.

“tail” resembles the posteriorly extended cloaca, proximally attended by Nobelian rods and strengthened by vascularized tissue that is engorged with blood during copulation. This gives the ventral cloacal surface a pinkish colour (Noble & Putnam 1931; Duellman & Trueb 1994). To insert the posterior pointing “tail” into the female vent, the male first flexes his pelvis at a right angle to the vertebral column. Contraction of the paired *Musculi compressores cloacae* (Duellman & Trueb 1994) bend the intromittent organ ventrally, with the male vent pointing anteriorly (Slater 1931). Keratinised spines are present within the male cloacal orifice, but whether they function to enhance the attachment of the male to the female remains unclear (Noble & Putnam 1931; Metter 1964).

Additionally, internal fertilisation including an amplexus and cloacal apposition occurs in a few anurans, such as several species of viviparous African dwarf toads *Nectophrynoides* (Wake 1980; Wake & Dickie 1998) and *Nimbaphrynoides* (Sandberger et al. 2010), and in two species of Caribbean *Eleutherodactylus*, the viviparous *E. jasperi* (Dewry & Kirkland 1976; Wake 1978) and the oviparous-direct developing *E. coqui* (Townsend et al. 1981). Mating has only been observed in couples of *E. coqui* in a special amplexic position called reverse hind leg clasp, that is initiated by the female (Townsend & Stewart 1986). Males do not clasp, and the female rests her hind legs on top of the male’s legs. This behaviour might be correlated with terrestrial reproduction and internal fertilization. It is also thought to be present in the viviparous *E. jasperi* but has not yet been observed (Wake 1978). Within the African Bufonidae, all species of *Nectophrynoides* (and also *Altiphrynoides malcolmi* and *Nimbaphrynoides occidentalis*, former members of *Nectophrynoides*, see Frost et al. 2006) practice internal fertilization. *Altiphrynoides* and *Nimbaphrynoides* both show a dimorphism of the male and female vent, and an inguinal amplexus in a unique belly-to-belly position has been reported as well (Grandison 1978).

As in the internally fertilising *Ascaphus* spp., males of the East African toad *Mertensophryne micranotis* (Bufonidae) exhibit modifications of the cloacal region (Duellman & Trueb 1994). They have small conical spines around the rim of the vent and at the entrance to the cloacal tube restricted to the ridges of the puckered vent (Grandison 1980, see also Fig 1C). Males and females keep a very tight cloacal contact during mating. Although the cloacal spines play a role to ensure a close apposition of the vents, to secure internal fertilisation, there is no evidence for a direct interlocking mechanism in the furrows of the female vent (Grandison & Ashe 1983).

Another potential record of internal fertilisation is provided for the neotropical Pumpkin Toadlet *Brachycephalus*

*ephippium* (Pombal et al. 1994). During mating, males shift from an inguinal to an axillary amplexus to optimally allow positioning of the vents, and thus maximize fertilization of the relatively large eggs (5.1 to 5.3 mm). A further record of viviparity in fanged frogs (*Limnonectes* spec.) from Sulawesi probably also involves internal fertilisation (Emerson 2001).

It can be hypothesised that (1) many terrestrially breeding species with large direct-developing clutches are internal fertilizers and (2) if additional viviparous species are encountered they will also show internal fertilisation. Thus, internal fertilisation and viviparity in anurans might be more widespread than currently recognized (see also Wake 1978).

Data on the reproductive biology, including the mating behaviour, of many species is still lacking (Duellman & Trueb, 1994; Wells 2007). Life history data from around 23 % of the currently known species is missing, as listed in the data deficient category of the IUCN (Stuart et al. 2008).

## Urodela

The majority of the 590 species of urodeles exhibit internal fertilization, whilst only males of the basal families Hynobiidae, Cryptobranchidae, and presumably Sirenidae, fertilise eggs externally (summarised in Duellmann & Trueb 1994; Wells 2007). The complex and elaborate courtship behavior of most salamanders includes the deposition of a spermatophore by the male, which is subsequently received by the female. A true intromittent organ in salamanders is lacking, although direct sperm transfer can be found in one species – the Corsican brook newt *Euproctus montanus*, a lotic breeding endemic of the island of Corsica. The cloaca of the male brook newt resembles a conical protuberance (Fig 1F). The cloacal chamber hosts a “pseudopenis”, a broad conspicuous papilla, which can be evaginated during mating (Brizzi et al. 1995; Carranza & Amat 2005). The male grasps the female during amplexus, holding her tail with his jaws and wrapping his tail around her trunk, whilst his backward projecting cloaca is positioned close to that of the female. A deep groove along the ventral surface of the pseudopenis, which is aligned with the cloacal tube, guarantees a guided, unidirectional flux of cloacal products. Thus, sperm mixed with secretory products is transferred directly into the female’s cloaca. The Salamandroidea that practice internal fertilization possess a distinct set of male cloacal glands necessary for spermatophore production (Sever 2002). The glands are hormonally controlled and hypertrophied during the breeding season, often causing a sexual dimorphism in cloacal shape. However, in *Eupro-*



*tus montanus*, cloacal glands are reduced or partly lacking (Brizzi et al. 1995; Sever 2002). Males of six salamandrid genera possess a so-called “pseudopenis”, a projection of the dorsal roof which nearly fills the entire anterior chamber of the cloaca. It is involved in shaping and expulsion of the spermatophore (Halliday 1998), but cannot be everted as in the Corsican brook newt (Brizzi et al. 1995; Carranza & Amat 2005).

Sexes of most species, regardless of the mode of fertilisation, show a sexual dimorphism in cloacal shape (Figs 1E–F). Usually, the male cloaca appears larger and more swollen than the female one. This is caused by the activity of the glands mentioned above (see also Sever 2002). Species that breed in the water and show elaborate courtship dances or walks, such as some members of the family Salamandridae, produce courtship pheromones, which are fanned towards the female using the tail. Salamanders that mate terrestrially also use courtship pheromones secreted from specialised glands to attract females. Pheromone-producing cloacal glands are therefore highly influenced by sexual selection (e.g. Sever 2002; Houck et al. 2008). Usually, female cloacae are less prominent, but they may also possess up to three types of cloacal glands in Salamandroidea, mainly accounting for sperm storage (spermathecae), a unique feature among vertebrates (Sever 1994). Females may retain and mix viable spermatozoa from multiple matings in the spermathecae for longer periods (e.g. Steinfartz et al. 2006). Female *Eurycea* fertilise eggs from stored sperm up to eight months after insemination, female *Notophthalmus viridescens* effectively store sperm for up to six months, and female *Salamandra salamandra* are reported to store sperm for up to two years (Sever et al. 1996; Stebbins & Cohen 1997; Sever & Brizzi 1998).

Additionally, the shape of female cloacae can be adapted to a specific substrate for oviposition and type of water body. Females of stream-breeding species, such as *Calotriton asper*, sometimes have a conically shaped cloaca for egg deposition and safe attachment between stones and in crevices (e.g. Carranza & Amat 2005).

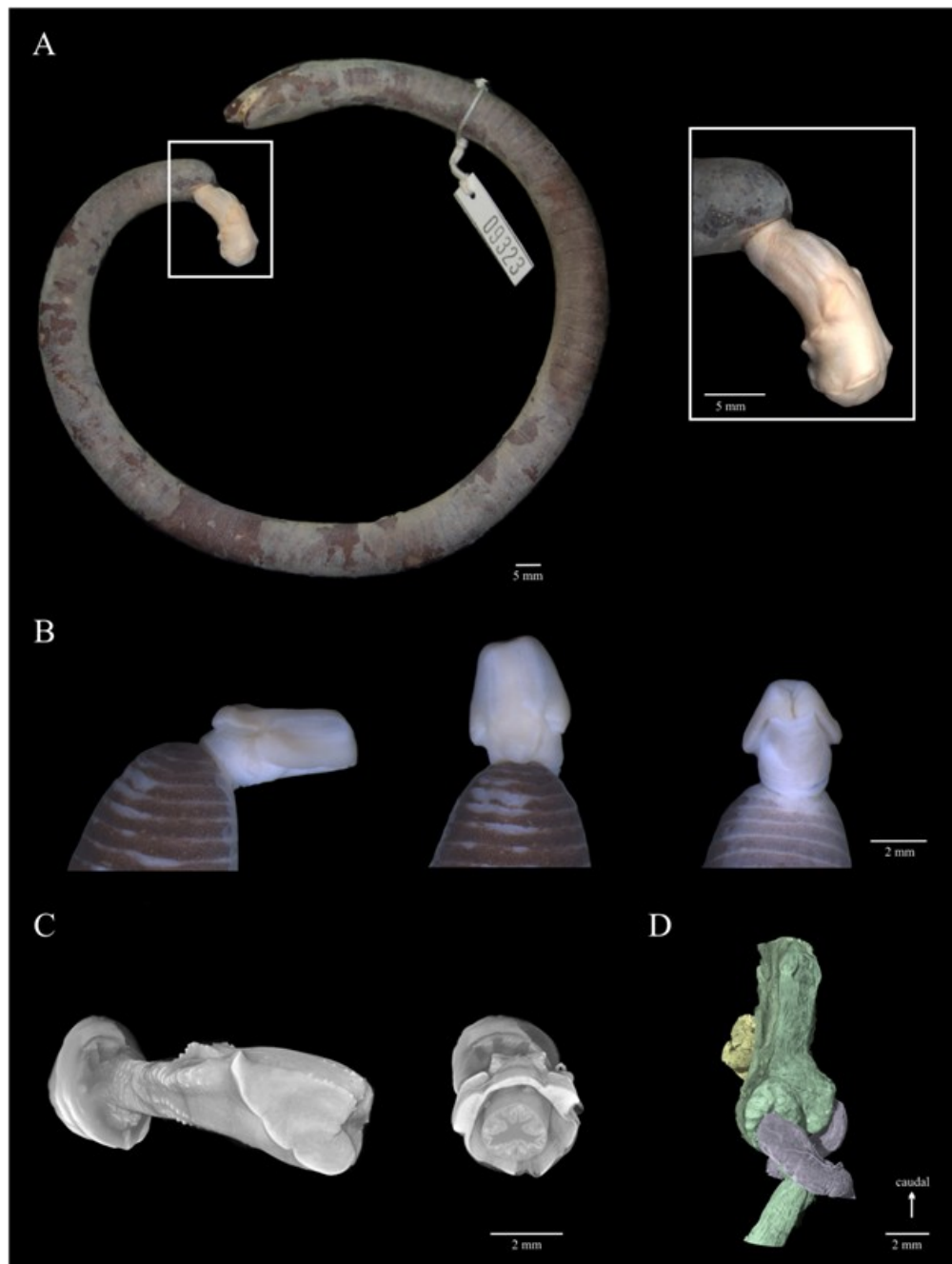
Lotic breeders such as *Calotriton* ssp. often engage in an amplexus directly transferring the spermatophore into the female cloaca (Fig 1G). It ensures direct and rapid spermatophore uptake, and thus reduces energy wasting, which can hardly be avoided during aquatic breeding where the male and the female often have no physical contact. Breeding patterns including an amplexus are common in salamandrids. Multiple ways of female capture are known, such as the cephalic capture of *Notophthalmus* ssp. (see Fig 1D), the dorsal capture of *Taricha*, or the ventral capture performed by fire salamanders (Stebbins & Cohen 1997). The mating amplexus may last up to several hours,

depending on the species. Salamanders of the family Ambystomatidae mate in the water, and the males guide females to spermatophore-uptake using a “tail-nudging-walk”, except in *Ambystoma gracile*, *A. laterale*, *A. jeffersonianum* and *A. macrodactylum*, which capture females in an amplexus (Duellmann & Trueb 1994; Verrell & Krenz 1998). In contrast, some plethodontids perform a unique “tail-straddling-walk” behaviour (e.g. Arnold 1977).

### Gymnophiona

In contrast to all salamanders (with the exception of *Euproctus montanus*) and frogs (with the exception of *Ascapus* ssp.), the male caecilian cloaca is evertible through the vent and operates as an intromittent organ or phallus, a unique structure among tetrapods (Tonutti 1931, see also Fig 2A). Presumably all ca. 190 caecilian species (oviparous and viviparous) practice internal fertilisation with the help of the phallodeum (Tonutti 1931; Wake 1972; Gower & Wilkinson 2002), which is inserted into the female vent during copulation (e.g. Kupfer et al. 2006a).

The caecilian vent is simply surrounded by several folds, which are variably arranged among the groups and display sexual dimorphism in some species, such as members of the Typhlonectidae (e.g. Taylor 1968; Kupfer 2007). In contrast, the cloaca is highly complex and diverse. The male caecilian cloaca is an elongated tube divided into two distinct chambers. The cranial urodeum is rather simply built, bearing longitudinal ridges, and connects to the intestine and the urogenital ducts, which enter after performing a U-bend (Sawaya 1942; Gower & Wilkinson 2002). An extraordinary feature is the presence of Müllerian ducts, which become glandular during reproductive activity, and secrete a fluid containing lipids and sugars necessary for sperm motility (e.g. Wake 1981). The caudal phallodeum is more broadly built and the inner structure is very different. The ridges are arranged in a more complex pattern (running transversely). In adults it is often equipped with tuberosities or crests, which give the phallodeum a characteristic morphology and gives rise to an extraordinary variation in shape (Wiedersheim 1879; Tonutti 1931, 1933; Wake 1972; Exbrayat 1991; Gower & Wilkinson 2002, see also Fig 2), that is important for caecilian systematics (Müller et al. 2005). East-African scolecomorphid caecilians even have cartilaginous spicules (Wake 1998). In many species, pouchy dorsolateral appendixes – so called “blind sacs” – extend anterior to the phallodeum. During eversion, the luminal surface of the phallodeum represents the outer structure of the phallus, with the urodeum lying in-between (see Tonutti 1931; Gower & Wilkinson 2002, see also Fig. C right).



**Fig. 2.** Genital morphology of caecilian amphibians. (A) male *Chthonerpeton indistinctum* (Gymnophiona: Typhlonectidae) showing an everted phallus, MHNM 09323, right – detail. (B) *Geotrypetes seraphini* (Gymnophiona: Caeciliidae), lateral (left), dorsal (central) and ventral (right) view of the everted phallus, AK 01149. (C) *Typhlonectes natans* (Gymnophiona: Typhlonectidae), SRμCT-Scan of the everted phallus. Right – virtual clipping, frontal view. (D) SRμCT-Scan of female cloaca (*Ichthyophis cf. kohtaoensis*). Dorsolateral view, virtual cut of cloacal sheath, cranial part and blind sacs, green - cloaca, violet – oviducts, yellow – bladder. Abbreviations: MHNM = Museo Nacional de Historia Natural Montevideo Uruguay, AK = Alexander Kupfer collection.



To retract the cloaca within the body after copulation, caecilians possess a specific muscle (musculus retractor cloacae), which is also found in some females (Wilkinson 1990).

The female cloaca of caecilians has received little attention (e.g. *Hypogeophis rostratus* Tonutti 1931; *Typhlonectes compressicauda*, Exbrayat 2006), the only dedicated morphological study was presented by Wake (1972), proposing a functional association between the specific male and female morphologies. The female cloaca is supposed to be non-eversible (Wilkinson 1990), therefore displaying a different morphology. Generally it is shorter than in males, and the urogenital ducts lack a copulatory loop (see Fig. 2D). There is also evidence for a bisection of the female cloaca (Exbrayat 1991; Kühnel et al. submitted). The cranial chamber is homologous to the male urodeum. The caudal chamber is marked by a different arrangement of longitudinal cloacal folds mostly lacking tuberosities, and therefore easily recognised. Nothing is at present known about how far the male phallus inserts into the female cloaca, and if special structures corresponding to the male ornamentation are present, helping in fixation during copulation.

Copulations in caecilians have rarely been observed. Data are available for two aquatic/semiaquatic species, the typhlonectids *Typhlonectes compressicauda* and *Chthonerpeton indistinctum*. Pairs of *C. indistinctum* copulated for between 30 minutes and 5 hours (Barrio 1969) and those of *T. compressicauda* for between 75 minutes and 3 hours (Murphy et al. 1977; Billo et al. 1985). Observations on copulations in terrestrial caecilians have, to the best of our knowledge, only been presented for the Indian ichthyophiid *Ichthyophis beddomei* (Bhatta 1999) and *Ichthyophis* cf. *kohtaoensis* (Kupfer et al. 2006a). Bhatta reports on a copulation lasting for about 40 or 45 minutes, an observation fitting well with the duration time of about 45 minutes that was observed in *Ichthyophis* cf. *kohtaoensis* (Kupfer et al. 2006a).

Caecilians show a remarkable diversity of reproductive modes associated with parental care (e.g. Wake 1977; Himstedt 1996; Wilkinson & Nussbaum 1998). Oviparous caecilians guarding egg clutches in terrestrial chambers (e.g. Sarasin & Sarasin 1887–1890) either have the presumed ancestral amphibian life cycle with aquatic larvae, or show direct development of juveniles with no aquatic larval stage (e.g. Brauer 1897). Females of viviparous species retain fertilised eggs. Embryogenesis is completed within the oviducts, and after hatching the foetuses feed mainly intrauterinely on the hypertrophied oviductal lining (e.g. Parker 1956; Welsch et al. 1977). After a long gestation period, the females give birth to fully metamorphosed, precocial young with the adult-type morphology

(e.g. Billo et al. 1985; Exbrayat & Delsol 1985). Recently, a novel form of parental investment, maternal dermatotrophy, a.k.a. skin feeding, where altricial young feed externally on the mother's hyperthrophied skin, has been described (Kupfer et al. 2006b; Wilkinson et al. 2008).

## SUMMARY AND PERSPECTIVES

In addition to their remarkable diversity of reproductive modes, amphibians also show large variation in their reproductive morphology. Many morphological peculiarities are related to the evolution of internal fertilisation, and ultimately to viviparity. In relation to fertilisation and sperm transfer, different strategies have evolved within the three amphibian orders, ranging from cloacal apposition in anurans to a true copula via a highly complex male intromittent organ in caecilians. Amphibians offer a prime system for comparative studies of evolutionary reproductive biology. Research on the reproductive or genital morphology should include modern methodology, such as 3D reconstruction and soft tissue synchrotron radiation based X-ray microtomography (SRμCT, see Fig. 2 C–D). Because amphibian diversity is steadily increasing (although at the same time many species are declining or even going extinct) we envisage that many more unexpected reproductive strategies and morphologies remain to be discovered.

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## CHAPTER 2

### **Sexual dimorphism and age of Mediterranean salamanders**

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## ABSTRACT

Sexual size dimorphism (SSD) is widespread among ectothermic vertebrates. Particularly, the 'True Salamanders and Newts' (Amphibia: Urodela: Salamandridae) are suitable for studying intra- and interspecific patterns of SSD, because they exhibit diverse reproductive modes including oviparity and viviparity combined with variable morphologies and body shapes. However, only a few studies have quantitatively addressed sexual dimorphism of their overall bauplan.

We analysed SSD for two Mediterranean species of the 'True Salamander' clade (*Salamandra algira* and *Mertensiella caucasica*) and equilibrated the morphometric approach to individual age by using skeletochronology. In species that have a short breeding season and live at high altitudes, such as Mediterranean amphibians, the fecundity-advantage hypothesis should predict female-biased SSD in order to maximise the reproductive success.

Both species were monomorphic in body size (snout-vent-length) but showed a significant male-biased dimorphism in arm and leg length likely related to the particular mating system, which involves an amplexus during spermatophore transfer. Arm length appeared sexually dimorphic during ontogeny both in *S. algira* and *M. caucasica*. A review on SSD indicated monomorphy of body size as a common lineage specific pattern among the 'True Salamander' clade, but also the presence of other traits such as sexually dimorphic limb proportions.

**Keywords:** Amphibia, salamanders, life history, sexual dimorphism, skeletochronology.

## INTRODUCTION

Sexual size dimorphism (SSD) accounts for differences in body size between males and females. In his seminal work on sexual selection Darwin (1871) recognized this phenomenon as common within the animal kingdom and summarised possible proximate causes of SSD. SSD can be considered an adaptation to distinct reproductive roles of males and females, resulting in a difference in adult body size between the two sexes (Fairbairn 1997). As a general pattern male-biased dimorphism is likely to occur in endothermic vertebrates (Fairbairn 1997, Isaac 2005). However, among poikilothermic animals, such as amphibians, male size rarely exceeds female size, *e.g.*, 90 % of anurans and 61 % of salamanders show a female-biased dimorphism (Shine 1979, Kupfer 2007). Female size is often positively correlated with fecundity, hence large females are able to produce larger clutches and in some cases also larger eggs or offspring (Shine 1988, Blanckenhorn 2005). Additionally, sexual dimorphism of traits not directly linked to reproduction can be caused by ecological differences between the sexes (Shine 1989), *e.g.*, the intersexual variation of adult head size is caused by ecological niche divergence (*e.g.*, Fairbairn 2007).

Although renowned for highly variable life histories (summarised in Wells 2007) our understanding of SSD patterns in amphibians is still incomplete in comparison with other vertebrates (see Fairbairn et al. 2007, Kupfer 2007).

The 'True Salamanders and Newts' (Urodela: Salamandridae) are highly suitable for studying intra- and interspecific patterns of sexual size dimorphism, as 56 % of the species exhibit female-biased and 15 % male-biased size dimorphism (Kupfer 2007) and show variable reproductive modes including oviparity and viviparity combined with variable morphologies and body shapes. Sexual dimorphism is found in numerous members of the family Salamandridae. In most cases, the dimorphism is discrete and has been demonstrated qualitatively; *i.e.*, the presence of male secondary sexual characteristics (*e.g.*, Malmgren & Thollesson 1999) or differences in head size (*e.g.*, Serra-Cobo et al. 2000) or tail length (*e.g.*, Inger 1947). However, few studies have quantitatively assessed sexual dimorphism of the overall bauplan of salamandrids using standard morphometrics and equilibrated their findings to age. Age should be included into the analyses to fully account for effects on SSD patterns,

but also to follow dimorphic traits during ontogeny (see also Monnet & Cherry 2002 and Kupfer 2007).

We qualitatively test the hypothesis that sexual dimorphism is found in the overall body shape or bauplan within two genera of the Salamandridae, *i.e.*, the viviparous, heavy-bodied *Salamandra algira* and the slender elongated, oviparous *Mertensiella caucasica* both inhabiting Mediterranean regions. We also equilibrated the morphometric approach to individual age by using skeletochronology. In species with reduced fecundity and higher offspring quality that live at high altitude or have a short breeding season (for example viviparous or lotic-breeding species), female-biased SSD should be favoured to compensate the lower fecundity. The breeding season of amphibians inhabiting the Mediterranean region is restricted to the rainy winter months. In pond breeding Mediterranean newts and frogs female-biased sexual dimorphism has been reported (*e.g.*, Jakob et al. 2003, Leskovar et al. 2006). Consequently, we would hypothesise a female-biased SSD in both Mediterranean salamanders.

## **MATERIAL AND METHODS**

### *Study animals*

The North African Fire Salamander, *Salamandra algira*, is a terrestrial salamander, living in habitats located in Mediterranean regions of Morocco, Algeria and Spain (Ceuta). Four subspecies are described (see Beukema et al. 2013): *S. a. algira* (Algerian populations), *S. a. spelaea* (Beni Snassen Massif, Morocco (also comp. Escoriza et al. 2006)), *S. a. tingitana* (Morocco [comp. Donaire-Barroso & Bogaerts 2003]) and *S. a. splendens* (north-eastern Middle Atlas Mountains, western and central Rif Mountains in Morocco). *S. algira* is viviparous and gives birth to aquatic larvae or fully developed juveniles (Donaire-Barroso & Bogaerts 2001, Donaire-Barroso et al. 2001).

The Caucasian salamander *Mertensiella caucasica* occurs at high altitudes in western Georgia and adjacent parts of North-East Turkey (Griffiths 1996, Franzen 1999). It is oviparous and reproduces in streams and its life cycle includes an aquatic, lotic larval stage (Klewen 1991).

*Morphometrics and analysis*

Thirty morphometric characters were selected for the analysis of sexual dimorphism (defined and summarised in Table 1). Arm and leg diameters were measured vertically to the body axis. Both diameter characters were measured in *S. algira* only. All measures were taken by one author using digital callipers to the nearest 0.01 mm (Rok International Industry, Ltd.).

**Table 1.** Description of measurements of morphometric body and head characters.

| Character            | Definition   |
|----------------------|--|
| <b>Body measures</b> |  |
| OAL                  | Overall length   |
| SVL                  | Snout-vent-length from the tip of the snout to the posterior edge of the cloaca                |
| TL                   | Tail length from the posterior edge of the cloaca to the tip of the tail                       |
| TH                   | Tail height  |
| UAL                  | Upper arm length from the posterior margin of the front leg (axilla) to the angle of the elbow |
| LAL                  | Lower arm length from the angle of the elbow to the wrist                                      |
| LAL2                 | Lower arm length measured until the tip of the longest finger                                  |
| TAL                  | Total arm length from the axilla to the tip of the longest finger                              |
| ULL                  | Upper leg length from the anterior margin of the hind leg (groin) to the angle of the knee     |
| LLL                  | Lower leg length from the angle of the knee to the ankle                                       |
| LLL2                 | Lower length measured until the tip of the longest toe   |
| TLL                  | Total leg length from the groin to the tip of the longest toe                                  |
| CL                   | Cloacal length   |
| CW                   | Cloacal width  |
| CHW                  | Chest width from axilla to axilla  |
| BWM                  | Body width at mid-body   |
| GG                   | Distance from groin to groin   |
| DEX                  | Distance of extremities from axilla to groin   |
| UAD                  | Upper arm diameter   |
| LAD                  | Lower arm diameter   |
| ULD                  | Upper leg diameter   |
| LLD                  | Lower leg diameter   |
| <b>Head measures</b> |  |
| HL                   | Head length  |
| HW                   | Head width at the angle of the jaw   |
| ED                   | Diameter of the eye  |
| ON                   | Orbit-naris-distance from the anterior edge of the eye to the nostril                          |
| IO                   | Interorbital-distance from eye to eye (measured from the centre of the eye)                    |
| IN                   | Internarial-distance from nostril to nostril   |
| ES                   | Eye-snout-distance from the anterior edge of the eye to the tip of the snout                   |
| IC                   | Intercanthal-distance from the anterior edge of the eye to the other                           |

Altogether 42 males and 17 females of *Salamandra algira* originating from near Taza (Morocco), clearly identified as adults, were taken for analysis (see Appendix A for specimen numbers). The colouration and the sampling locality identified the animals as *S. a. splendens* (see Beukema et al. 2013). Specimens were sexed via externally visible sexual characters (*e.g.*, swollen cloaca) and also by inspection of the gonads employing a small ventrolateral incision. The *Mertensiella caucasica* sample included 26 females and 22 males from the Zigana pass (Trabzon province, North-Eastern Turkey, see Appendix A for specimen numbers). All specimen were sexed through externally visible characters (*e.g.*, swollen cloaca; protuberance on the dorsal surface of the tail base of males).

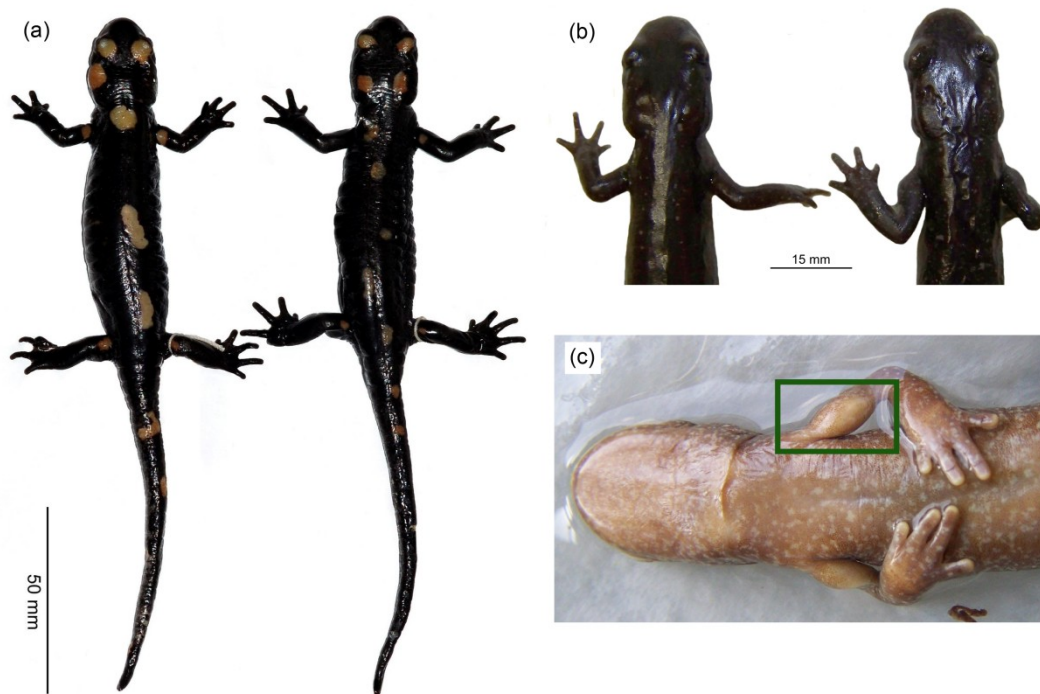
The sexual dimorphism index (SDI, after Gibbons and Lovich 1990) was calculated by dividing the mean snout-vent-length (SVL) of the larger sex by the mean snout-vent-length of the smaller sex and subtracting one ( $SVL_{\text{large}}/SVL_{\text{small}}-1$ ) to obtain a first conception of sexual size dimorphism. All characters measured were tested for normality using the D'Agostino and Pearson omnibus normality test. Univariate t-tests (two-sided at  $\alpha \leq 0.05$ ) were used to test for dimorphism between sexes. In addition, SSD was assessed by an analysis of covariance (ANCOVA) using snout-vent-length as co-variable to adjust the characters to body size. Correlations of body measures and age were carried out using Spearman correlation analysis. All statistical tests were conducted using Microsoft Excel 2007, GraphPad Prism 4.0c and Minitab 10.51 for Macintosh computers. Specimens were photographed using a Nikon D50 digital SLR or a Kodak ZD710 digital camera.

### *Histology and skeletochronology*

Ageing analysis in vertebrates, including amphibians, is traditionally carried out using skeletochronology (for a review see Smirina 1994). Skeletochronology visualizes periodical skeletal growth, *i.e.*, using cross sections of bone material: "growth marks" (bright and broad bone material due to active osteogenesis) and "lines of arrested growth" (LAGs; distinctive thin bone layers caused by stagnation of growth) to estimate individual age (*e.g.*, Alcobendas & Castanet 2000). To assess the age of the salamanders, the longest toe of the hind leg was clipped off from *S. algira* and *M. caucasica* specimens. The phalanges were embedded in paraffin wax. Cross sections (thickness: 7  $\mu\text{m}$ ) were produced using a microtome (HM 360 and

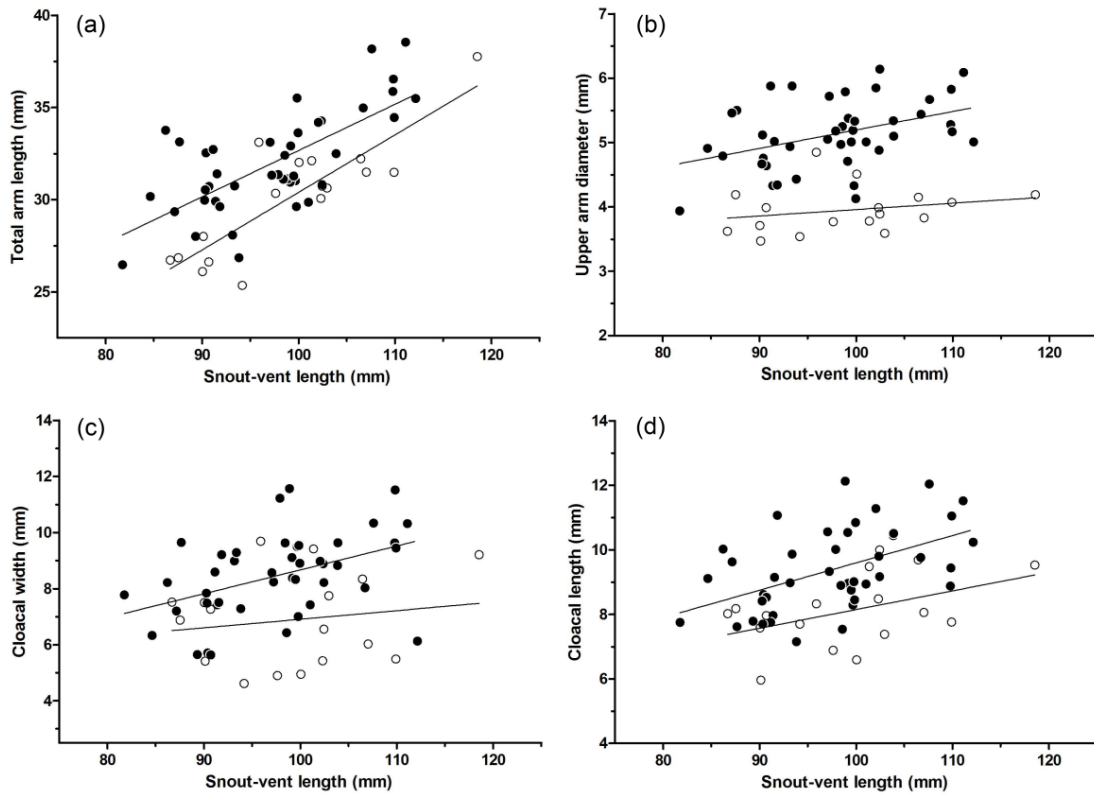
Zeiss Stemi 1000) and stained using kresyl-violet (comp. Scholz et al. 2010). LAGs on at least 20 serial cross-sections per specimen were counted three times by one author using an optical microscope (Zeiss Axioskop with a mounted Pixelink PL-B622CF camera). The histological sections are stored at the State Museum of Natural History Stuttgart (SMNS, Germany).

All age data were tested for normality using the D'Agostino and Pearson omnibus normality test. To allow comparisons between distinct age groups, specimens were sorted into four age groups (1+ to 5; 5+ to 10; 10+ to 15; 15+). SVL as well as total arm length (TAL) were compared within these groups (t-test, two-sided,  $\alpha \leq 0.05$ ) and the respective SDI was also calculated for selected body characters within the age classes.



**Figure 1.** Sexual dimorphism in Mediterranean salamanders. (a) Habitus of *Salamandra algira*. Males (right) and females (left) do not differ in general body size, but show several other dimorphic characters (e.g., note the enlarged forelimbs in the male). (b) Habitus of *Mertensiella caucasica*. Males (right) and females (left) do not differ in general body size, but show several other dimorphic characters (e.g., note the enlarged forelimbs and upper arm muscles of the male). (c) Ventral view of a male *M. caucasica* and demonstrating extremely enlarged upper arm muscles.





**Figure 2.** Sexual dimorphism of *Salamandra algira*. Some selected dimorphic characters are shown: (a) Total arm length TAL (b) Upper arm diameter UAD (c) Cloacal width CW (d) Cloacal length CL. Males have significantly larger sizes for each of these traits.

## RESULTS

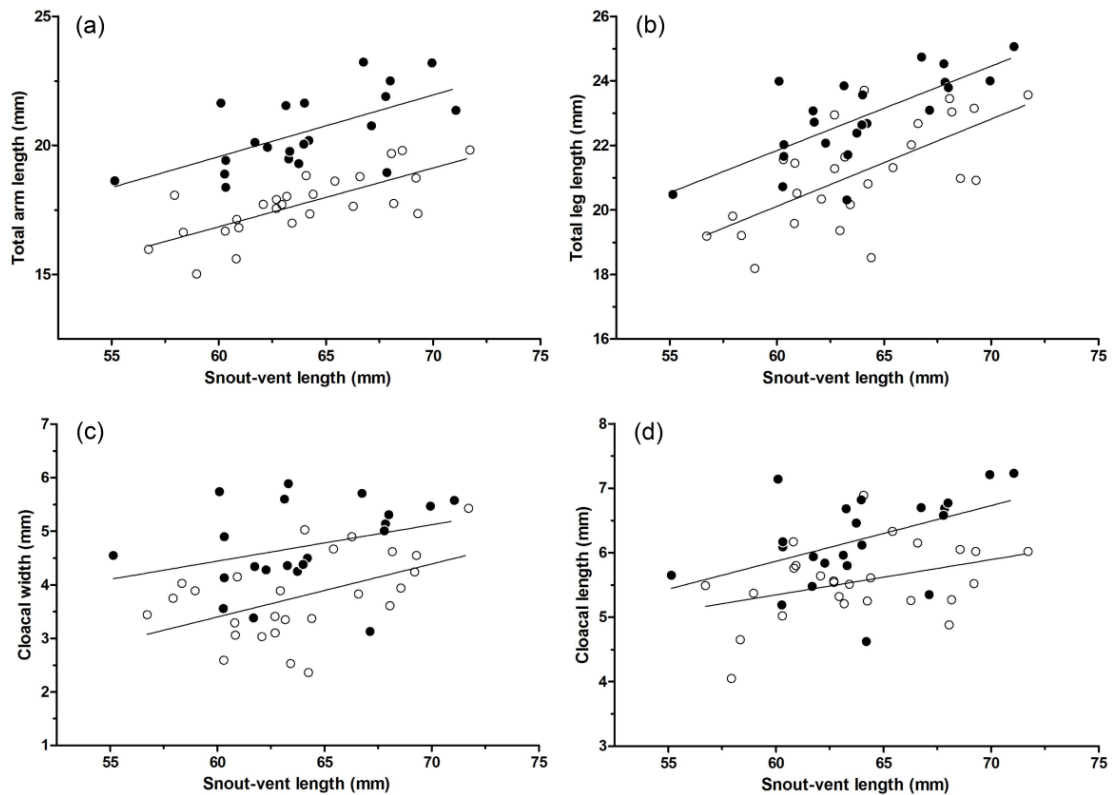
### *Patterns of sexual size dimorphism*

The sexes of *Salamandra algira* did not differ in general body size (see Figure 1a). The size dimorphism index (SDI) was 0.0385 (only implying a weak tendency towards a female-biased dimorphism). However, t-tests showed significant intersexual differences in arm length and diameter and in cloacal proportions (see Figure 2 and Table B1 in Appendix B). When adjusted to SVL, significant differences in arm lengths, arm diameters, as well as in lower leg diameter and cloacal proportions (see Table 2) were uncovered (see also Figure 1a, Figure 2), which were all male-biased.

No difference in general body size was also detected in *Mertensiella caucasica*. The SDI revealed a sexual dimorphism slightly shifted towards females (SDI = 0.0017). Similarly, significant intersexual male-biased differences were found regarding arm

and leg length as well as in cloacal characters (Figure 1b/c, Figure 3 and Table B2 in Appendix B).

When adjusted to SVL the significant SSD patterns persisted, except for lower leg length (see Table 2). Enlarged upper arm diameters in males were clearly visible externally (see Figure 1b/c).



**Figure 3.** Sexual dimorphism of *Mertensiella caucasica*. Some selected dimorphic characters are shown: (a) Total arm length TAL (b) Total leg length TLL (c) Cloacal width CW (d) Cloacal length CL. Males have significantly larger sizes for each of these traits.

### Body size and age

Males of *S. algira* were on average 13.3 years old (range: 5 - 20 years, median: 13.5 years) and females had an average age of 11.4 years (range: 5 - 19 years, median: 11 years). Body size and age of *S. algira* were not significantly related.

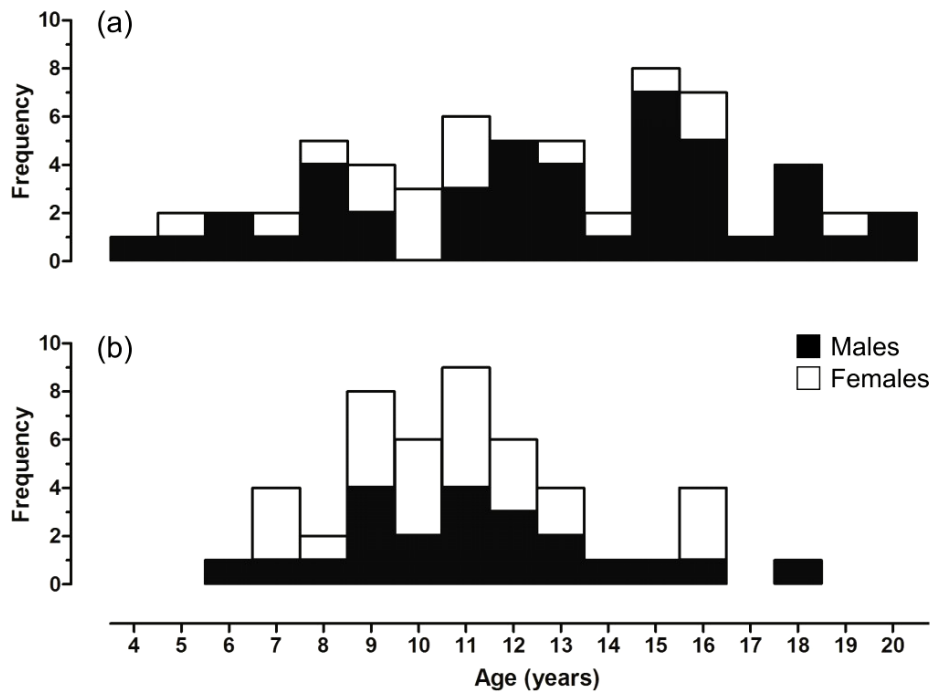
Males of *M. caucasica* were on average 11.2 years old (range: 6 - 18 years, median: 11 years) and females averaged an age of 10.8 years (range: 7 - 16 years, median: 11 years). All morphometric traits except for tail length ( $p \leq 0.05^*$ ,  $r = 0.47$ ) and the resulting overall length ( $p \leq 0.05^*$ ,  $r = 0.53$ ) in females were not related to age.

**Table 2.** Analysis of sexual size dimorphism in *Salamandra algira* (n = 42 males, n = 17 females) and *Mertensiella caucasica* (n = 22 males, n = 26 females). Results of one-factor ANCOVA comparing 29 (*S.a.*) or 25 (*M.c.*) traits of sexes relatively to snout-vent-length (SVL). F-values and significance levels are presented for each species.

| Character adjusted for SVL | <i>Salamandra algira</i> |         | <i>Mertensiella caucasica</i> |         |
|----------------------------|--------------------------|---------|-------------------------------|---------|
|                            | F                        | P       | F                             | P       |
| <b>Body measures</b>       |                          |         |                               |         |
| OAL                        | 0.13                     | 0.721   | 4.09                          | 0.05    |
| TL                         | 0.13                     | 0.721   | 4.09                          | 0.05    |
| TH                         | 2.65                     | 0.109   | 0.72                          | 0.402   |
| UAL                        | 5.49                     | < 0.05  | 90.45                         | < 0.001 |
| LAL                        | 9.66                     | < 0.01  | 14.54                         | < 0.001 |
| LAL2                       | 23.86                    | < 0.001 | 28.25                         | < 0.001 |
| TAL                        | 17.20                    | < 0.001 | 93.47                         | < 0.001 |
| ULL                        | 2.55                     | 0.116   | 21.7                          | < 0.001 |
| LLL                        | 0.20                     | 0.653   | 0.06                          | 0.806   |
| LLL2                       | 0.56                     | 0.459   | 16.44                         | < 0.001 |
| TLL                        | 2.26                     | 0.138   | 31.96                         | < 0.001 |
| CW                         | 15.42                    | < 0.001 | 15.2                          | < 0.001 |
| CL                         | 19.74                    | < 0.001 | 11.39                         | < 0.001 |
| BWM                        | 1.07                     | 0.306   | 3.62                          | 0.064   |
| DEX                        | 0.86                     | 0.359   | 1.19                          | 0.174   |
| UAD                        | 84.39                    | < 0.001 | -                             | -       |
| LAD                        | 6.47                     | < 0.01  | -                             | -       |
| ULD                        | 2.12                     | 0.151   | -                             | -       |
| LLD                        | 4.40                     | < 0.05  | -                             | -       |
| CHW                        | 0.75                     | 0.392   | 1.37                          | 0.249   |
| GG                         | 0.66                     | 0.419   | 0.38                          | 0.539   |
| <b>Head measures</b>       |                          |         |                               |         |
| HW                         | 0.50                     | 0.482   | 1.44                          | 0.237   |
| HL                         | 0.55                     | 0.462   | 0.02                          | 0.237   |
| ED                         | 0.02                     | 0.895   | 0.72                          | 0.400   |
| ON                         | 0.44                     | 0.508   | 0.50                          | 0.485   |
| IO                         | 0.02                     | 0.898   | 0.55                          | 0.463   |
| IN                         | 0.30                     | 0.585   | 0.01                          | 0.920   |
| ES                         | 0.06                     | 0.805   | 0.00                          | 0.995   |
| IC                         | 0.64                     | 0.428   | 0.39                          | 0.538   |

See Figure 4 for age frequency histograms of both species and for examples of cross sections see Figure C1 in the Appendix C.

Among age classes, male *S. algira* grew slightly faster in SVL until the age of 5, after 5+ females caught up or even outran males, but these differences were not significant. This relation appeared not significant in *M. caucasica* too, regardless of the small sample size in younger age classes. Regarding TAL, male *S. algira* and *M. caucasica* showed enlarged forearms at younger age and the difference remained significant at ages of above 10 ( $p < 0.01^{**}$ ) and 15 ( $p < 0.05^{*}$ ) in *M. caucasica* (see Figure 5).

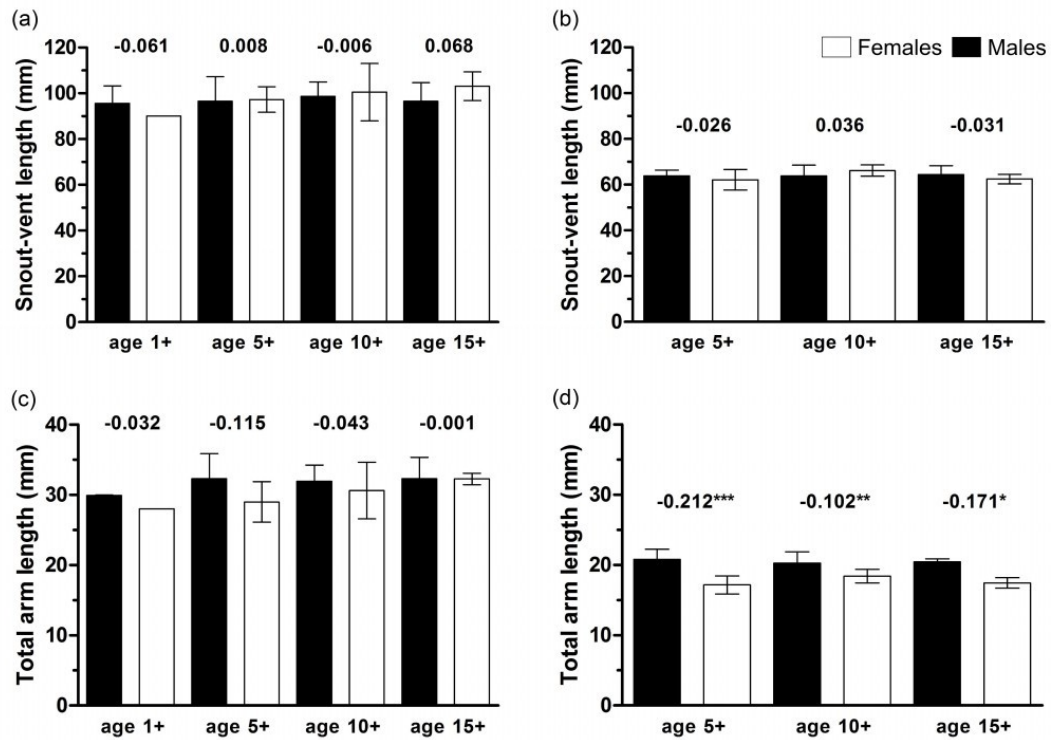


**Figure 4.** Age frequency histograms for (a) *Salamandra algira* and (b) *Mertensiella caucasica*.

## DISCUSSION

### *Dimorphism, fecundity and mating systems*

Both Mediterranean salamanders did not show marked intersexual differences in body size. Natural selection should favour large females, when female size is positively correlated with fecundity (see Shine 1988). Indeed many amphibians show a female-biased dimorphism in body size regardless of their reproductive mode (*e.g.*, Gomez-Mestre et al. 2012, Han & Fu 2013). For viviparous species with reduced fecundity and higher offspring quality that live at high altitude or have a short breeding season, the fecundity-advantage hypothesis predicts female-biased SSD. Females are also the larger sex in species with reduced fecundity but larger clutch volume (*e.g.*, Bruce 2000, Kupfer 2009). Both in viviparous frogs (Castanet et al. 2000) but also in some viviparous caecilians (Scholz et al. 2010) female body size exceeds that of males. Interestingly, the lack of a body size dimorphism of the salamanders studied here nicely shows that diverse selection pressures work together to shape the sexual dimorphism of the terrestrial salamanders.



**Figure 5.** Age and SSD in *Salamandra algira* and *Mertensiella caucasica*. (a) Development of SVL (mean ± SD) and SSD in *S. algira*. (b) Development of SVL (mean ± SD) and SSD in *M. caucasica*. (c) Development of TAL (mean ± SD) and SSD in *S. algira*. (d) Development of TAL (mean ± SD) and SSD in *M. caucasica*. SSD is indicated above each bar as SDI. Significant intersexual differences are marked with \* ( $p \leq 0.05$ ), \*\* ( $p \leq 0.01$ ), \*\*\* ( $p \leq 0.001$ ).

However, the sexes differed in several other morphological traits that were detected when sexes were adjusted for snout-vent-length. Male *S. algira* have significantly longer forearms than females. All members of the genus *Salamandra* are terrestrial, viviparous and their courtship behaviour is well adapted towards terrestriality (e.g., Klewen 1991, Veith et al. 1998). Males grasp females during a sexual embrace termed “amplexus” (e.g., Himstedt 1965, Klewen 1991, Thiesmeier 2004) in which males fix females whilst depositing a spermatophore subsequently picked up by the female. Since male spermatophore production is costly, selection favours traits reducing its waste. Therefore, the ventral amplexus of Fire salamanders is a reliable evolutionary stable mating strategy, because it minimizes the distance between the cloacae of the sexes to fasten spermatophore pickup and ultimately internal fertilisation (Halliday 1990). Enlarged male forelimbs are also advantageous for successfully monopolizing females during mating and to prevent male interference

(e.g., Stebbins & Cohen 1997, Houck & Arnold 2003). The same applies for males of *M. caucasica*, because highly significant arm length differences can be attributed to the courtship that resembles that of *Salamandra* in its basic pattern, apart from that courtship takes place in lotic habitats (e.g., Arnold 1987, Schultschik 1994). Forelimb sexual dimorphism often occurs in amphibian species that mate in an amplexus, for example male anurans (overview in Wells 2007) such as *Rhinella marina*, *Lithobates catesbeianus* and *Rana temporaria*, having much more robust forelimbs than females. In particular, adductor and flexor forelimb muscles size might play an important role for mating success (Peters & Aulner 2000, Lee 2001, Navas & James 2007). Our results suggest that the adductor and flexor muscles of the forelimb are sexually dimorphic in *S. algira* as well, as there is a highly significant intersexual difference in upper and lower arm diameter. This indicates that the upper parts of the forelimbs might provide the major force during amplexus, and the muscles involved might be for example the M. humeroantebrachialis, which flexes the elbow joint, the M. anconaeus humeralis lateralis and medialis, which extends the elbow joint and the M. coracobrachialis longus, which retracts the humerus (see Francis 1934, Duellman & Trueb 1994 for morphological description of salamander muscles). However, the exact functional morphology remains to be studied experimentally. On the other hand, we do not have information about seasonal or permanent aspects of sexual dimorphic musculature. Both Mediterranean salamander species lead a secretive life during summer and autumn and breed only during a short season in winter and early spring (e.g., Beukema et al. 2013). *S. algira* were collected during the breeding season in December accounting for the high abundance of males in the sample. We must assume that muscle size varies seasonally, but the comparative summer aspect could not be studied. Potentially, the enhancing effects of testosterone might cause a permanent dimorphism, but an androgen-mediated hypertrophy of the muscles likely exists during breeding as known for many anurans (e.g., Peters & Aulner 2000, Wells 2007). Among terrestrially mating urodeles, male golden-striped salamanders *Chioglossa lusitanica* (Arntzen 1999) and Caucasian salamanders *M. caucasica* (Franzen 1999) exhibit swollen upper arms in conjunction with the amplexus. Since there is intersexual difference in body size it seems that forelimb length and muscle size influence male reproductive success in both species to a greater extent than overall body size. It

provides a better ability to resist sexual interference by competing males, because it is not easy for a rival male to dislodge its' opponent from an amplexus, if it has a powerful grip for proper fixation of the female. Although *S. algira* is known to fight for resources (*e.g.*, refuges) in male-male combat (Bogaerts & Donaire-Barroso 2005), this behaviour does not affect male body size, unlike in other salamanders showing male rivalry such as the newt *Ommatotriton ophryticus* (Raxworthy 1989). Fighting in *Salamandra* also includes intensive use of the forelimbs whilst climbing onto the opponent and during aggressive embrace (*e.g.*, Kästle 1986, Thiesmeier 2004, Bogaerts & Donaire-Barroso 2005) quite similar to a mating amplexus, giving males with longer and more robust forelimbs an advantage. Male *S. algira* have stronger legs as well and likely males may outcompete rivals in fights whilst standing on stronger hind legs (see Kästle 1986 for details of rivalry fights in *S. salamandra*). In oviparous *M. caucasica* we found a significant male-biased dimorphism in hind leg length and in contrast to *S. algira*, all characters were sexually dimorphic. Two hypotheses might explain the observation. Firstly, males need a secure position during courtship in lotic habitats (see Schultschik 1994 for a detailed description of courtship behaviour). Stronger legs could prevent drifting away and even losing the female while mating. Secondly, male Caucasian salamanders rub their tail tubercle against the female cloaca (and possibly insert it) to ensure that the female is ready for sperm transfer and to guide the female exactly over the spot, where a spermatophore will be deposited (see also Schultschik 1994, Sever et al. 1997). Enlarged legs may represent a benefit for these movements in lotic habitats or on land.

As known from other salamandrids, both *S. algira* and *M. caucasica* males can be distinguished from females on base of the cloaca. The male cloaca is equipped with a complex set of glands producing material for spermatophores and releasing courtship pheromones. Cloacal shape is a sexually selected trait that increases male reproductive success (Sever 2003) and therefore males have a more swollen cloaca than females (Verrell 1989, Halliday 1990). Sexual hormones, which are associated with maturation and sexual activity, control the development of cloacal glands, being most hypertrophied in the breeding season (Sever 2003). Other sexually dimorphic traits, such as head size or tail length, were not found in *S. algira* and *M. caucasica*. Courtship of both species neither involves biting, nor tail fanning or wrapping as known from other salamandrids (summarised in Kühnel et al. 2010).

A literature review (including only studies of statistically analysed data with large sample sizes) on sexual dimorphism in the genera *Salamandra*, *Lyciasalamandra*, *Mertiensiella* and *Chioglossa* revealed a lack of dimorphism in body size (snout-vent length) as a common lineage specific pattern among the 'True salamander' clade (see Figure 6).

However, in some viviparous species females appear larger because of their higher body mass during pregnancy (e.g., *Salamandra infraimmaculata*). The evolution of other dimorphic traits such as limb size and associated muscles, likely influenced by sexual selection (due to a highly specific mating system), is more relevant among the “true salamanders”. For example, male *S. salamandra* are actively searching and waiting for mates during rainy nights (e.g., Himstedt 1965, Greven 2003), driving sexual selection pressures on these courtship related traits. It also seems important for males to ensure effective mating and sperm transfer, as *S. salamandra* females mate with several males, which do not sire a females' clutch equally (Steinfartz et al. 2006).

### *Dimorphism and age*

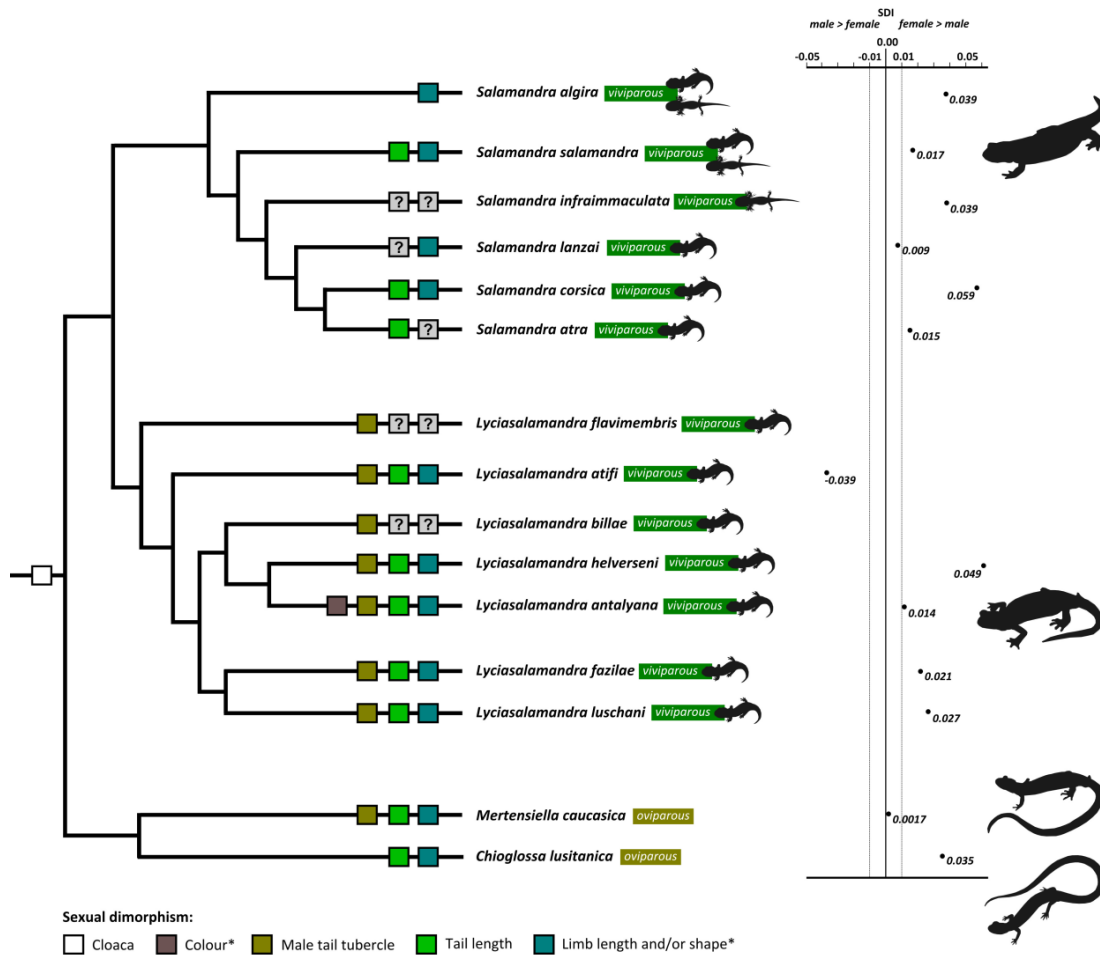
Our results on body size and age agree well with other studies (e.g., Dolmen 1982, Castanet & Smirina 1990, Francillon-Viellot et al. 1990, Caetano & Castanet 1993, Smirina 1994) showing that body size cannot predict individual age, as body size overlaps within a given age class. Both *S. algira* and *M. caucasica* showed no correlation between age and size, making it impossible to estimate individual age just by size. In contrast, Üzum (2009) found a weak correlation between age and SVL and body mass in the latter species. Similarly, we reported that age, tail length and the resulting overall length are weakly related only in female *M. caucasica*. A potential effect of age on body traits needs to be tested in larger samples. Another point is how reliable ageing via skeletochronological methods is for very old individuals. Skeletochronology of phalanges underestimated the age of long-lived Alpine newts *Mesotriton alpestris*, because the bone-remodelling processes and LAG rapprochement contributed to a reduced reliability (Wagner et al. 2011). Especially high altitude populations of salamanders were likely affected (Eden et al. 2007, Wagner et al. 2011). Our study approach agreed well with Wagner et al. (2011), who suggested reducing the thickness of cross sections up to 7 µm to allow a higher



resolution. But if assumedly very old individuals do not grow any further (Wagner et al. 2011), it is likely that both *S. algira* and *M. caucasica* might live well beyond the ages reported herein.

Generally, the large body size ranges in the age classes reflect individual differences of growth rates. Growth can be influenced by changing food availability and quality, foraging behaviour, population density, predator density, metabolism rate, as well as by differences in habitat and climate, which in turn affect the activity levels of the salamanders (e.g., Alcobendas & Castanet 2000, Arntzen 2000, Miaud et al. 2001). However, these conditions would affect both sexes equally, which is why they do not explain sexual dimorphism.

Although many factors may influence sexually dimorphic traits, the body size differences at maturity are most important (Halliday & Verrell 1988, Francillon-Viellot et al. 1990, Miaud et al. 2001, Marzona et al. 2004, Seglie et al. 2010). Resources previously only invested in growth are now split up due to a trade-off between growth and reproduction. Consequently, individual growth rates of both sexes are reduced or growth halts completely. Numerous studies have shown, that the timing of sexual maturity is sex-specific ("sexual bimaturity"; see reviews in Kupfer 2007, Wells 2007) and that growth rates are sex-specific, too. It is possible that both sexes reach sexual maturity simultaneously, but size differences usually are caused by divergent growth rates during the juvenile and subadult phase (see also Hasumi 2010). More studies are needed to analyse the proximate effects of salamander life histories and age on sexual dimorphism. Which background may influence size and sexual dimorphism of a given species must be studied more specifically using a multifactorial approach.



**Figure 6.** Phylogenetic relationships with an illustration of the distribution of sexual dimorphism and reproductive modes of 'True Salamanders' (following the phylogenetic hypotheses after Weisrock et al. 2006, Zhang et al. 2008). \*Sexual dimorphism of limbs can be found in length (predominantly of forelimbs) and/or shape (especially swollen male upper arms; the degree of seasonality of the expressed traits needs further validation for all taxa), \*Colour dimorphism: occurs only within *Lyciasalamandra* (*L. irfani*, *L. arikani*, *L. yehudani* were not included in the tree, but exhibit sexual dichromatism). The analysis of SSD among the 'True Salamanders' is based on literature (for a full reference list and recommended reading see Appendix D).

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## **APPENDIX A**

### **Specimen Register**

ZMH (Zoological Museum Hamburg), ZSM (Bavarian State Collection of Zoology Munich), ZFMK (Zoological Research Museum Alexander Koenig Bonn).

#### *Salamandra algira*

ZSM 75/2013 - ZSM 127/2013

#### *Mertensiella caucasica*

ZFMK 16020, 6022, 35778, 37975, 41434, 47474, 47475, 51926, 51959 - 51961, 51998 - 52004, 52007 - 52009, 52012, 83120, 83851, 83852, 86136, 86312, 86317, 86320 & 86389; ZMH A00314 - A00322, A00324 & A00325; ZSM 1208/2005, 2209/2005, 2527/2005, 517/1999, 518/1999 & 519/1999.

**APPENDIX B - Table B1/B2**

**Table B1.** Analysis of sexual size dimorphism in *Salamandra algira* (n = 42 males, n = 17 females). The means (M), standard deviations (SD), minima and maxima (range) of 30 characters are listed. Results of t-tests (two-tailed,  $\alpha = 0.05$ ) are shown in the last column.

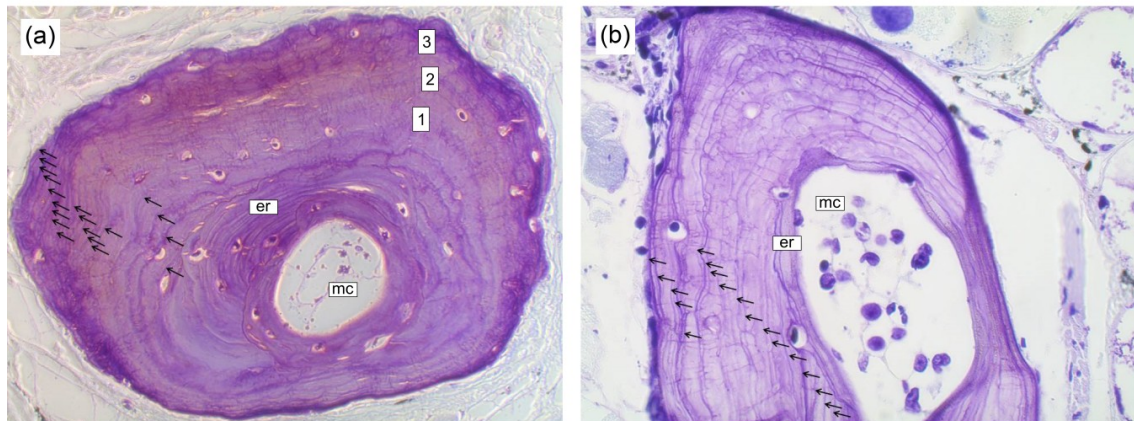
| Character     | Males (n = 42) |                 | Females (n = 17) |                 | <i>P</i> |
|---------------|----------------|-----------------|------------------|-----------------|----------|
|               | M ± SD         | Range           | M ± SD           | Range           |          |
| Body measures |                |                 |                  |                 |          |
| SVL           | 95.38 ± 7.91   | 81.75 - 118.53  | 99.05 ± 7.87     | 86.69 - 107.03  | n.s.     |
| OAL           | 172.10 ± 16.23 | 132.53 - 215.31 | 175.19 ± 15.34   | 151.43 - 215.31 | n.s.     |
| TL            | 74.50 ± 9.15   | 44.87 - 94.43   | 76.14 ± 8.15     | 64.74 - 96.78   | n.s.     |
| TH            | 6.50 ± 0.90    | 4.98 - 10.97    | 7.05 ± 0.53      | 6.55 - 7.91     | n.s.     |
| UAL           | 11.35 ± 1.27   | 9.09 - 14.39    | 10.85 ± 1.34     | 8.1 - 14.51     | n.s.     |
| LAL           | 11.01 ± 1.22   | 8.57 - 13.99    | 10.52 ± 1.21     | 8.38 - 13.66    | < 0.05   |
| LAL2          | 20.00 ± 1.96   | 17.38 - 24.44   | 19.25 ± 1.95     | 16.41 - 23.25   | < 0.05   |
| TAL           | 32.04 ± 2.97   | 26.47 - 38.54   | 30.09 ± 3.02     | 25.35 - 37.76   | < 0.05   |
| ULL           | 11.12 ± 1.43   | 7.97 - 14.79    | 10.72 ± 1.34     | 8.53 - 12.86    | n.s.     |
| LLL           | 11.81 ± 1.48   | 8.92 - 15.6     | 12.03 ± 1.44     | 9.84 - 15.75    | n.s.     |
| LLL2          | 23.02 ± 1.96   | 20.15 - 27.24   | 22.96 ± 2.08     | 19.18 - 29.16   | n.s.     |
| TLL           | 34.14 ± 2.91   | 29.9 - 41.02    | 33.68 ± 2.95     | 28.71 - 42.02   | n.s.     |
| CW            | 8.46 ± 1.68    | 5.64 - 11.57    | 6.88 ± 1.70      | 4.62 - 9.69     | < 0.001  |
| CL            | 9.17 ± 1.35    | 7.54 - 12.04    | 8.09 ± 1.34      | 5.96 - 10.0     | < 0.001  |
| BWM           | 18.37 ± 2.35   | 11.92 - 24.11   | 15.32 ± 2.38     | 15.85 - 23.87   | n.s.     |
| DEX           | 47.19 ± 4.94   | 38.46 - 59.98   | 48.96 ± 4.86     | 41.94 - 59.18   | n.s.     |
| UAD           | 5.12 ± 0.45    | 3.94 - 5.83     | 3.95 ± 0.69      | 3.47 - 4.85     | < 0.001  |
| LAD           | 4.54 ± 0.54    | 3.59 - 5.56     | 4.28 ± 0.51      | 3.75 - 5.25     | n.s.     |
| ULD           | 5.95 ± 0.88    | 4.0 - 7.15      | 5.73 ± 0.94      | 4.79 - 7.29     | n.s.     |
| LLD           | 5.02 ± 0.56    | 3.91 - 6.27     | 4.98 ± 0.59      | 4.13 - 6.36     | n.s.     |
| CHW           | 14.65 ± 1.58   | 10.55 - 18.92   | 15.32 ± 1.61     | 13.25 - 17.43   | n.s.     |
| GG            | 12.30 ± 1.18   | 10.40 - 16.77   | 13.24 ± 1.20     | 11.52 - 14.98   | n.s.     |
| Head measures |                |                 |                  |                 |          |
| HW            | 16.08 ± 1.18   | 13.58 - 19.67   | 16.32 ± 1.09     | 14.87 - 19.67   | n.s.     |
| HL            | 22.74 ± 1.97   | 17.15 - 25.77   | 23.32 ± 1.89     | 18.42 - 27.9    | n.s.     |
| ED            | 3.97 ± 0.56    | 3.07 - 5.56     | 4.11 ± 0.57      | 3.35 - 4.93     | n.s.     |
| ON            | 4.14 ± 0.64    | 3.12 - 5.42     | 4.05 ± 0.64      | 2.94 - 5.34     | n.s.     |
| IO            | 13.21 ± 1.63   | 3.08 - 16.03    | 13.60 ± 0.94     | 11.86 - 16.03   | n.s.     |
| IN            | 6.28 ± 0.59    | 4.67 - 7.32     | 6.25 ± 0.59      | 5.26 - 7.70     | n.s.     |
| ES            | 8.25 ± 0.71    | 6.43 - 9.91     | 8.27 ± 0.73      | 7.3 - 10.4      | n.s.     |
| IC            | 9.50 ± 0.80    | 7.47 - 11.15    | 9.44 ± 0.82      | 8.11 - 11.33    | n.s.     |

**Table B2.** Analysis of sexual size dimorphism in *Mertensiella caucasica* (n = 22 males, n = 26 females). Means (M), standard deviations (SD), minima and maxima (range) of 30 characters are listed. Results of t-tests (two-tailed,  $\alpha = 0.05$ ) are shown in the last column.

| Character     | Males (n = 22) |                 | Females (n = 26) |                | <i>P</i> |
|---------------|----------------|-----------------|------------------|----------------|----------|
|               | M ± SD         | Range           | M ± SD           | Range          |          |
| Body measures |                |                 |                  |                |          |
| SVL           | 63.9 ± 3.76    | 55.14 - 71.06   | 63.76 ± 3.91     | 56.72 - 71.72  | n.s.     |
| OAL           | 149.48 ± 15.22 | 125.55 - 181.45 | 144.45 ± 12.19   | 128.43 - 168.7 | n.s.     |
| TL            | 85.58 ± 12.61  | 62.74 - 110.39  | 80.14 ± 10.07    | 64.64 - 104.63 | n.s.     |
| TH            | 4.71 ± 0.66    | 3.65 - 6.89     | 4.92 ± 0.79      | 3.64 - 6.54    | n.s.     |
| UAL           | 8.15 ± 0.69    | 7.14 - 9.68     | 6.51 ± 0.63      | 5.49 - 7.91    | < 0.001  |
| LAL           | 6.68 ± 0.58    | 5.48 - 7.74     | 5.92 ± 0.8       | 4.01 - 6.99    | < 0.001  |
| LAL2          | 12.35 ± 0.9    | 10.9 - 14.32    | 11.2 ± 0.95      | 9.46 - 13.9    | < 0.001  |
| TAL           | 20.5 ± 1.44    | 18.37 - 23.23   | 17.7 ± 1.2       | 15.02 - 19.82  | < 0.001  |
| ULL           | 7.55 ± 0.62    | 6.21 - 8.49     | 6.86 ± 0.67      | 5.67 - 8.35    | < 0.001  |
| LLL           | 7.49 ± 0.71    | 6.16 - 8.86     | 7.38 ± 0.85      | 6.02 - 9.05    | n.s.     |
| LLL2          | 15.31 ± 1.02   | 13.5 - 17.33    | 14.28 ± 1.31     | 12.1 - 16.41   | < 0.01   |
| TLL           | 22.87 ± 1.36   | 20.31 - 25.06   | 21.13 ± 1.61     | 18.19 - 23.71  | < 0.001  |
| CW            | 4.71 ± 0.79    | 3.13 - 5.89     | 3.77 ± 0.79      | 2.36 - 5.43    | < 0.001  |
| CL            | 6.2 ± 0.69     | 4.62 - 7.23     | 5.55 ± 0.57      | 4.05 - 6.89    | < 0.001  |
| CHW           | 7.95 ± 1.02    | 5.73 - 9.63     | 7.56 ± 1.05      | 5.68 - 10.33   | n.s.     |
| BWM           | 9.4 ± 1.38     | 6.76 - 11.44    | 10.04 ± 1.46     | 7.36 - 12.92   | n.s      |
| GG            | 7.24 ± 0.71    | 5.68 - 8.45     | 7.34 ± 0.8       | 6.2 - 9.98     | n.s.     |
| DEX           | 31.92 ± 2.61   | 27.24 - 36.75   | 32.63 ± 3.34     | 27.04 - 40.14  | n.s.     |
| Head measures |                |                 |                  |                |          |
| HW            | 9.79 ± 0.58    | 8.9 - 10.71     | 9.57 ± 0.62      | 7.92 - 10.59   | n.s.     |
| HL            | 13.69 ± 0.84   | 12.28 - 15.74   | 13.66 ± 0.94     | 11.63 - 15.57  | n.s.     |
| ED            | 2.77 ± 0.37    | 1.93 - 3.33     | 2.66 ± 0.28      | 2.14 - 3.36    | n.s.     |
| ON            | 2.96 ± 0.37    | 2.44 - 3.62     | 2.99 ± 0.33      | 2.37 - 3.8     | n.s.     |
| IO            | 8.35 ± 0.67    | 7.1 - 9.6       | 8.19 ± 0.68      | 6.87 - 9.9     | n.s.     |
| IN            | 4.34 ± 0.35    | 3.74 - 5.14     | 4.32 ± 0.54      | 3.18 - 5.8     | n.s.     |
| ES            | 5.87 ± 0.59    | 4.03 - 6.98     | 5.86 ± 0.44      | 5.21 - 7.0     | n.s.     |
| IC            | 6.39 ± 0.69    | 5.08 - 7.99     | 6.47 ± 0.51      | 5.4 - 7.4      | n.s.     |

## APPENDIX C

### Figure C1



**Figure C1.** Selected cross sections of phalanges for skeletochronological assessment; er = endosteal resorption, mc = medullary cavity. (A) *Salamandra algira*, 19 year old female, 200 x magnifications. (B) *Mertensiella caucasica*, 18 year old male, 400 x magnifications.

## APPENDIX D

### References and recommended literature for Figure 6

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## CHAPTER 3

### **Age and fecundity in *Salamandra algira* (Urodela: Salamandridae)**

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## ABSTRACT

Amphibians are characterised by indefinite growth. Their body size reflects a trade-off between growth and reproduction. Consequently, growth rates decrease or even cease after maturation. Furthermore, the sexes often mature at different ages (sexual bimaturity). We examined fecundity patterns of the terrestrial salamander *Salamandra algira* (Salamandridae) and tested if age, body size and the fecundity of both sexes are connected and how these reproductive traits interact. We revealed positive correlations for female size, age and fecundity traits, i.e. egg number and volume. The male number of testes lobes was also positively correlated to age. Our study provides baseline data on a rarely studied terrestrial salamandrid. Further collection-based research is needed to obtain more valuable data aiding the understanding of life history evolution of the Salamandridae.

**Keywords.** Fecundity, salamanders, Urodela, *Salamandra*, skeletochronology.



## INTRODUCTION

Amphibians are potentially capable of indefinite growth (HALLIDAY & VERELL 1988, VERRELL & FRANCILLON 1986, KUPFER 2007). However, often growth rate is low early on, increases with advanced development and finally decreases or ceases completely due to several limiting factors of intrinsic and extrinsic nature. A growth decrease often coincides with sexual maturity, when resources are either split between reproduction and growth, or only invested in reproduction (HALLIDAY & VERRELL 1988, MIAUD et al. 2000, MIAUD et al. 2001, MARZONA et al. 2004). Sexual maturity in amphibians is sex-specific (the phenomenon of sexual bimaturity) and does not only affect the general growth pattern, but also the degree of sexual size dimorphism (SSD). Ecological factors, such as climate and altitude, also play important roles for maturation (HALLIDAY & VERRELL 1988). Several studies have shown that animals grow more slowly at high altitude and that they can become larger, older and even delay sexual maturity (e.g. CAETANO & LECLAIR 1996, MIAUD et al. 2000, MARUNOUCHI et al. 2000, MIAUD et al. 2001, AMAT et al. 2010). Within species, activity phases in high altitude populations are restricted due to a cooler and raw climate, compared to those of lowland populations (ARNTZEN 2000). This leads to delayed metamorphosis and maturation, giving the individuals a chance to invest more resources in growth before the growth-reproduction trade-off arises. Early metamorphosis and maturation reduces body size and could affect female fecundity, because these traits are linked. Larger females are able to produce more offspring and thus to enhance their reproductive success (FAIRBAIRN 2007). To what extent age influences male fecundity is quite unclear, but BRUCE et al. (2002) found that the differentiation and the number of testes lobes increases with age in *Desmognathus ocoee*, *D. monticola* and *D. quadramaculatus* and DOLMEN (1982) detected the same for *Triturus cristatus*.

The North African Fire Salamander (*Salamandra algira*, BEDRIAGA 1883) inhabits Mediterranean regions of Morocco, Algeria and Spain (Ceuta). The high-altitude populations of the Rif- and Middle-Atlas Mountains were recently described as new subspecies *S. a. splendens* (BEUKEMA et al. 2013) and they are particularly interesting for fecundity studies, as altitude was often reported to influence the reproduction of salamanders (e.g. TILLEY 1973). *S. algira* is viviparous and gives

birth to aquatic larvae (lecithotrophic viviparity) or even fully developed juveniles (matrotrophic viviparity) in *S. a. tingitana* (DONAIRE-BARROSO & BOGAERTS 2001, DONAIRE-BARROSO et al. 2001). Viviparous species have a reduced fecundity (clutch size) as an effect of egg retention (DUELLMAN & TRUEB 1994, WELLS 2007), but their offspring benefits from enhanced survival, because the development of the eggs outside the females' body is omitted. We examined *S. algira* specimen for fecundity patterns of both sexes and we additionally determined their age using skeletochronology and measured body size in order to study the influence of age and size on fecundity in both males and females. It might be predicted that females might compensate their lower fecundity with a larger clutch volume, especially in high-altitude populations, where growth and maturation might be slowed and thus larger body sizes could be reached (as precondition to have larger clutch volumes). How far male fecundity increases with age will be examined in the following as well.

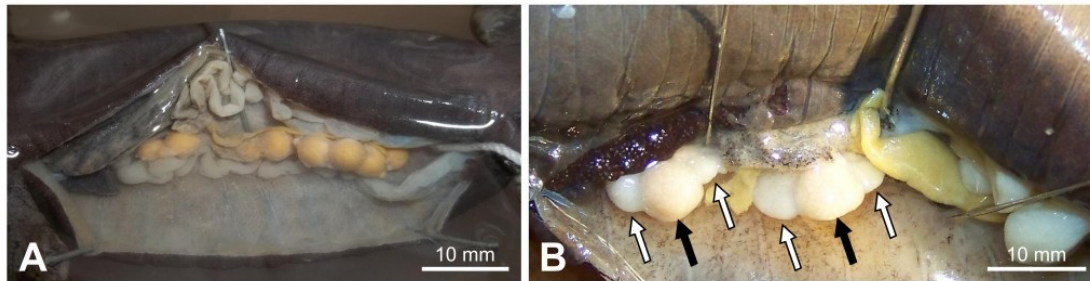
## MATERIAL AND METHODS

### *Animal dissection and statistics*

We analysed specimens of *Salamandra algira* housed in the Bavarian State Collection of Zoology Munich (ZSM, see Appendix 1 for specimen numbers). Because of their colouration and collection locality (Taza, Morocco) we referred to them as *S. a. splendens* (BEUKEMA et al. 2013). Specimens were sexed via externally visible sexual characters (e.g. swollen cloaca) and also by the inspection of the gonads employing a ventrolateral incision. Body size of both sexes was accessed by measuring snout-vent-length (SVL in mm). All salamanders (females n = 15, males n = 21) were carefully dissected and the following data was collected (see Fig. 1 for dissection situs): in females the clutch size (number of eggs in each ovary) was counted and egg diameter was measured with a digital calliper (ROK International Industry Co., Ltd.) under a stereomicroscope to the nearest 0.01 mm. In males the number of testes lobes was counted. The egg volume was calculated following DZIMINSKI & ALFORD (2005):

$$V_{\text{egg}} (\text{mm}^3) = \frac{4}{3} \pi (\sqrt{A}/\pi)^3 \text{ and } A = \text{egg}\varnothing^2$$

The clutch volume was calculated by multiplying clutch size and egg volume. The correlation of fecundity traits and age or SVL was carried out using Spearman correlation analysis and linear regression. All statistical tests were conducted with Prism 5.01 and Microsoft Excel 2007 for Windows 7.



**Figure 1.** Viscera of *Salamandra algira*. **A** Situs of an adult female with fully yolked ova in one ovary. **B** Situs of an adult male. Two testes segments are shown: segments that are in retention phase are marked with black arrows; anterior and posterior lobes, which are in regeneration and active during the coming breeding season are marked with white arrows.

### *Histology and skeletochronology*

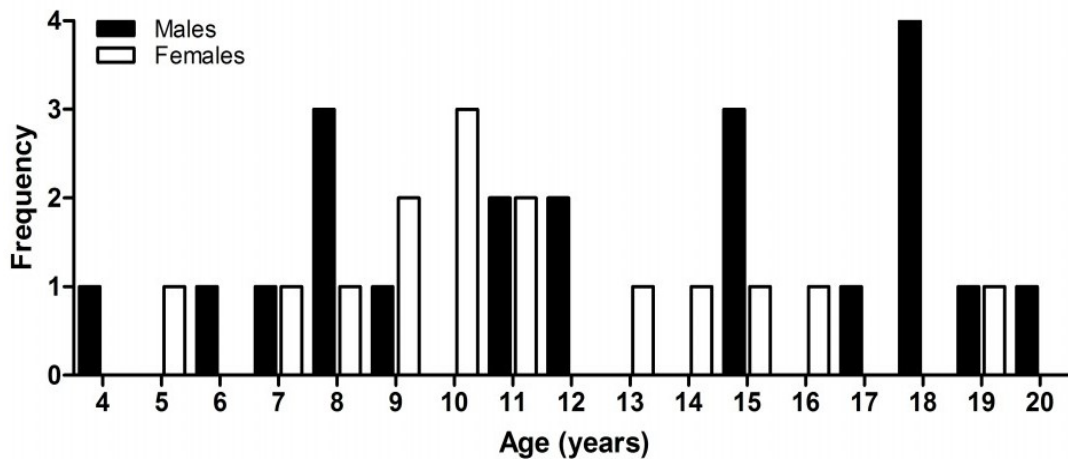
To assess the age of the salamanders, the longest finger (third finger) was clipped off from all *S. algira* specimens. They were embedded into paraffin wax and cross sections (thickness: 7  $\mu$ m) were produced using a microtome (HM 360 and a mounted Zeiss Stemi 1000). All cross sections were stained using kresyl-violet (comp. SCHOLZ et al. 2010). The lines of arrested growth (LAGs) were counted three times using an optical microscope (Zeiss Axioskop with a mounted Pixelink PL-B622CF camera).

## RESULTS

### *Female fecundity and age*

Female SVL ranged from 86.7 to 109.9 mm (mean  $98.0 \pm 7.6$  mm) and they were on average 11.1 years old ( $\pm 3.7$  years, range: 5-19 years; see an age distribution histogram in Fig. 2).

The mean number of eggs in female ovaries was  $12.9 (\pm 4.1)$ , range: 7-22 eggs). The mean diameter of eggs was  $3.9 \pm 0.4$  mm (range: 3.1-4.4 mm). The resulting egg volume was  $8.4 \pm 2.4$  mm<sup>3</sup> (range: 3.9-11.9 mm<sup>3</sup>) and clutch volume was  $106.9 \pm 40.0$  mm<sup>3</sup> (range: 27.3-187.5 mm<sup>3</sup>). The egg distribution was asymmetric in the left and right ovaries in 12 of the 15 females (left side of ovary: min. 3 to max. 13 eggs; right side of ovary: min. 3 to max. 11 eggs). The comparison of the fecundity data with age and SVL in female salamanders revealed a significant correlation between egg and clutch volume with age, i.e., the volume of both the eggs ( $r^s = 0.5171$ ,  $p \leq 0.05^*$ ) and of the whole clutch ( $r^s = 0.5350$ ,  $p \leq 0.05^*$ ) increases with age. This also holds for the correlation of SVL and egg number ( $r^s = 0.6655$ ,  $p \leq 0.01^{**}$ ), which increased with increasing body size (Fig. 3A-C). In general, all clutch characteristics were positively correlated with age and SVL, except for the SVL-egg volume correlation. The linear regression analysis confirmed these results.



**Figure 2** Age frequency histogram for male ( $n = 21$ ) and female ( $n = 15$ ) *Salamandra algira*.

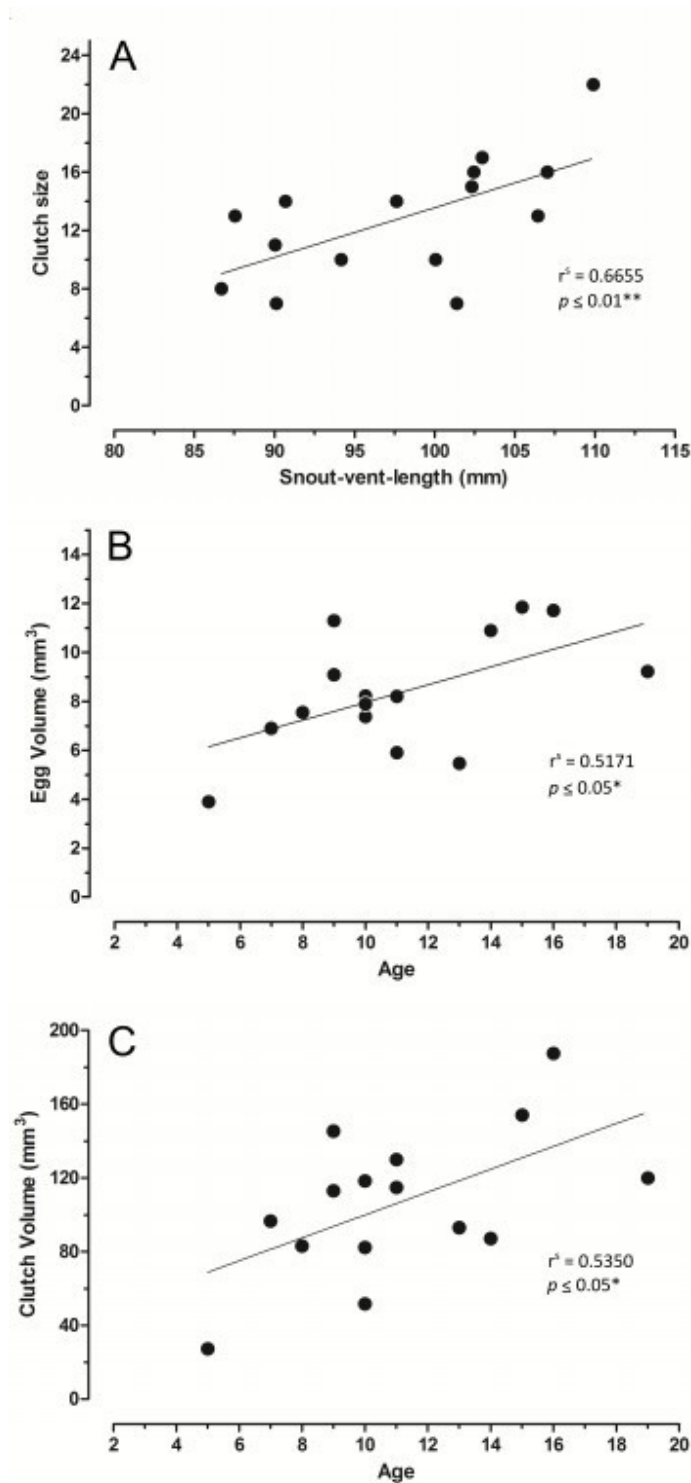
#### *Male testes lobes and age*

Male SVL ranged from 81.8 to 112.2 mm (mean  $96.0 \pm 8.0$  mm) and males were on average 12.8 years old ( $\pm 4.9$  years, range: 4-20 years; see an age distribution histogram in Fig. 2). Counting the testes lobes revealed a mean number of lobes of  $5.2 \pm 1.8$  for both sides combined, (range: 2-8 lobes; 0-4 on left and right side, respectively). Twelve males had the same number of lobes on each side, but the

remaining nine individuals showed an asymmetric distribution pattern, e.g. two lobes on one side and one on the other or even four lobes on one side and none on the other. Age was significantly positive related to the number of testes lobes ( $r^s = 0.5459$ ,  $p \leq 0.05^*$ , Fig. 4). Although the correlation of SVL and the number of testes lobes was not significant, a tendency towards a positive relation was found ( $r^s = 0.1535$ ), which was confirmed by a linear regression analysis.

## DISCUSSION

Reproductive traits, such as egg and clutch size, are highly important to evaluate female reproductive success. Large female size is often correlated with a higher fecundity (e.g. KUPFER 2007, WELLS 2007). The fecundity analysis of female *Salamandra algira* revealed an increasing clutch size in relation to a larger body size. On the other hand, female body mass is not related to reproductive traits such as litter size or total litter mass (KOPP & BAUR 2000). Positive relations of body size and fecundity were shown in other salamanders. For example, larger females with big eggs as well as a larger clutch sizes have been reported in *Lissotriton vulgaris* (VERRELL & FRANCILLON 1986) and *Triturus carnifex* (CVETKOVIĆ et al. 1996). Further examples for this relation are also known from plethodontid salamanders, e.g. *Batrachoseps* and *Aneides* (ANDERSON 1960), *Desmognathus ochrophaeus* (MARTOF & ROSE 1963), *Bolitoglossa rostrata* (HOUCK 1977), *Plethodon cinereus* (FRASER 1980) and the ambystomatid *Ambystoma talpoideum* (SEMLITSCH 1985). Moreover, the individual body size and thus fecundity traits are associated with age. The volume of eggs and of the whole clutch of female *S. algira* increases with age (a similar result was found for *Hynobius kimurae*, which has an increased egg diameter at older age [MISAWA & MATSUI 1999], as well as for *Ambystoma talpoideum* [SEMLITSCH 1985] and *Lissotriton vulgaris* [VERRELL & FRANCILLON 1986]). In general, the tendency of older and larger females to bear larger eggs is conspicuously. Sexual maturity is sex-specific (sexual bimaturity; see KUPFER 2007, WELLS 2007) and females often mature later than males (comp. e.g. TILLEY 1980). Since age, size and fecundity are interrelated and a larger size and thus more or larger eggs are advantageous for female reproductive success, a delayed female maturity compared to males should be favourable.

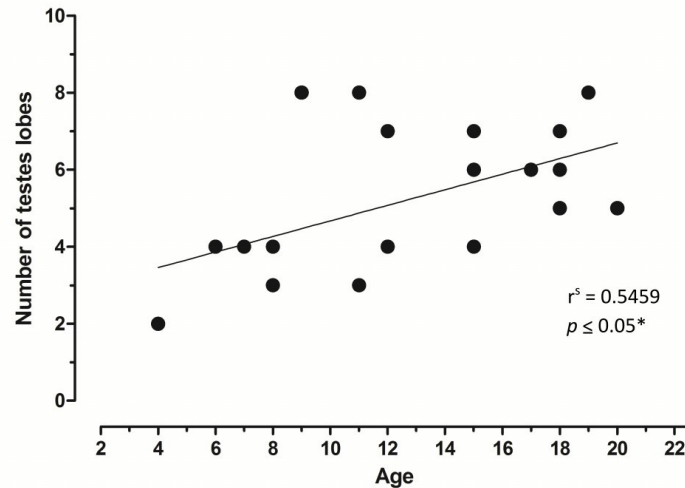


**Figure 3** Relations of age and fecundity traits in female *Salamandra algira* (n = 15).

**A** Bivariate correlation of snout-vent length (SVL) and clutch size.

**B** Bivariate correlation of age and egg volume.

**C** Bivariate correlation of age and clutch volume.



**Figure 4** Bivariate correlation of age and testes lobe number of male *Salamandra algira* (n = 21).

Females have comparatively high reproductive costs (e.g. MIAUD et al. 2001, SEGLIE et al. 2010) and an early maturation decreases the body size caused by the trade-off between growth and reproduction. This might influence the reproductive success negatively, because only large and numerous oocytes maximize the offspring number and fitness (SHINE 1979, ARNTZEN 2000, MARZONA et al. 2004), what is especially true for viviparous amphibians, as their fecundity is generally lowest compared to species that have aquatic or terrestrial eggs (DUELLMAN & TRUEB 1994, WELLS 2007). Furthermore, a lot of extrinsic factors can influence the fecundity. For example, populations from different altitudes are known to differ in reproductive and growth traits, especially as they have lower metabolic rates and shorter activity seasons and mature at a more advanced age (e.g. TILLEY 1973, WELLS 2007). The influence of altitude and temperature even led to a shift from lecithotrophic viviparity to matrotrophic viviparity within different populations of *S. a. tingitana* (BEUKEMA et al. 2010). We examined *S. algira* from a higher elevation region. They are dependent on shorter reproductive seasons during the cooler winter months, but how far they have a delayed maturity needs to be tested (in comparison with other populations). However, the given age data might suggest a delayed maturity, as only older males and females (starting at age 4+ and 5+) were present in the sample, which was collected during the breeding season in December. The absence of younger, immature salamander age groups is common in population studies (THIESMEIER 2004).

For males, the effects of body size are not so clear - their reproductive costs are relatively low and it might not be mandatory to delay sexual maturity to reach a larger body size. Therefore, selection seems to drive body size in different directions in the two sexes, but it is very important to consider the reproductive strategies and mating system of terrestrial salamanders. Males of *Salamandra* ssp. have been reported to engage in rivalry fights and do by all means profit from being larger and thus able to outcompete opponents (e.g. KÄSTLE 1986, BOGAERTS & DONAIRE-BARROSO 2005). These reports need to be considered, as well as the fact that rivalry and territoriality is not rare in urodeles (MATHIS et al. 1995). Male *S. algira* are also involved in rivalry fights (BOGAERTS & DONAIRE-BARROSO 2005) and a larger size should be an advantage (also during courtship and mating), but the sexes of *S. algira* do not show sexual dimorphism in general body size (REINHARD et al., submitted). Selection favors both large male and female size in *S. algira*, finally leading to a lack of sexual size dimorphism. Contrarily, the evolution of other dimorphic traits, such as enlarged limbs in males, is more important (REINHARD et al., submitted).

Age is related to testes lobe number in males. The relation has been demonstrated previously in some newt species such as *L. vulgaris* and *T. cristatus* (DOLMEN 1982, MALMGREN & THOLLESSON 1999) or *Cynops pyrrhogaster* (MARUNOUCHI et al. 2000). As females often prefer larger and older males (female choice, see HALLIDAY 1977), age and body size seem to be highly useful fitness indicators. This can be ascribed to both successful survival strategies and courtship success, as well as to an increased individual reproductive output due to a higher testes lobe number (and likely sperm number).

In general, it is advisable to consider age, fecundity traits, sexual dimorphism analysis and even ecological parameters as a whole to get an extensive insight into the mating systems of amphibians, as many parameters are tightly linked and mutually dependent. Further studies of additional terrestrial salamanders, either collection-based or in the field, would be very useful to proceed with understanding the evolution of life histories and reproductive strategies.



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## **APPENDIX**

Specimen of *Salamandra algira* examined:

ZSM 76/2013, 79/2013, 80/2013, 82/2013 - 84/2013, 86/2013, 91/2013, 94/2013 - 96/2013, 98/2013, 99/2013, 102/2013, 104/2013, 106/2013, 109/2013 - 112/2013, 115/2013 - 117/2013, 119/2013, 120/2013, 125/2013.

## CHAPTER 4

### **Sexual dimorphism in a French population of the Marbled Newt *Triturus marmoratus* (Urodela: Salamandridae)**

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## **ABSTRACT**

Amphibians have developed a large set of life-history strategies and demonstrate an impressive diversity of reproductive patterns, when compared to other vertebrates. Selection pressures on males and females to get a maximum of reproductive success and therefore produces different forms of sexual dimorphism. Here, we studied the pattern of sexual dimorphism of the marbled newt *Triturus marmoratus* from a French Population and included 27 body- and head-related characters into our analysis.

More than the half of these characters were sexually dimorphic between the sexes and most of the differences increased, when the data was adjusted for general body size before analysis. Thus, an adjustment for body size variation and the use of larger specimen series is important for the assessment of sexual dimorphism.

**Keywords.** Sexual dimorphism, salamanders, Urodela, *Triturus*, morphometrics.

## INTRODUCTION

Sexual dimorphism is a common phenomenon among animals. Opposing selection forces equilibrate differently in the sexes of the same species and are responsible for a variety of phenotypic differences (BLANCKENHORN 2005, FAIRBAIRN 2007). Sexual differences can be permanent or seasonal, whilst seasonal differences are often influenced by hormones and are tightly connected to reproduction (SHINE 1990, MALMGREN & THOLLESSON 1999). Some traits can be easily observed, for example body size and shape, as well as colouration (MALMGREN & THOLLESSON 1999). Concerning body size, examples of females or males that weigh or grow up to several times as much as their conspecific mates can be found (e.g. in some fishes, spiders or marine invertebrates; see BLANCKENHORN 2005). Female-biased sexual size dimorphism is common within invertebrates and poikilothermic vertebrates, whereas males are larger in most mammals and birds (ANDERSSON 1994, ABOUHEIF & FAIRBAIRN 1997, BLANCKENHORN 2005). Sexual and fecundity selection are the major forces that drive the evolution of sexual dimorphism, but ecological factors, e.g. resource utilization through niche partitioning, can contribute as well (SHINE 1989, MALMGREN & THOLLESSON 1999) and cause, for example, differences in head morphology due to differences in feeding strategies (MALMGREN & THOLLESSON 1999). If males are generally larger, they have an advantage in male-male combat or resource protection and this in turn increases their mating and thus reproductive success (e.g. KUPFER 2007). Females also profit from a large body size, as a lot of studies have shown that clutch size or the size of eggs and offspring, and consequently their reproductive success, increases with female size in poikilothermic animals (reviewed in BLANCKENHORN 2005). Thus, sexual dimorphism is often seen as a life-history adaptation (e.g. ENDLER 1983, MALMGREN & THOLLESSON 1999) and should be considered in the context of the species' biology, as natural and sexual selection are working together to determine the kind and the degree of sexual dimorphism (FICETOLA et al. 2013).

Amphibians show a unique diversity of life-history strategies (DUELLMAN & TRUEB 1994, KUPFER 2007). This makes them ideal for studies of sexual size dimorphism (SSD), which is important for understanding the evolution of mating systems. SSD is present in all amphibian orders (KUPFER 2007) and among salamanders most species



exhibit a female-biased size dimorphism, whereas male-biased dimorphism mainly occurs in species with aggressive or territorial behaviour (SHINE 1979). The large bodied Eurasian salamandrid genus *Triturus* is known for a high degree of sexual dimorphism (e.g. ANDERSSON 1994, MALMGREN & THOLLESSON 1999), which does not only include general body size but also body shape and secondary sexual characters, such as colouration or the development of dorsal crests (e.g. ANDERSSON 1994, MALMGREN & THOLLESSON 1999, KUPFER 2007).

To further contribute to the understanding of sexual dimorphism and the underlying mechanisms, we explored sexual differences in a French population of the large bodied marbled newt *Triturus marmoratus*. We discuss the evolution of sexually dimorphic traits in the context of the ecology and reproductive biology of the species and its close relatives.

## **MATERIAL AND METHODS**

### *Study animal*

*Triturus marmoratus* is widely distributed in South-west Europe, especially on the Iberian Peninsula and in large parts of Western France (GRIFFITHS 1996, HERRERO et al. 2003a). It has been elevated to species level as no signs of present or past hybridization were found (GARCÍA-PARIS et al. 1993, 2001). Beforehand, it was dealt as the nominate form *T. m. marmoratus* besides its sister subspecies *T. m. pygmaeus* (now *T. pygmaeus*), that occurs on the South-west Iberian peninsula and is much smaller than *T. marmoratus* (HERRERO et al. 2003a).

Marbled newts reproduce annually in stagnant or slow flowing waters or even in temporary pools with no specific association to a special vegetation type (GRIFFITHS 1996, HERRERO et al. 2003a). It is oviparous and adults leave the water after the end of the reproductive period (HERRERO et al. 2003a).

Sexual dimorphism is quite pronounced. Females are often reported to be the larger sex (e.g. FRANCILLON-VIEILLOT et al. 1990, HERRERO et al. 2003a). Furthermore, males are easily distinguishable from females, because they develop a crest on the back and dorsal tail, which regresses at the end of the annual reproductive period. However, the dorsal crest remains visible as a coloured stripe in terrestrial adult males (GRIFFITHS 1996). We have studied sexual dimorphism in a large series of

museum specimens housed at the Zoologische Staatssammlung München (ZSM, Bavarian state collection) of the Marbled newt *Triturus marmoratus*. Generally, measurements of large specimen series housed in natural history collections are key to analyse body trait differences between the sexes.

### *Morphometrics and analysis*

Twenty-seven morphometric characters were selected for analysis of body shape and sexual dimorphism in *T. marmoratus*. Body characters used were: overall length (OAL), snout-vent-length (SVL) from the tip of the snout to the posterior edge of the cloaca, tail length (TL) from posterior edge of the cloaca to the tip of the tail, tail height at mid-tail (TH), upper arm length from the posterior margin of the front leg (axilla) to the angle of the elbow (UAL), lower arm length from the angle of the elbow to the wrist (LAL), lower arm length measured until the tip of the longest finger (LAL2), total arm length from the axilla to the tip of the longest finger (TAL), upper leg length from the posterior margin of the hind leg (groin) to the angle of the knee (ULL), lower leg length from the angle of the knee to the ankle (LLL), lower leg length measured until the tip of the longest toe (LLL2), total leg length from the groin to the tip of the longest toe (TLL), cloacal length (CL), cloacal width (CW), body width at mid-body (BWM), chest-width (CHW) from axilla to axilla, distance from groin to groin (GG) and the distance of extremities (DEX) from axilla to groin. Additionally, crest height (CH) was measured for the males. The following head measures were used: head length (HL), head width at the angle of the jaw (HW), diameter of the eye (ED), orbito-narial-distance (ON) from the anterior edge of the eye to the nostril, interorbital-distance (IO) from one eye to the other in the centre of the eye, internarial-distance (IN) from nostril to nostril, eye-snout-distance (ES) from the anterior edge of the eye to the tip of the snout and intercanthal distance (IC) from one anterior edge of the eye to the other. All measures were taken to the nearest 0.01 mm using a digital caliper (Rok International Industry, Ltd.).

Altogether 35 males (ZSM 1831/2006 - 1836/2006, 1876/2006 - 1897/2006, 1910/2006 - 1916/2006) and 67 females (ZSM 1837/2006 - 1875/2006, 1898/2006 - 1909/2006, 1917/2006 - 1932/2006) from Banyuls-sur-mer (Pyrenees, southern France), which were clearly identified as adults, were used for analysis. Specimens

were sexed via externally visible sexual characters. The sexual dimorphism index (SDI) was calculated by dividing the mean snout-vent-length of the larger sex with the mean snout-vent-length of the smaller sex and subtracting one ( $SVL_{\text{large}}/SVL_{\text{small}} - 1$ ) following GIBBONS AND LOVICH (1990) to obtain a first conception of sexual size dimorphism (if the male measure is larger, the result needs to be multiplied with -1 again). All characters measured were tested for normality using the D'Agostino and Pearson omnibus normality test. Univariate t-tests (two-sided at  $\alpha \leq 0.05$ ) were used to test for dimorphism between sexes. In addition, SSD was assessed by an analysis of covariance (ANCOVA) using snout-vent-length as co-variable to adjust the characters to body size. All statistical tests were conducted using Microsoft Excel 2007, GraphPad Prism 5.01 and MedCalc 12.7.1.0 for Windows 7.

## RESULTS

The sexes of the marbled newt are characterised by a significant difference in body size. The size dimorphism index (SDI) after GIBBONS AND LOVICH (1990) was 0.049 for SVL, expressing that the females were about 5 % larger than males in our sample (the SDI for OAL accounts for even 0.078 and thus nearly 8 %). The parametric testing showed significant intersexual differences for the following traits: SVL, OAL, TL, TH, LAL2, ULL, LLL2, TLL, CW, CL, BWM, DEX, HW, HL, ON, IO and IN (see Table 1).

When adjusted to SVL, the ANCOVA approved some previous results, but it also revised some others and even identified some cryptic ones (see Table 2). This shows how important a proper adjustment of the measurement data is and that above all, larger differences in general body size can hide or falsify SSD results, when the data is not properly adjusted. A female may, for example, have smaller limbs in relation to general body size and a male may have larger limbs in relation to general body size, but the larger female body size might cover this aspect.

**Table 1.** Analysis of sexual size dimorphism in *Triturus marmoratus* (n = 35 males, n = 67 females). The means (M), standard deviations (SD), minima and maxima (range) of 27 characters are listed. Results of t-tests (two-tailed,  $\alpha = 0.05$ ) are shown in the last column.

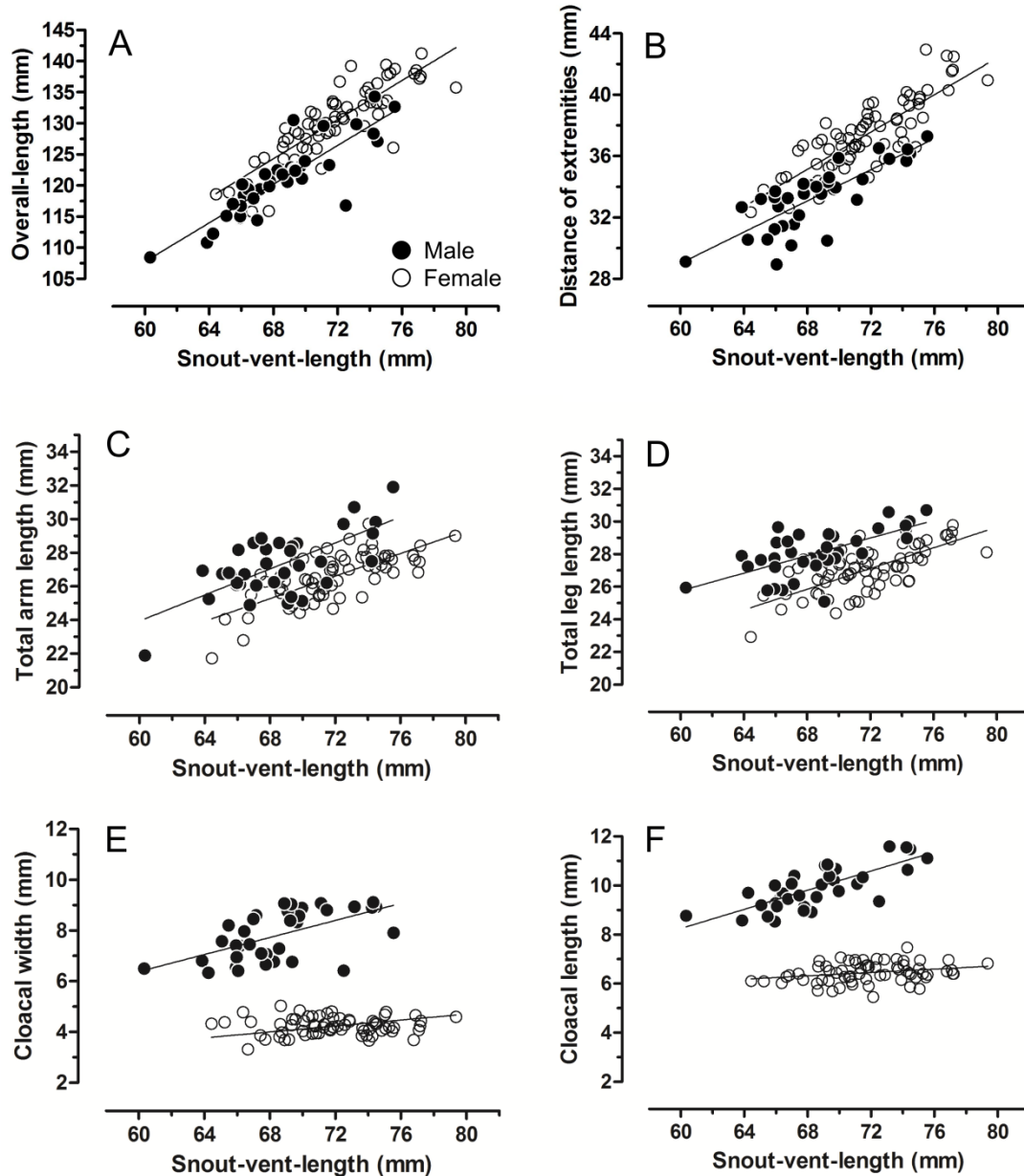
| Character   | Males (n = 35) |                 | Females (n = 67) |                 |          |
|-------------|----------------|-----------------|------------------|-----------------|----------|
|             | M ± SD         | Range           | M ± SD           | Range           | <i>P</i> |
| <b>Body</b> |                |                 |                  |                 |          |
| SVL         | 68.53 ± 3.36   | 60.34 - 75.55   | 71.9 ± 3.13      | 64.43 - 79.36   | < 0.001  |
| OAL         | 121.03 ± 6.04  | 108.46 - 134.31 | 130.44 ± 5.75    | 115.82 - 141.23 | < 0.001  |
| TL          | 52.5 ± 3.55    | 44.31 - 61.27   | 58.54 ± 3.49     | 48.17 - 66.4    | < 0.001  |
| TH          | 14.8 ± 2.42    | 11.07 - 19.83   | 12.22 ± 1.8      | 9.3 - 17.69     | < 0.001  |
| UAL         | 10.22 ± 1.05   | 7.43 - 13.03    | 10.03 ± 0.8      | 7.58 - 12.07    | n.s.     |
| LAL         | 7.95 ± 0.5     | 7.06 - 9.3      | 7.85 ± 0.65      | 6.73 - 9.75     | n.s.     |
| LAL2        | 17.02 ± 1.17   | 14.47 - 19.68   | 16.55 ± 1.0      | 12.68 - 18.35   | < 0.05   |
| TAL         | 27.24 ± 1.9    | 21.9 - 31.91    | 26.58 ± 1.47     | 21.73 - 29.72   | n.s.     |
| ULL         | 9.02 ± 0.73    | 7.1 - 10.15     | 9.37 ± 0.64      | 7.52 - 10.72    | < 0.05   |
| LLL         | 8.24 ± 0.69    | 6.98 - 9.31     | 8.47 ± 0.71      | 6.94 - 9.97     | n.s.     |
| LLL2        | 19.03 ± 0.99   | 17.06 - 20.71   | 17.72 ± 1.0      | 14.56 - 19.68   | < 0.001  |
| TLL         | 28.05 ± 1.39   | 25.09 - 30.7    | 27.08 ± 1.4      | 22.92 - 29.79   | < 0.01   |
| CW          | 7.81 ± 0.97    | 6.34 - 9.12     | 4.24 ± 0.34      | 3.32 - 5.03     | < 0.001  |
| CL          | 9.91 ± 0.87    | 8.53 - 11.6     | 6.45 ± 0.4       | 5.46 - 7.48     | < 0.001  |
| CHW         | 11.38 ± 1.02   | 8.6 - 13.61     | 11.62 ± 0.8      | 9.94 - 13.49    | n.s.     |
| BWM         | 14.3 ± 1.51    | 10.54 - 18.85   | 15.12 ± 1.36     | 12.64 - 18.22   | < 0.01   |
| GG          | 9.53 ± 0.83    | 7.24 - 11.41    | 9.32 ± 0.54      | 8.1 - 10.49     | n.s.     |
| DEX         | 33.35 ± 2.12   | 28.94 - 37.29   | 37.51 ± 2.32     | 32.35 - 42.96   | < 0.001  |
| CRH         | 3.52 ± 1.05    | 1.84 - 6.18     | -                | -               | -        |
| <b>Head</b> |                |                 |                  |                 |          |
| HW          | 12.4 ± 0.71    | 10.71 - 14.42   | 12.84 ± 0.71     | 11.64 - 14.97   | < 0.01   |
| HL          | 13.69 ± 0.67   | 12.36 - 14.77   | 14.11 ± 0.66     | 12.58 - 15.76   | < 0.01   |
| ED          | 3.8 ± 0.34     | 3.11 - 4.56     | 3.73 ± 0.26      | 3.26 - 4.43     | n.s.     |
| ON          | 4.25 ± 0.34    | 3.45 - 5.08     | 4.4 ± 0.33       | 3.51 - 5.1      | < 0.05   |
| IO          | 8.98 ± 0.56    | 7.88 - 10.13    | 9.19 ± 0.46      | 8.19 - 10.13    | < 0.05   |
| IN          | 3.0 ± 0.26     | 2.59 - 3.54     | 3.2 ± 0.27       | 2.71 - 4.01     | < 0.001  |
| ES          | 6.31 ± 0.49    | 5.18 - 7.31     | 6.4 ± 0.44       | 5.52 - 7.43     | n.s.     |
| IC          | 7.01 ± 0.42    | 6.08 - 7.82     | 6.95 ± 0.35      | 6.21 - 7.71     | n.s.     |

| Character adjusted for SVL | F      | P       |
|----------------------------|--------|---------|
| <b>Body</b>                |        |         |
| OAL                        | 34.32  | < 0.001 |
| TL                         | 34.32  | < 0.001 |
| TH                         | 57.44  | < 0.001 |
| UAL                        | 13.13  | < 0.001 |
| LAL                        | 8.31   | < 0.01  |
| LAL2                       | 40.74  | < 0.001 |
| TAL                        | 46.46  | < 0.001 |
| ULL                        | 0.03   | 0.867   |
| LLL                        | 0.16   | 0.688   |
| LLL2                       | 112.91 | < 0.001 |
| TLL                        | 68.16  | < 0.001 |
| CW                         | 744.98 | < 0.001 |
| CL                         | 969.03 | < 0.001 |
| CHW                        | 1.96   | 0.164   |
| BWM                        | 0.77   | 0.382   |
| GG                         | 22.44  | < 0.001 |
| DEX                        | 52.49  | < 0.001 |
| <b>Head</b>                |        |         |
| HW                         | 0.63   | 0.431   |
| HL                         | 0.55   | 0.460   |
| ED                         | 9.28   | < 0.01  |
| ON                         | 1.37   | 0.245   |
| IO                         | 0.79   | 0.376   |
| IN                         | 2.36   | 0.127   |
| ES                         | 11.39  | < 0.001 |
| IC                         | 18.81  | < 0.001 |

**Table 2.** Analysis of sexual size dimorphism in *Triturus marmoratus* (n = 35 males, n = 67 females). Results of one-factor ANCOVA comparing 26 traits of sexes relatively to snout-vent-length (SVL). F-values and significance levels are shown in the second and third columns.

Female marbled newts have a larger SVL, TL and thus OAL than conspecific males, and the ratio of body and tail length is significantly different in both sexes (tail 43.4 % in males and 44.9 % in females,  $p < 0.001^{***}$ ; body 56.6 % in males and 55.1 % in females,  $p < 0.001^{***}$ ). Additionally, females have a larger DEX of 3.5 % (all following percent values are adjusted to SVL). All other significant differences are larger in males, affecting TH (males are on average 4.6 % larger), UAL (0.9 %), LAL (0.7 %), LAL2 (1.8 %), TAL (2.8 %), LLL2 (3.2 %), TLL (3.2 %), CW (5.5 %), CL (5.5 %), GG (0.9 %), ED (0.3 %), ES (0.3 %) and IC (0.5 %). The male

average dorsal crest height is 3.52 mm (SD = 1.05 mm) and varies from 1.84 mm up to 6.18 mm. Dorsal crest height did not correlate with male SVL ( $r = 0.21$ ,  $p = \text{n.s.}$ ). See graphics of selected characters in Figure 1.



**Figure 1.** Sexual dimorphism of *Triturus marmoratus*. Some selected characters are shown: (A) Overall-length OAL - females are larger (B) Distance of extremities DEX - females are larger (C) Total arm length TAL - males are larger (D) Total leg length TLL - males are larger (E) Cloacal width CW - males are larger (F) Cloacal length CL - males are larger.

## DISCUSSION

Studies of sexual size dimorphism provide information about the general intersexual divergence of the same species and allow insights into the impact of selective forces on the size of males and females. Here, we demonstrate the importance of comparative analysis in relation to general body size (snout-vent-length). This approach reduces the risk that shape differences remain undiscovered, especially if the body size difference is very large. The marbled newt, *T. marmoratus*, is characterized by a variety of sexual differences. Females are clearly larger than males, as it was shown in previous studies (e.g. FRANCILLON-VIEILLOT et al. 1990, HERRERO et al. 2003a, JAKOB et al. 2002). In general, females differ in body size and males in shape or rather body proportions.

JAKOB et al. (2002) reported a significantly longer lifespan for female marbled newts as well as delayed sexual maturity (minimum age of mature males was 2 years, for females 3 years). The growth rate of males is higher (JAKOB et al. 2002) and the early maturation contributes to a smaller adult size, because the increase of resource usage for gametogenesis decreases the availability for somatic growth (MARZONA et al. 2004). A delayed sexual maturity and thus a higher body size are advantageous for female fecundity. This is also reflected in the manifestation of sexual dimorphism in females, because they were larger in body size-related traits only, especially in snout-vent-length and distance of extremities. This is in concordance with the fecundity model, which predicts that larger females are able to produce more or larger offspring, which increases their reproductive success (FAIRBAIRN 2007) and is common among amphibians (DUELLMAN & TRUEB 1994) and in the Salamandridae (REINHARD & KUPFER 2013). Above all, the distance of extremities is directly correlated to the length of the pleuropertitoneal cavity, which is the space for maturing eggs, and a larger trunk is therefore beneficial for female reproductive success (KALEZIC et al. 1992, MALMGREN & THOLLESSON 1999). Trunk length in turn directly affects snout-vent-length. Distance of extremities is an important dimorphic measure in other newt species, such as *T. cristatus* (MALMGREN & THOLLESSON 1999, KUBIŠOVÁ et al. 2007), *T. dobrogicus* (KUBIŠOVÁ et al. 2007), *Lissotriton vulgaris* (MALMGREN & THOLLESSON 1999) and *L. montandoni* (BABIK & RAFIŃSKI 2004). We postulate that this trait may be found in all species of the

European newt complex, and also in other species with female-biased dimorphism, but this remains to be proven in the future (see a summary in Figure 2).

Males are characterized by divergent body proportions compared to females, especially concerning the tail, the extremities and the cloaca. The dorsal crest, which develops during the breeding season, is the most remarkable trait and its size range is quite large. Studies of mate choice have indicated, that females are likely to respond easier to high-crested males (e.g. for *T. cristatus*, MALMGREN & ENGHAG 2008 or *Lissotriton vulgaris*, GREEN 1991). Crest height in the marbled newt males is likely to be accentuated by female choice. In crested newts, the crest serves as an indicator of good condition and additionally increases the surface area of the male body improving cutaneous respiration, ultimately supporting endurance during courtship (MALMGREN & THOLLESSON 1999). Another important trait is the tail fin. Males also have a significantly larger tail, due to their heightened fin. They perform tail-lashes during courtship and the size of the fin can be decisive for pheromone transfer. A larger tail creates a stronger water flush, especially during amplifying male behavioural signals, such as the "Whip" or the "Whiplash" (see ARNTZEN & SPARREBOOM 1989 and SPARREBOOM & TEUNIS 1990 for further information on behavioural patterns during courtship), that potentially transfer pheromones more efficiently. The extensive courtship displays requires a firm foothold, so enlarged limbs are very useful. Especially the lower parts of the limbs are more sexually dimorphic and aid in stabilizing the courtship display. This improves the performance and can be observed in other species with a similar courtship behaviour as well, especially in *T. cristatus* (MALMGREN & THOLLESSON 1999). A longer distance between the male groins may indicate a slightly broader pelvic girdle. Thus, the femurs attach with a wider gap and additionally stabilize the posture during courtship. Direct measurements on dry bone material or non-invasive micro-CT scanning could provide further insights on this matter.

Furthermore, males of *T. marmoratus* have longer and wider cloacae than females. The male cloaca is equipped with a complex set of glands, which release courtship pheromones and produce the sperm containing stalk and cap for the spermatophores (SEVER 2003). Because this trait is sexually selected and directly increases the male reproductive success (MALMGREN & THOLLESSON 1999, SEVER 2003), the male cloaca is more swollen than the female one (HALLIDAY 1990, VERREL 1989). Sexual

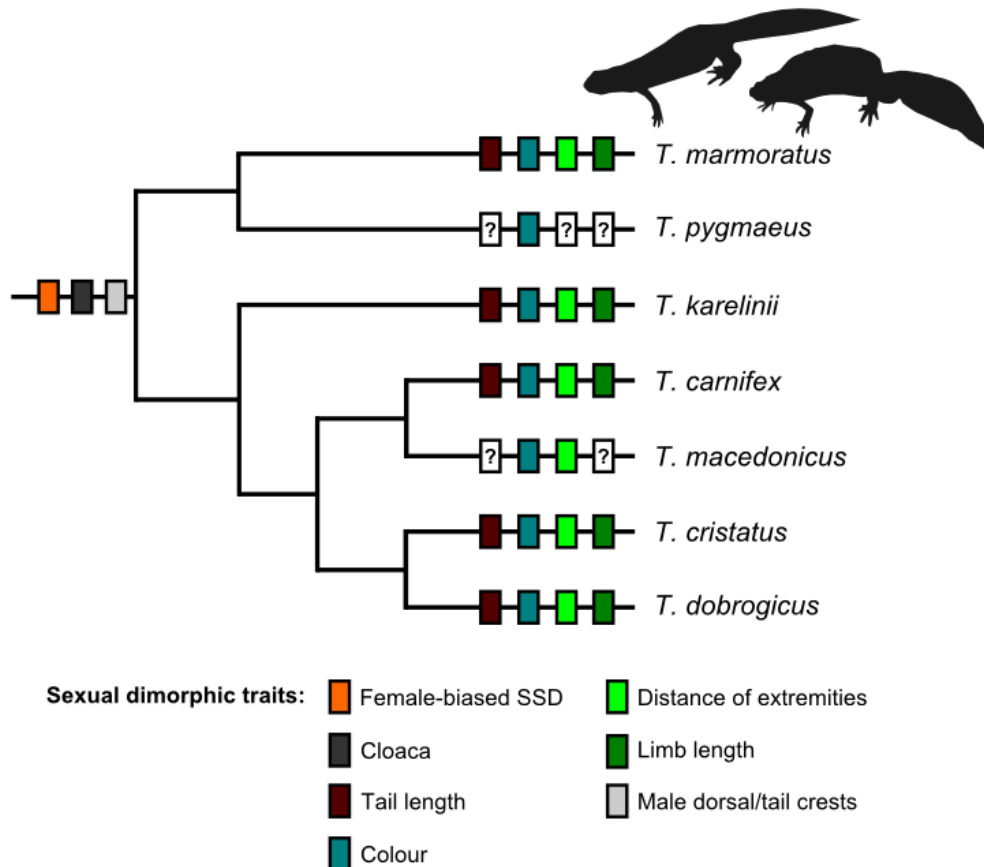


hormones, which are connected with maturation and sexual activity, control the development of the cloacal glands and are thus hypertrophied during the breeding season (SEVER 2003). Courtship pheromones are very important, as they can induce all typical features of female mating behaviour and responses preceding the final spermatophore pick-up (TREER et al. 2013). Interestingly, visual cues (once regarded as key cues, see HIMSTEDT 1979) are less important compared to olfactory cues during courtship in the newts *Ichthyosaura alpestris* and *Lissotriton helveticus*, because females responded to induced pheromones of their own species, but at the same time followed heterospecific female newts (TREER et al. 2013). This shows the significance of courtship pheromones, cloacal dimorphism, and its direct effect on male reproductive success.

Some head-related characters are sexually dimorphic as well, but only the shape (sexual shape dimorphism SShD) and not head size was affected. This absence of SSD, but the presence of SShD is exceptional (SCHWARZKOPF 2005, IVANOVIĆ & KALEZIĆ 2012), but can be found for instance also in other newts such as *L. vulgaris* (IVANOVIĆ & KALEZIĆ 2012). Whether skull characters differ in the two sexes and cause shape differences of external head characters needs to be further analysed.

According to the ecological model, differences in food resource usage might influence intersexual shape differences (e.g. SHINE 1989). In fact, LIZANA et al. (1986) found differences in food preferences between juvenile and adult Marbled newts and as well between the sexes in a population in Salamanca (HERRERO et al. 2003a), but to what extent ecology affects skull shape and geometry needs to be tested. Geometric morphometrics can be directly studied on dried skull material or better using non-invasive  $\mu$ -CT-scanning of ethanol preserved specimens (see also STOCK et al. 2003, IVANOVIĆ et al. 2013).

Because different selection pressures interact and directly influence the diverse manifestations of sexual dimorphism, they should always be considered in close association.



**Figure 2.** Summary of sexual dimorphic traits within the genus *Triturus*. The phylogeny was modified after WIELSTRA & ARNTZEN 2011. References are listed in the Appendix.

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## APPENDIX

### *References and suggested reading for Figure 2*

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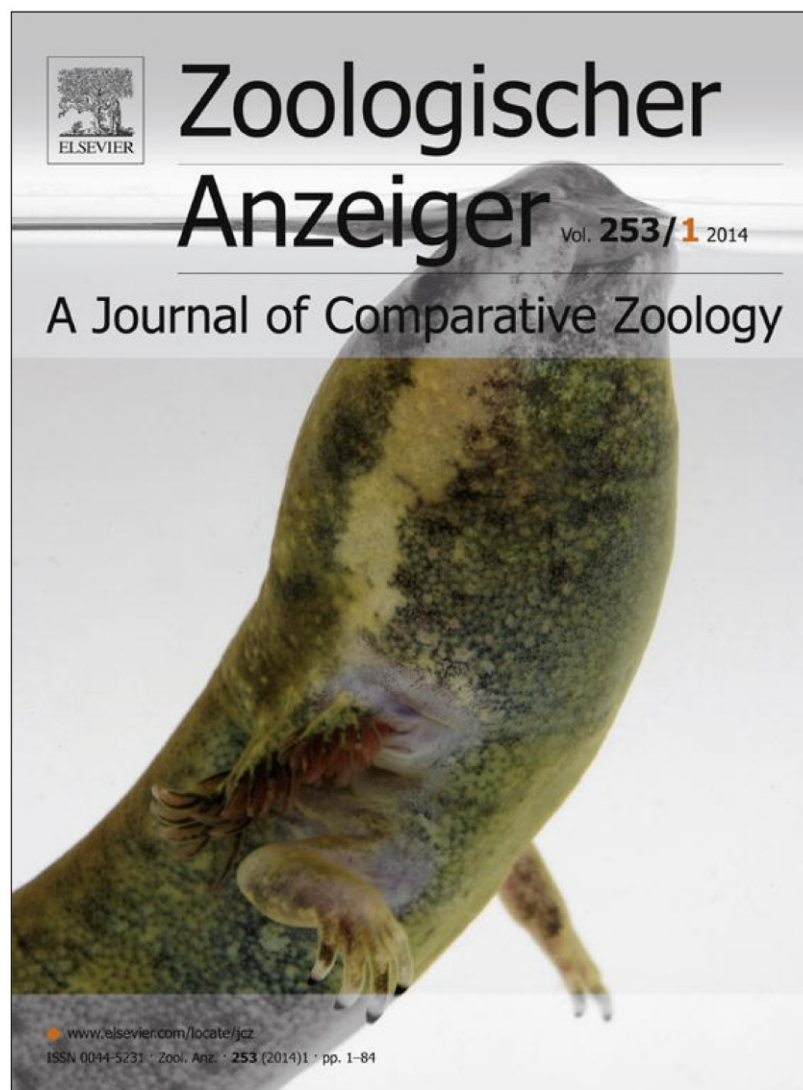


## CHAPTER 5

### **External fertilization and paternal care in the paedomorphic salamander *Siren intermedia* Barnes, 1826 (Urodela: Sirenidae)**

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## External fertilisation and paternal care in the paedomorphic salamander *Siren intermedia* Barnes, 1826 (Urodela: Sirenidae)

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### ABSTRACT

Parental care is widespread in the animal kingdom and enhances offspring survival. Amphibians exhibit an extraordinary diversity of care strategies, including guarding, transport and even feeding of young. Among amphibians, females are usually the carers, but here we present a case of male parental care in the aquatic salamander *Siren intermedia*, accompanied by records of external fertilisation. Sirenids are a phylogenetically distinct group within basal salamanders, of which the precise systematic position has long been debated. Our observations of external fertilisation and paternal care of *S. intermedia* lead us to conclude that apparently both internal fertilisation and maternal care evolved after the split between the more basal groups (Cryptobranchioidea and Sirenoidea) and all other salamanders (suborder Salamandroidea).

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### 1. Introduction

Parental care is a widespread, fitness-increasing behaviour shown by parents towards their offspring (Trivers, 1972; Gonzalez-Voyer and Kolm, 2010). Caring behaviour is connected to variable degrees of parental expenditure, i.e., parental investment, and may therefore reduce the survival and the ability to invest in additional offspring (Duellman and Trueb, 1994; Gonzalez-Voyer and Kolm, 2010). Especially amphibians exhibit an extraordinary diversity of parental care strategies (Duellman and Trueb, 1994), e.g., including egg-attendance, transport of eggs or larvae and even feeding of offspring, as known from dendrobatid frogs (Brown et al., 2010) and skin-feeding caecilians (Kupfer et al., 2006; Wilkinson et al., 2008). The variety of strategies to protect and nurture offspring seems to be higher in amphibians than in all other vertebrate classes and must have evolved independently in all three major orders of amphibians (Stebbins and Cohen, 1997).

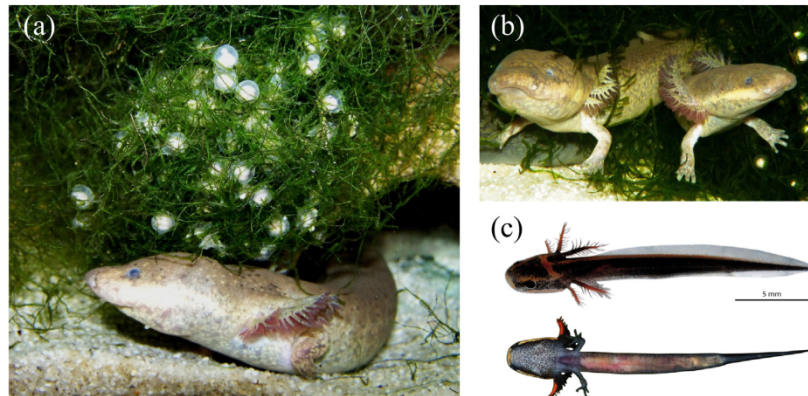
Most commonly females stay with terrestrial egg clusters enhancing their survivorship through aeration, moistening, predator protection and removal of dead or fungus-infected eggs (Duellman and Trueb, 1994). Although parental care behaviour such as egg guarding in salamanders is known from most families (e.g., Noble and Marshall, 1932; Hubbs, 1962; Nussbaum, 1969;

Godley, 1983; Russell et al., 2002; Nussbaum, 2003; see a summary in the online Appendix), parental care in aquatic breeders is rare. If present, usually the females of stream-breeding species are the carers, whereas pond-breeding species typically provide no care (Duellman and Trueb, 1994; Wells, 2007). Paternal care is frequent in frogs and toads, but much rarer in salamanders. For example, the external fertilising cryptobranchids are known to accomplish paternal care (Kawamichi and Ueda, 1998; Petranks, 1998).

The aquatic salamander family Sirenidae consists of two genera: *Siren* and *Pseudobranchius*. These obscure peribranchiate/neotenesous salamanders are considered the sister group of all modern salamanders/suborder Salamandroidea (e.g., Roelants et al., 2007; Pyron and Wiens, 2011). Sirenid reproductive biology, including the mode of fertilisation and the provision of parental care, has long been an enigma and subject of debate on the evolution of amphibians (e.g., Sever et al., 1996). Two conflicting hypothesis concerning the fertilisation mode exist for many decades. The external fertilisation hypothesis builds on the fact that female *Siren intermedia* Barnes, 1826 lack any dedicated cloacal structures for sperm storage (Sever et al., 1996; Sever, 2003) and males apparently miss cloacal glands for spermatophore production (Sever et al., 1996; Sever, 1991, 2003). On the other hand internal fertilisation is suggested within the family, too, because eggs laid in small clusters over a longer period in *Pseudobranchius striatus* (LeConte, 1824) apparently seem too difficult to be fertilised externally (Goin and Goin, 1962). We finally try to clear up this matter by reporting on paternal care and external fertilisation in *S. intermedia*.

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**Fig. 1.** Reproductive biology of *Siren intermedia*. (A) Male *S. intermedia* guarding eggs, singly placed in plant material. (B) Mating *S. intermedia* – note the pronounced sexual dimorphism in head size of the male (left) in comparison to the female (right). (C) Larva of *S. intermedia*, approximately seven days after hatching.

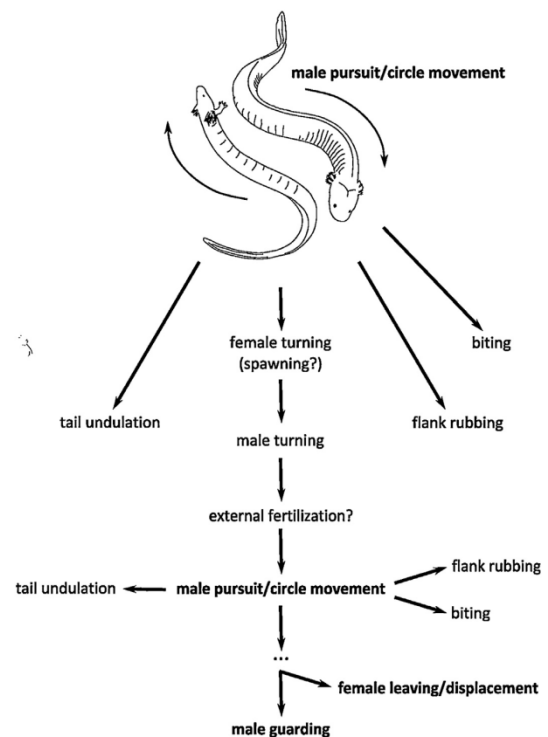
## 2. Materials and methods

Our observations on mating behaviour and parental care in *S. intermedia* were made in the laboratory. A breeding population of two males and two females were kept as pairs in large tanks (100 cm × 40 cm, 120 cm × 40 cm) and a second breeding set-up consisted of one male and three females. Sexes could be easily distinguished, because males have larger heads and are generally larger (mean total length  $411 \pm 12$  mm) than females ( $369 \pm 30$  mm, but see also in Godley, 1983; Petranka, 1998; Fauth and Resetarits, 1999). Usually individuals were housed separately, but for breeding purposes they were kept together during the winter months. The tanks were filled with tap water to a level of about 40 cm, filtered using a Hamburg mat filter and aerated by air pumps and air stones. A thin layer of fine aquarium sand covered the tank bottom, semicircular tube caves served as hiding places and a dense vegetation of floating java moss completed the aquarium setup. Water temperatures ranged from 22 °C to 24 °C in the summer to 12–15 °C during winter and spring. The light cycle was set at 11:13 daylight/night and completed by natural dusk/dawn through an opposite window. Animals were fed *ad libitum* a diet of earthworms, fish, shrimps, small locusts or crickets and blood worms. Courtship and parental care behaviour was documented using a Kodak ZD710 digital camera.

## 3. Results

### 3.1. Courtship

From November to January males occupied a shelter as a nest site, which they actively equipped with java moss (plucking the plant material and dragging it into the nest site, see also Fig. 1A) and defended it aggressively against conspecifics through biting. We observed the first courtship sequences at the nest site in late January, which lasted for several hours. Courtship included several repeating patterns (see Fig. 2; Video 1 in online appendix). The couple was steadily moving in circles within the nest site, coiling around each other and around the java moss. The male pursued the female in close contact to the females' cloaca and sometimes rubbed its head against the flank and the cloacal region of the female, slightly vibrating its head in slow shivering motions. During courtship both sexes waved their tail fins by undulating the tail tip. During oviposition the female turned on its back, positioned the cloaca near the top of the nest cavity and halted for



**Fig. 2.** Behaviour patterns of *Siren intermedia* during and after courtship. For behavioural terms follow Houck and Arnold (2003). Tail undulation and flank rubbing can invariably occur during the circle movement behaviour, but never during the turning-on-back movement.

several seconds. Then the male turned on its back and positioned the cloaca near the site of oviposition. The male stayed motionless for longer than the female, then abruptly jerked with the whole body and turned back into normal position, repeating the circle movement around the female. The couple repeated this behaviour



over and over again. Cloacal apposition was not observed, as the male touched the female cloaca steadily with its head. Eggs were directly coiled into the java moss (Fig. 1A).

The male behaved more aggressively later during courtship bouts by biting the female into its flanks and near the cloaca. It appeared as if the male started biting to force the courtship to continue, whilst the female made efforts to leave the nest site.

### 3.2. Paternal care

After the female completed oviposition and left the nest, the male stayed in close proximity to the eggs. Eggs placed in several layers into the java moss were situated in the middle of the nest and the male coiled around them (Fig. 1A). The total number of eggs could not be counted, because the risk of disturbing the behaviour was too high, but we estimated there were approximately 120–130 eggs with an ovum diameter of about 3 mm. If disturbed, the male showed varying degrees of aggression, i.e., intruding females were driven away by biting (see Video 2 in online Appendix), which sometimes left them heavily injured. Any other moving objects were attacked, too (e.g., tweezers with food – active feeding was therefore avoided after the first aggressive occurrence). Next to clutch defence, the male showed other behaviours likely increasing embryo survival. The male constantly moved the egg mass, circled around it and aerated the eggs through vigorous tail fanning. Additionally, the male continuously cleaned the nest by swallowing aquarium sand and releasing it out through its gill slits (see Video 3 in online Appendix). The development of the larvae took approximately 35 days. Paternal care continued after the larvae hatched (see larva in Fig. 1C) and the male aggressively defended the larvae. Both the male and the larvae left the nest occasionally but returned to the nest cavity. Larvae were associated with the male for up to one week after hatching. Even if the male had the chance to choose between different hiding sites after disturbance, it returned to its nesting cavity and to the larvae (several hiding sites were provided, but ignored by the male, except for the original nesting site).

## 4. Discussion

Although amphibians have evolved a variety of parental care strategies, usually females of both aquatic and terrestrial breeding urodeles provide care after oviposition (Wells, 2007) (comp. Table 1), whereas paternal care as observed in the aquatic salamander species *S. intermedia* is rare and exceptional for urodeles and likewise only occurs in lotic cryptobranchids (hynobiids are reported to have paternal care, but it should rather be considered as scramble competition between males – comp. Tables 1 and S1 (in the online Appendix and the text below), whereas further post-hatching care apart from *Siren* is only known from the plethodontid *Hydromantes strinati* (Aellen, 1958) (see Oneto et al., 2010).

**Table 1**  
Summary of the number of species exhibiting parental care among the Urodela.

| Family/taxon         | Maternal care<br>(n species) | Paternal care (n<br>species) | Biparental care<br>(n species) |
|----------------------|------------------------------|------------------------------|--------------------------------|
| Hynobiidae (54)      | 1 <sup>a</sup>               | 8 <sup>a</sup>               | 1 <sup>a</sup>                 |
| Cryptobranchidae (3) | –                            | 3                            | –                              |
| Sirenidae (4)        | 2 <sup>a</sup>               | 1                            | –                              |
| Ambystomatidae (32)  | 1                            | –                            | –                              |
| Dicamptodontidae (4) | 4                            | –                            | –                              |
| Salamandridae (94)   | 5                            | –                            | –                              |
| Proteidae (6)        | 4                            | 1 <sup>a</sup>               | 1 <sup>a</sup>                 |
| Rhyacotritonidae (4) | 1 <sup>a</sup>               | –                            | –                              |
| Amphiumidae (3)      | 2                            | –                            | –                              |
| Plethodontidae (431) | 36                           | 1 <sup>a</sup>               | 2 <sup>a</sup>                 |

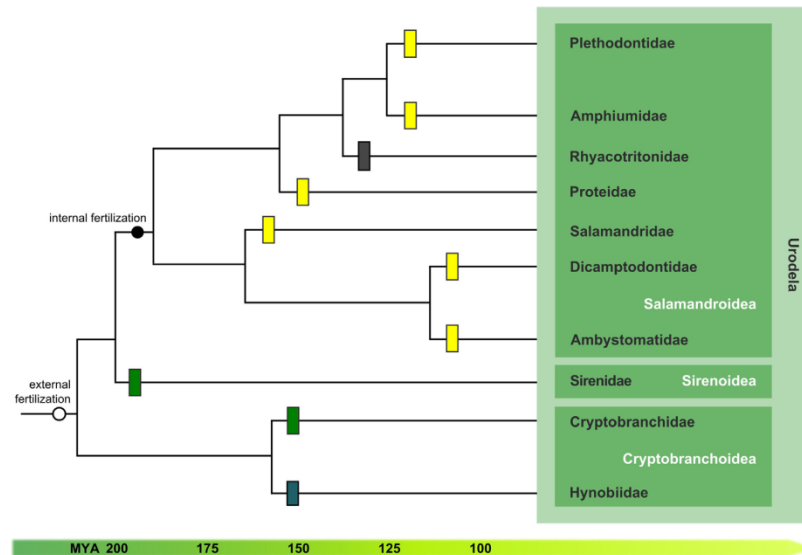
<sup>a</sup> Observations are based on uncertain or contradicting data and need further validation – see also Table S1 in Appendix for detailed information.

Earlier adult *S. intermedia* were observed staying with larvae (Hubbs, 1962), although the sex was not determined.

Parental care modes of vertebrates such as amphibians and fish show an evolutionary relationship between the sex of the carer and the mode of fertilisation (Ridley, 1978; Gross and Sargent, 1985). Amphibians and teleosts show some convergences in reproductive adaptations with both sexes involved (Gross and Shine, 1981). Male care is more likely to occur in external fertilising species (Ridley, 1978), whereas female care is common in internally fertilising species (Gross and Shine, 1981). Male parental care is predominant in fishes and 76% of this care is linked to external fertilisation and territorial defence of the spawning site (Gross and Sargent, 1985). For amphibians, the correlation between fertilisation mode and the sex showing care is highly significant, too (Gross and Shine, 1981). We provide the first direct evidence of external fertilisation in a sirenid salamander. External fertilisation has been assumed previously, because males lack dedicated cloacal glands for spermatophore production and females lack spermathecae (see Salthé, 1967; Sever et al., 1996; Sever, 2003), as known from internally fertilising salamanders. Ultsch (1973) also concluded that *Siren lacertina* has external fertilisation, because field-collected females laid unfertilised eggs. If they had been able to store sperm, they should have laid fertilised eggs. In external fertilising species the male has to follow the female for fertilisation if eggs are placed singly or in small clusters (e.g., Indiviglio, 2010), but the “egg-laying-fertilising” alternations during close contact observed in *S. intermedia* would more successfully secure fertilisation and the interlacing of the eggs into the plant material ensures the singly laid eggs to adhere as a compact mass, also facilitating external fertilisation. The tail-fanning behaviour of the male towards the eggs might additionally enhance sperm dispersal. *Pseudobranchius* species, belonging to the second genus of the family Sirenidae, are known to lay their eggs singly or in small clumps (Salthé, 1967; Ultsch, 1973; Petranksa, 1998; Kowalski, 2004), which seems to rule out external fertilisation, but females lack spermathecae, too, as seen in *Siren* (Sever, 1991; Wells, 2007).

Male nest building activity and aggression prior to courtship clearly indicate territoriality. If one sex occupies a territory including a nest site before courtship and fertilisation, it may be selected to care for the offspring as a consequence of its territoriality, confirming the “association hypothesis” (Gross and Shine, 1981; Nussbaum, 2003). Females of external fertilising species therefore lay their eggs inside the males’ territory and can leave it afterwards (Ridley, 1978), as confirmed for *S. intermedia*.

Sexual size dimorphism (SSD) in salamanders is usually female-biased (Shine, 1979), but in *Siren* males are not only the larger sex, they also have significantly larger heads and enlarged masseter muscles compared to females (Godley, 1983; Petranksa, 1998; Fauth and Resetarits, 1999) (see Fig. 1B). As males are engaged in parental care, male size likely relates to the successful defence of territories and clutches against predators and might have advantages in direct combat. Natural selection should favour larger males size, if it serves efficient offspring protection. A similar adaptive cause for male-biased SSD is known for the giant African bullfrog, *Pyxicephalus adspersus* Tschudi, 1838, a very large and compact ranid, where females reach around 50% of the male snout-vent-length (about 200 mm) and males defend eggs and tadpoles (Cook et al., 2001). Within the phylogenetically basal Cryptobranchioidea male-biased sexual dimorphism of some *Hynobius* species engaged in male scramble competition for eggs includes enlarged hindlimbs and heads, e.g., *Hynobius nigrescens*, Stejneger, 1907 (Hasumi and Iwasawa, 1990; Park and Park, 2000) during the breeding season. Despite being involved in territorial contests and paternal care male giant salamanders *Andrias* and hellbenders *Cryptobranchus* show no pronounced sexual dimorphism, except for a seasonal cloacal swelling (Shine, 1979; Makowsky et al., 2010; Sparreboom, 2012).



**Fig. 3.** Phylogenetic relationships (modified after Pyron and Wiens, 2011), modes of parental care and fertilisation of extant salamanders. Modes of parental care are shown in coloured squares: blue – parental care absent or paternal (male) care, green – paternal care, yellow – maternal (female) care, grey – parental care absent. Numbers at nodes represent divergence times in Mya, taken from TimeTree (Hedges et al., 2006). (For interpretation of the colour legend used in this figure, the reader is referred to the pdf version of this article.)

Extensive care for eggs in *Siren* included active cleaning of the nest site to improve hygienic conditions and possibly to prevent infections from pathogens, e.g., through aquatic fungi. Although not recorded, it is likely that males also remove dead or infected eggs to prevent further infection.

As sirens can occur in high densities with up to 2.17 individuals per m<sup>2</sup>, egg predation is very likely and was confirmed several times (Scroggins and Davis, 1956; Collette and Gehlbach, 1961; Frese et al., 2003; Hampton, 2009). Since embryonic development of large eggs is slower than that of small eggs (Nussbaum, 1987), egg predation is prevented during paternal care. Males showing bite marks especially after oviposition (Hampton, 2009) results from nest defence against conspecifics. Usually it is inefficient for lentic breeders to increase investment into large eggs and proportionally larger hatchlings, because the longer developmental time of large eggs imposes higher embryonic mortality (Nussbaum, 1987). In *S. intermedia*, however, an increased maternal investment into egg size is secured by paternal care, increasing both embryonic and larval survival.

The phylogenetic position of sirenid has long been debated, but more recent phylogenetic hypothesis places them as sister group to the Salamandroidea (e.g., Roelants et al., 2007; Pyron and Wiens, 2011). Paternal care behaviour in combination with external fertilisation of *S. intermedia* supports the current phylogenetic hypothesis (e.g., Pyron and Wiens, 2011) from an ethological perspective. Hynobiids are thought to resemble the most recent common ancestor of all salamanders (Zhang et al., 2006, see also Fig. 3). Although males of some hynobiid salamanders have been reported in association with egg sacs, male guarding likely prevents multiple insemination and sperm competition in a scramble competition mating system, rather than serving the defence against predators. Cryptobranchid and sirenid salamanders share paternal care behaviours (comp. Kawamichi and Ueda, 1998; Petranka, 1998) and an external fertilisation mode. Males are territorial, usually care for egg clutches, whereas females leave after spawning.

Future studies focusing on the significance of paternal care for the genetic mating system of sirenid salamanders should apply molecular markers, e.g., polymorphic microsatellites. The evolutionary shift from paternal to maternal care among the suborder Salamandroidea is likely linked to the evolution of internal fertilisation via a spermatophore in males in combination with female spermathecae.

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#### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jcz.2013.06.002>.

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## APPENDIX

**Table S1** Summary of species that exhibit parental care among the Urodela. ♀ Female care, ♂ Male care, = biparental care. Some observations are based on uncertain or contradicting data and need to be checked (marked with a ?). In the case of the Hynobiidae, it seems that male paternal care is a post-insemination guarding behaviour, not a true paternal egg guarding, as there is a scramble competition situation among males.

| Species                             | ♀  | ♂  | =  | References  |
|-------------------------------------|----|----|----|---|
| <b>Hynobiidae</b>                   |    |    |    |   |
| <i>Hynobius arisanensis</i>         |    |    | *? | Hou et al., 2010  |
| <i>Hynobius kimurai</i>             |    | *  |    | Nussbaum, 1985, 2003; Sparreboom, 2012  |
| <i>Hynobius leechi</i>              |    | *  |    | Park & Park, 2000   |
| <i>Hynobius maoershanensis</i>      |    | *  |    | Sparreboom, 2012  |
| <i>Hynobius naevius</i>             |    | *  |    | Nussbaum, 1985, 2003  |
| <i>Hynobius nebulosus</i>           |    | *  |    | Thorn, 1962; Nussbaum, 2003; Sparreboom, 2012                                 |
| <i>Hynobius retardatus</i>          |    | *  |    | Thorn, 1962; Sparreboom, 2012   |
| <i>Hynobius stejnegeri</i>          | *? |    |    | Sparreboom, 2012  |
| <i>Hynobius yangi</i>               |    | *  |    | Sparreboom, 2012  |
| <i>Ranodon sibiricus</i>            |    | *? |    | Thorn, 1994; Sparreboom, 2012   |
| <b>Cryptobranchidae</b>             |    |    |    |   |
| <i>Andrias davidianus</i>           |    | *  |    | Nussbaum, 2003; Wells, 2007; Sparreboom, 2012                                 |
| <i>Andrias japonicus</i>            |    | *  |    | Kerbert, 1904; Nussbaum, 1985   |
| <i>Cryptobranchus alleganiensis</i> |    | *  |    | Smith, 1906; Ridley, 1978; Nussbaum, 1985                                     |
| <b>Sirenidae</b>                    |    |    |    |   |
| <i>Siren intermedia</i>             | *? | *  |    | Noble & Marshall, 1932; Hubbs, 1962; Godley, 1983; Nussbaum, 1985; this study |
| <i>Siren lacertina</i>              | *? |    |    | Ultsch, 1973  |
| <b>Amphiumidae</b>                  |    |    |    |   |
| <i>Amphiuma means</i>               | *  |    |    | Weber, 1944; Ryan, 1977   |
| <i>Amphiuma tridactylum</i>         | *  |    |    | Hay, 1888; Fontenot, Jr., 1999  |
| <b>Dicamptodontidae</b>             |    |    |    |   |
| <i>Dicamptodon aterrimus</i>        | *  |    |    | Nussbaum, 1969  |
| <i>Dicamptodon copei</i>            | *  |    |    | Nussbaum, 1985, 2003  |
| <i>Dicamptodon ensatus</i>          | *  |    |    | Nussbaum, 1969, 1985, 2003; Ryan, 1977  |
| <i>Dicamptodon tenebrosus</i>       | *  |    |    | Nussbaum, 1969  |
| <b>Plethodontidae</b>               |    |    |    |   |
| <i>Aneides aeneus</i>               | *  |    |    | Canterbury & Pauley, 1994   |
| <i>Aneides ferreus</i>              | *  | *? |    | Dunn, 1942; Storm, 1947   |
| <i>Aneides lugubris</i>             | *  | *? |    | Ritter & Miller, 1899   |
| <i>Atylodes genei</i>               | *  |    |    | Sparreboom, 2012  |
| <i>Batrachoseps wrighti</i>         | *  |    |    | Tanner, 1953  |
| <i>Bolitoglossa diminuta</i>        | *? |    |    | Robinson, 1976  |
| <i>Bolitoglossa pesrubra</i>        | *  | *  |    | Vial, 1968  |
| <i>Desmognathus apalachicola</i>    | *  |    |    | Nussbaum, 2003  |

---continued---

|                                   |          |  |
|-----------------------------------|----------|--|
| <i>Desmognathus auriculatus</i>   | *        | Petranka, 1998; Nussbaum, 2003                               |
| <i>Desmognathus brimleyorum</i>   | *        | Trauth, 1988; Nussbaum, 2003                                 |
| <i>Desmognathus carolinensis</i>  | *        | Nussbaum, 2003   |
| <i>Desmognathus fuscus</i>        | *        | Petranka, 1998; Nussbaum, 2003                               |
| <i>Desmognathus monticola</i>     | *        | Nussbaum, 1985, 2003; Bruce, 1990                            |
| <i>Desmognathus ochrophaeus</i>   | *        | Tilley, 1972; Forester, 1981; Bruce, 1990;<br>Nussbaum, 2003 |
| <i>Desmognathus ocee</i>          | *        | Nussbaum, 2003   |
| <i>Desmognathus orestes</i>       | *        | Petranka, 1998; Nussbaum, 2003                               |
| <i>D. quadramaculatus</i>         | *        | Nussbaum, 1985, 2003; Petranka, 1998                         |
| <i>Desmognathus santeetlah</i>    | *        | Jones, 1986  |
| <i>Desmognathus welteri</i>       | *        | Nussbaum, 1985, 2003; Petranka, 1998                         |
| <i>Desmognathus wrighti</i>       | *        | Petranka, 1998   |
| <i>Ensatina eschscholtzii</i>     | *        | Stebbins, 1954; Petranka, 1998                               |
| <i>Eurycea bislineata</i>         | *        | Nussbaum, 1985, 2003; Petranka, 1998                         |
| <i>Eurycea cirrigera</i>          | *        | Niemiller & Miller, 2007                                     |
| <i>Eurycea junaluska</i>          | *        | Petranka, 1998; Nussbaum, 2003                               |
| <i>Eurycea longicauda</i>         | *        | Nussbaum, 1985, 2003; Petranka, 1998                         |
| <i>Gyrinophilus porphyriticus</i> | *        | Nussbaum, 1985, 2003   |
| <i>Hemidactylium scutatum</i>     | *        | Harris & Ludwig, 2004; Wells, 2007                           |
| <i>Plethodon cinereus</i>         | *        | Bachmann, 1984   |
| <i>Plethodon dorsalis</i>         | *        | Wilkinson et al., 1993; Petranka, 1998                       |
| <i>Plethodon glutinosus</i>       | *        | Petranka, 1998   |
| <i>Plethodon nettingi</i>         | *        | Lannoo, 2005   |
| <i>Plethodon welleri</i>          | *        | Petranka, 1998   |
| <i>Pseudotriton montanus</i>      | *        | Nussbaum, 1985, 2003; Petranka, 1998                         |
| <i>Pseudotriton ruber</i>         | *        | Petranka, 1998   |
| <i>Speleomantes imperialis</i>    | *        | Sparreboom, 2012   |
| <i>Speleomantes strinatii</i>     | *        | Oneto et al., 2010   |
| <b>Proteidae</b>                  |          |  |
| <i>Necturus beyeri</i>            | *        | Nussbaum, 2003   |
| <i>Necturus lewisi</i>            | *        | Lannoo, 2005   |
| <i>Necturus maculosus</i>         | *        | Eycleshymer, 1906; Nussbaum, 1985, 2003                      |
| <i>Proteus anguinus</i>           | *? *? *? | Nussbaum, 1985, 2003   |
| <b>Rhyacotritonidae</b>           |          |  |
| <i>Rhyacotriton kezeri</i>        | *?       | Nussbaum, 1969   |
| <b>Ambystomatidae</b>             |          |  |
| <i>Ambystoma opacum</i>           | *        | Croshaw & Scott, 2005  |
| <b>Salamandridae</b>              |          |  |
| <i>Euproctus platycephalus</i>    | *        | Alcher, 1981   |
| <i>Pachytriton brevipes</i>       | *        | Thiesmeier & Hornberg, 1992                                  |
| <i>Pachytriton labiatus</i>       | *        | Nussbaum, 2003; Sparreboom, 2012                             |
| <i>P. caudopunctatus</i>          | *        | Sparreboom, 2012   |
| <i>Salamandrina perspicillata</i> | *        | Boscherini & Romano, 2011                                    |



## ADDITIONAL REFERENCES FOR TABLE S1

Some references of Table S1 are listed in the reference section of the main text and additional references that occur only in Table S1 are listed below.

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## CHAPTER 6

### **Mating and maternal investment of the viviparous caecilian amphibian *Typhlonectes natans* (Amphibia: Gymnophiona: Typhlonectidae)**

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## ABSTRACT

The reproductive modes and strategies of amphibians are highly diverse. The limbless, tropical caecilians both are oviparous or viviparous and exhibit varying degrees of parental investment and highly unusual investment strategies, such as maternal dermatophagy and intrauterine feeding.

Here, we examined mating and maternal investment of the aquatic viviparous caecilian *Typhlonectes natans* in a laboratory population. Litter sizes ranged from three to eight and litter masses ranged from 22 to 65.7 g. Newborns reached up to 43 % of the maternal total length. Despite this high maternal investment, annual breeding was observed. Although fecundity is reduced in viviparous species, as clutch size is decreased in comparison to many oviparous species, the lower fecundity of viviparous taxa is compensated via uterotrophy, leading to increased offspring size and quality.

Although captive care and breeding of caecilians in the laboratory have some obstacles, it is a very useful way to collect reproductive biology data on these usually secretive amphibians and can contribute to our understanding of their evolutionary reproductive biology.

**Keywords:** Caecilian reproduction, *Typhlonectes*, maternal investment, Gymnophiona.

## INTRODUCTION

Caecilians are snake-like, burrowing amphibians of the old and new world tropics and their biology is as fascinating as it is poorly known. A variety of studies have dealt with frog or salamander biology, but more than half of the caecilian species are considered as "data deficient" in terms of their conservation status (compare IUCN 2013, see AmphibiaWeb 2014 for up-to-date species count), what can be mainly ascribed to their secretive lifestyles.

Nevertheless, past studies dealing with caecilian biology have shown diverse modes of reproduction, as well as different ways of parental care and investment. For example, egg guarding is a common behaviour in oviparous species (both with aquatic larvae or direct development) (see *e.g.*, Wake 1993, Kupfer et al. 2004). Furthermore, some species provide nutrients for their offspring besides yolk (Wake 1993) and hence increase their survival. This accounts particularly for viviparous species with reduced fecundity due to decreased ovum sizes and numbers (Wake 1993). The embryos are retained in the oviduct and after the yolk is exhausted, the fetuses feed on the hypertrophied epithelia of the maternal oviduct, using specialized fetal teeth for abrading the tissue (comp. Parker 1956, Parker & Dunn 1964, Wake 1977, 1993). The fetal dentition differs markedly from that of the adults and is changed to the adult formation at birth (Wake 1977, Wake 1993, Parker & Dunn 1964). In this way, the newborn are precocial and large enough to get along by themselves right after birth. However, some caecilian species (oviparous as well as viviparous) have altricial juveniles, which retain the fetal dentition after hatching or birth and use it to scrape off maternal skin for further nourishment during the prolonged phases of maternal care (O'Reilly et al. 1998, Kupfer et al. 2006a, Kupfer et al. 2008, Wilkinson et al. 2008, Kouete et al. 2012, Wilkinson et al. 2013).

The caecilians of the South American family Typhlonectidae are secondarily aquatic (Wilkinson & Nussbaum 1999, Kupfer et al. 2006b), all of their members (13 species) are viviparous and additional nourishment is provided via intrauterine feeding (Kupfer et al. 2006b, AmphibiaWeb 2014).

We examined the Rio Cauca Caecilian *Typhlonectes natans*, which is originally distributed in Colombia (Rio Cauca and Rio Magdalena) and in Northwest-Venezuela (Wilkinson & Nussbaum 1999), and monitored the breeding in a

laboratory population over four subsequent years. Maternal investment must be very high, as newborn juveniles are precocial and independent. Hence, we tried to assess how female size and condition could influence offspring size and number, since among amphibians larger females often have larger or more numerous offspring (Shine 1979, Fairbairn 1997, *e.g.*, salamanders - Salthe 1969, Reinhard & Kupfer 2013). We also analysed the effects of litter size and to what extent sibling body measures vary within litters and in connection with maternal size.

## **MATERIAL AND METHODS**

### *Captive Care*

All observations on mating behaviour and reproduction of *Typhlonectes natans* were made in the laboratory. The breeding population was kept in several tanks with at least one male and several females in each tank. The tanks were large (min. 80 x 40 cm for up to 5 individuals) and equipped with various hiding places (artificial caverns from the fish-keeping store or simple clay pots), plants (especially *Anubias* with hard leaves, which are preferred to coil around) and floating islands, which were used for resting outside the water every once in a while. The water levels ranged from 35 - 50 cm (depending on tank size) and the water was filtered using Hamburg mat filters (a strong filter is necessary for keeping the water clear and thus to prevent skin irritations and infections, as the water pollution due to the animals is relatively high). Since these caecilians always try to use the spots behind the filter as additional hiding place, it has proved to be advantageous to tightly sew the filter mat cylindrically around the pump, to prevent injury or poor control possibilities. In addition to filtering, about 20 - 30 % of the water was changed weekly and refilled with fresh, tempered tap water. The bottom of the aquarium was kept blank for better cleaning. Mandatory in keeping caecilians is a tight-fitting, heavy lid, as they are able to escape through small slits and even to raise and crawl out under too light weighted lids. For this reason, we preferably used steel mesh as lid material or put stones on the edges of the lids. Water temperatures ranged from 25 - 27 °C in summer and 21 - 25 °C in winter (additional heating with customary aquarium heaters was necessary). The light cycle was set at 11:13 daylight/night and completed by natural dusk/dawn through opposite windows. Animals were fed ad libitum a diet

of earthworms, chopped fish and prawns, freshwater shrimps (*Gammarus*), blood worms, mussels and occasionally axolotl-pellets (Axobalance), which are suitable for caecilians as well - due to the similar feeding ecology of aquatic newts and aquatic caecilians.

The breeding groups were housed together all year round. Splitting of the groups was only necessary in cases of biting or diseases. The sexes of adult *T. natans* are easily distinguishable via the size and the shape of the cloacal disk (see Warbeck 2002).

#### *Data collection and statistics*

Breeding activity and births were monitored from 2010 to 2013. Total length (TL), body-width at mid-body (BW) of the mothers and the offspring were recorded directly after birth, as well as their weight. Additionally, body mass and sizes of the young were monitored throughout their growth. Mass was measured with a Kern CM 320-IN pocket balance and sizes were measured from photos using ImageJ 1.45s. The body volume of females and young was calculated using a formula, simplifying the body as uniform cylindrical:

$$V = \frac{\pi}{4} * BW^2 * TL$$

All calculations and statistical tests (linear/nonlinear regression, Spearman correlation, coefficient of variation) were carried out with Excel 2007 and Prism 5.01 for Windows 7. All data were tested for normal distribution using the D'Agostino & Pearson omnibus normality test in Prism.

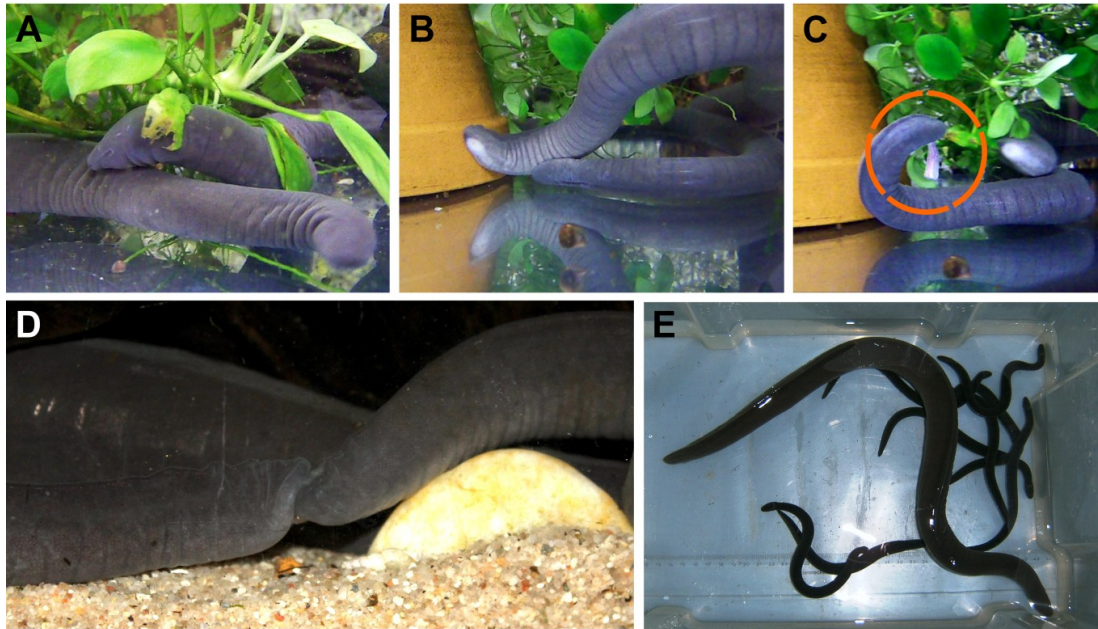
## **RESULTS**

### *Mating behaviour*

Mating sequences were observed once in January 2010 and once in November 2012. In the weeks before, the males showed an increased degree of aggression with heavy biting (see also Warbeck 2002), what made it necessary to remove the 'submissive' males from the tank to prevent severe injury, letting only the most aggressive, 'dominant' male stay with the females. It was eye-catching that some of the females were hanging limp in the water or lying motionless on the tank ground. Normally,



males and females are resting most of the time in their hiding places (particularly coiled together, see also Warbeck 2002) and leaving them only to breathe and for feeding or active swimming (sharing of the same burrows or resting places is also common in *T. compressicauda* in the wild, see Moodie et al. 1978). Remarkably, females had the posterior parts of their body curled up slightly dorsally, so that their cloaca was exposed, likely to signal receptiveness. The single male kept with the females was swimming rapidly and constantly moving towards the females, whilst slowly "sniffing" all along the females (Figure 1A). If anything (other tank mates or even floating plant material) touched the female cloaca, they were quickly jerking with the end of their bodies. During the sniffing, the male was over and over again swimming apart from the female, likely disturbed by other tank mates. After that, the male moved backward, whilst feeling the ground, passing objects and conspecifics with the cloacal disk. When the male touched the females' body, it was "scanned" that way very slowly (with the female not moving, see Figure 1B) and when reaching the females' cloaca, the male suddenly everted its phallodeum for insertion into the female cloaca. These attempts were repeated very often, as most trials failed (see Figure 1C). Thus, mating attempts can take several hours or might even take days until successful copulation (Figure 1D), because they were also interrupted both by males and females and the insertion of the phallodeum was often not successful. Females sometimes showed bite marks approximately on mid-body during breeding time, likely suggesting the possibility of a mating bite, though this has not been observed yet. The copulation itself can last up to several hours, during which the mating partners remain relatively still or swim slowly together.



**Figure 1.** Reproduction of *Typhlonectes natans*. (A) The male consequently sniffs all along the females' body. (B) The male uses its cloacal disk to "scan" the females' body. (C) Unsuccessful copulation trial: the males' phallodeum is still everted. (D) A successful copulation. Left: male, right: female. Photo © B. Jermann (E) After a gestation period of about seven months, three to eight precocial juveniles are born.

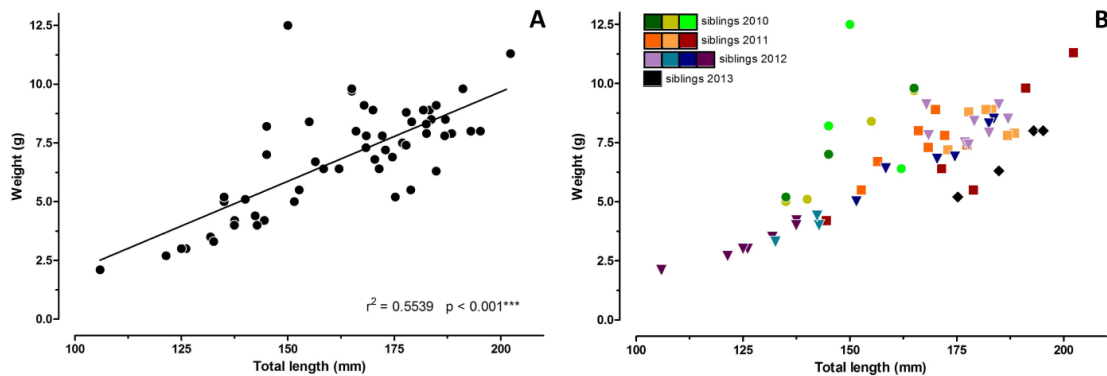
#### *Birth and maternal investment*

In 2010, three of five females had litters, three again in 2011, four in 2012 and only one in 2013. See an extract of mother/offspring data, for which mother data is present (pregnant females stayed together with non-pregnant females and birth happened earlier than expected in three cases, so that the mothers were not distinguishable from the other females after birth), in Table A in the appendix. Therefore, breeding can be annually, at least in captivity (see Figure 1E for a picture of a mother with her newborn offspring). Gestation periods lasted up to approximately seven months.

The birth process (until all young are born) lasted up to several hours or even days (there can be breaks of some days between the young). The young are born with the head and the elongated, sac-like gills first. The gills can weigh one third of the newborn, *e.g.*, a newborn of 170 mm total length (TL), body mass (BM) 8.9 g had gills with a mass of 3.2 g. The gills fall off shortly after birth and sometimes the

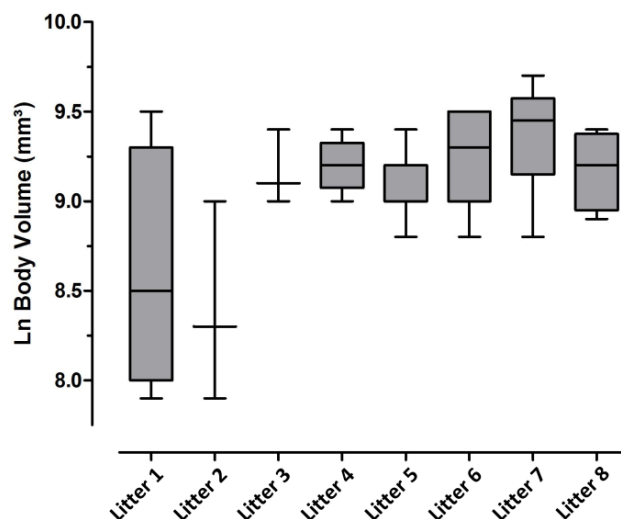
young actively try to strip them off, but a white scar still indicates the former gill placement dorsally on the head several days or even weeks after birth.

Litter sizes ranged from three to eight young. The mean body size at birth was 163 mm (SD  $\pm$ 15.7 mm, range: 105.9 - 202.3 mm, n = 42) and mean body width at midbody was 7.9 mm (SD  $\pm$  1.3, range: 4.3 - 10.8 mm, n = 42). The average body mass at birth was 6.9 g (SD  $\pm$  1.5, range: 2.1 - 12.5 g, n = 42). See graphics of size and weight at birth in Figure 2.



**Figure 2.** (A) Graphic representation of the *Typhlonectes natans* offspring size and weight at birth (n = 57). (B) Size and body mass at birth of the *T. natans* offspring sorted in sibling cohorts (n = 57).

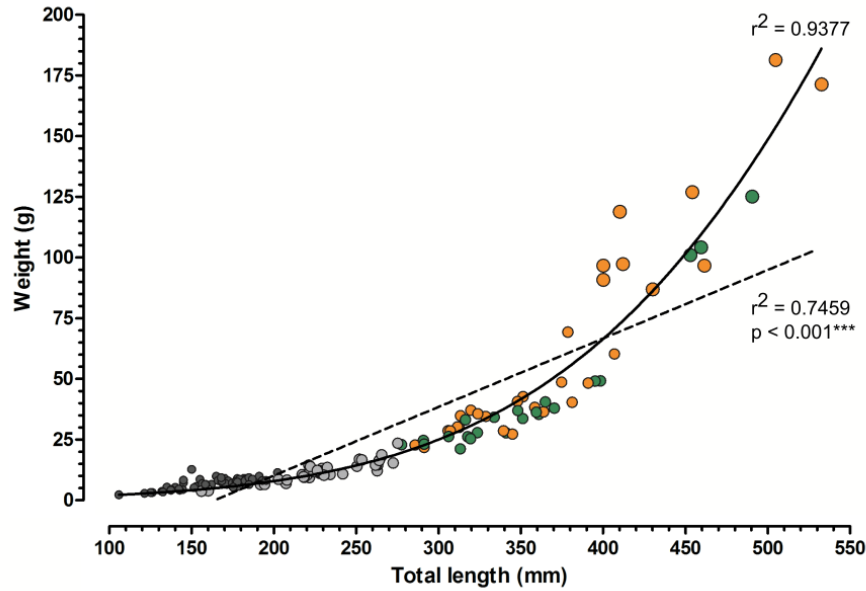
The newborn reached an average size of 37.2 % of the mother TL (range: 32.8 - 43 %) and an average body mass of 6.5 % of the mothers' mass (range: 3 - 14.4 %). If the weight of the juveniles is summed up to litter weight, an average mass of 40.1 g was obtained (SD  $\pm$  15.8, range: 22 - 65.7 g), what equals 34.4 % of the mothers' weight (range: 16.1 - 53.1 %). Litter volume was 5 % of the mothers' volume (range: 2.5 - 7.3 %). Although it was possible that siblings had different birth sizes and masses, they were always within a similar range, whereas birth masses fluctuated more than size (compare Figure 2B). The coefficient of variation (CV) ranged between 3.2 % and 10.3 % for sibling sizes and between 8.1 % and 34.7 % for sibling mass. The coefficient of variation range was highest for sibling body width (19 - 73.7 %) and thus for sibling body volume (13.2 - 71.9 %, see Figure 3). The variation of sibling weight was highest in smaller litters ( $p < 0.01**$ ) and the same applied to the variation of sibling body volume in smaller litters ( $p < 0.01**$ ).



**Figure 3.** Box and whiskers plot (minimum to maximum) of the *Typhlonectes natans* offspring body volume ( $n = 42$  newborn included). The variation of sibling body volume is highest in smaller litters (compare with Table A in the appendix).

The female body volume and the litter volume were not significantly related (in both correlation models) and the same applies to female and litter mass (see Table 1). Similarly, female body size, volume and weight had no significant effect on the number of young.

However, a significant correlation existed between the TL of the mothers and the mean TL of their young, whereas female TL and mean mass of the young were not significantly correlated. Maternal TL also correlated significantly with the litter volume, but not with litter weight. Another weak significant correlation was found between female weight and mean offspring total length, but only with linear regression, as Spearman correlation was not significant, but had a relatively high  $r^s$ -value. Correlations of female volume and mean offspring total length, mean offspring volume and mean offspring weight were not significant. The same applies to the correlations of female mass and litter volume/mass, as well as mean offspring volume/weight. But as all Spearman results lie between zero and one, all those characters tend to increase together, even if they were not significant. See all Spearman and linear regression results in Table 1. See Figure 4 for a growth estimation curve of *T. natans* from birth to adult size.



**Figure 4.** Growth estimation curve including total length (mm) and weight (g) of newborn, subadult and adult *Typhlonectes natans* (n = 151 measurements). Sexing is possible starting at total lengths of approx. 250 mm and a body mass of approx. 25 g (before this limit, the male and female cloacae look similar). Males are marked in green, females in orange, newborns in dark grey and not sexable subadults in light grey. Given are the results for both linear and nonlinear regression.

**Table 1.** Summary of the results of the linear regressions (first line per section) and Spearman correlations (second line per section) for the entire litter and mother data or individual offspring and mother data.

|                                | Female volume        | Female total length                 | Female weight                |
|--------------------------------|----------------------|-------------------------------------|------------------------------|
| <i>Litter</i>                  |                      |                                     |                              |
| <b>Volume</b>                  | n.s.<br>$r^s = 0.69$ | $p < 0.05^*$<br>$r^s = 0.9^{**}$    | n.s.<br>0.43                 |
| <b>Body mass</b>               | n.s.<br>$r^s = 0.4$  | n.s.<br>0.38                        | n.s.<br>0.62                 |
| <i>Offspring (individuals)</i> |                      |                                     |                              |
| <b>Ø Total length</b>          | n.s.<br>$r^s = 0.6$  | $p < 0.01^{**}$<br>$r^s = 0.9^{**}$ | $p < 0.05^*$<br>$r^s = 0.71$ |
| <b>Ø Volume</b>                | n.s.<br>$r^s = 0.69$ | $p < 0.05^*$<br>$r^s = 0.9^{**}$    | n.s.<br>$r^s = 0.43$         |
| <b>Ø Body mass</b>             | n.s.<br>$r^s = 0.5$  | n.s.<br>$r^s = 0.7$                 | n.s.<br>$r^s = 0.75$         |
| <b>n</b>                       | n.s.<br>$r^s = 0.46$ | n.s.<br>$r^s = 0.48$                | n.s.<br>$r^s = 0.58$         |

## DISCUSSION

### *Mating behaviour*

To the best of our knowledge, the mating behaviour of *T. natans* was never reported before, only of its sister species *T. compressicauda*. Murphy et al. (1977) reported that the copulation of *T. compressicauda* lasted several hours, corresponding well with our observation for *T. natans*. Murphy et al. (1977) did not observe initial stages of the courtship, but it is likely that it is similar in *T. natans* and *T. compressicauda*. Apparently, *T. natans* males lack any specific cloacal glands (Kühnel 2013). Pheromone production might be carried out with the aid of skin glands located in the epidermis, what could explain the male sniffing prior to the copulation attempts. Chemical communication plays an important role for *T. natans* and females signal their receptivity with pheromones released directly into the water (Warbeck 2002). Choice tests showed, that receptive females prefer unrelated and foreign males over males they had already mated with or related males (Warbeck 2002). The receptivity of the females even leads to increased aggression in males, leading to heavy biting, a behaviour already observed (Warbeck 2002). Males seem to have larger heads (SR, pers. obs.), a likely sexually selected trait, since the males engage in direct combat. However, sexual dimorphism needs to be tested in larger samples, using comparative body-size-adjusted analyses (see Kupfer 2009). The tactile scanning of the female via the male cloacal disk likely functions to find the female cloaca and to ensure a proper insertion of the phallodeum, although many mating attempts fail. How far the scanning process or possible mating bites provide stimuli for the female remains unknown. We observed jerking reactions of the female when it was touched, so that it presented its cloacal regions due to a craniodorsal flexion. Thus, the touching of the male might additionally induce a better accessibility of the female cloaca. The mating process is basically similar to that of snakes. In snakes, pheromones, which attract males, occur in the dorsal skin of the females and after the male has approved a receptive female, tactile signals predominate the courtship and tactile alignment followed by intromission attempts occur, with the female gaping or presenting its cloaca (see Vitt & Caldwell 2014 for a summary of mating behaviours in snakes, see also Kupfer et al. 2006c for further observations of caecilian mating behaviour).

### *Maternal investment*

Alternative pathways of parental investment, such as maternal dermatotrophy (Kupfer et al. 2006a, Kupfer et al. 2008, Wilkinson et al. 2008, Kouete et al. 2012) or intrauterine feeding (e.g., Parker 1956, Wake 1977), are found frequently within the phylogenetically derived caecilians, the Teresomata (see San Mauro et al. 2014), and have a considerable effect on offspring quality.

We also found a high degree of parental investment in the aquatic caecilian *Typhlonectes natans*. The offspring-female-ratio of size (mean TL) and mass (litter) exceeded 30 % and was up to 43 % concerning the individual newborn size when compared to maternal size and up to 53 % regarding total litter mass compared to maternal mass. The size of the individual young at birth and the total litter volume correlated positively with maternal size, meaning that larger females have larger offspring. Maternal mass only had a slight impact on the size of the individual young at birth. In contrast, Moodie et al. (1978) reported a positive correlation of maternal length and the number of fetuses of *T. compressicauda* from the Amazon basin (with a modal number of ten fetuses per female) and no significant correlation of maternal size and foetal size or foetal size and litter size.

A reliable breeding of caecilians in the laboratory is complicated, but easier with aquatic than with terrestrial species (SR, pers. obs.). We were able to successfully monitor eight births during the last four years, but continued breeding (and adding more data) could improve our present findings and presumably strengthen the significance levels we recorded so far.

All the other relations might well become significant with an increased data basis, as they already tended to increase together. Only the litter sizes of larger females likely do not change when adding more data, as female size does not necessarily influence clutch sizes in viviparous caecilians (Wake 1980, 1993), but this needs to be validated for *T. natans* in the future. Hence, larger females are likely to have few high-quality precocial offspring rather than larger litters. Investment into juveniles is also very high in the larger sister species *T. compressicauda*, with offspring sizes of up to 60 % of the mothers' lengths (Wake 1977). Other viviparous species with precocial offspring have similar mother/offspring ratios, e.g., the terrestrial *Schistometopum thomense* with offspring sizes of 57 % of the maternal length or 50 % maternal length in *Gymnopsis multiplicata* (Wake 1977). The highest number of

young born in the course of this study in one litter was eight, similar to O'Reilly & Ritter (1995). Warbeck (2002) reported up to nine juveniles per female. For *T. compressicauda* Exbrayat & Delsol (1985) reported a mean litter number of four juveniles per female, whereas Wake (1977) found up to nine fetuses in the oviducts. Likely a reduced fecundity due to the reduction of offspring number is vitally compensated through a higher offspring size and quality and therefore a higher chance of survival. The juveniles of *T. natans* and other Typhlonectids are precocial (e.g., Barrio 1969, Billo et al. 1985), can independently feed on their own (from a greater variety of food sources) and are likely less vulnerable as prey themselves.

Newborn siblings varied in birth mass and body volume and interestingly, smaller litters varied more in these parameters than larger ones. We would have predicted the opposite: larger litters vary more than smaller ones, because the competition for uterine space or nutrients is higher when more siblings are present. However, the opposite is the case and it would be interesting to know the distribution of fetuses within the oviducts. O'Reilly & Ritter (1995) found that half of twelve dissected gravid female *T. natans* had an uneven distribution of fetuses in their uteri. Two out of three dissected females of *T. natans* also showed an uneven distribution of intrauterine fetuses (AK, pers. obs.), but obviously the young did not vary much in total length or mass. We would predict for an asymmetric foetal disposition that a single foetus has more resources in one oviduct than two or three in the other. But how sibling competition or asymmetric foetus distribution functions in *T. natans* needs to be further validated. The mean ovarian clutch size of *T. natans* is 39 (n = 4 females; AK, pers. obs.), hence the litter size might possibly be reduced by oophagy of degrading, unfertilized eggs. Oophagy is known from the salamander *Salamandra atra*, which ovulates 28 - 104 eggs, but gives birth to only 1 - 2 young (Greven 1998, Vitt & Caldwell 2014), and from some subspecies of *S. salamandra* (Greven 1998, Buckley 2007). Nevertheless, this strategy could likely exist in other live-bearing species. In general, intrauterine feeding of the fetuses on the oviductal epithelium is accomplished via a specialized foetal dentition, which is resorbed before birth or shortly afterwards (Wake 1977, Wilkinson 1991). Moodie et al. (1978) found the digestive tracts of all fetuses of *T. compressicauda* completely filled with cellular material, especially intact sheets of epithelium. This provides a strong nutrient supply for the growing offspring and considering the high



female/offspring ratio, the cost for reproduction must be very high, likely decreasing the female body condition during pregnancy, especially in the wild, as observed in oviparous caecilians guarding egg clutches (Kupfer et al. 2004) or perform post-hatching parental care (Kupfer et al. 2008). Wake (1977) also reports that the yolk supply of *T. compressicauda* fetuses is resorbed at lengths of approx. 30 mm and that they increase their size 6.6 times until they are born with approx. 200 mm. In the laboratory, the caecilians are fed regularly with high energy food and can rest most of the day. Shortly before parturition females are especially slow and have sometimes trouble to reach the water surface for breeding due to their highly increased size, but a body condition decrease was not detectable (presumably as a captive breeding artefact).

Reproductive biology data are still scarce for many caecilian species and more studies are needed to properly reconstruct the evolution of the caecilian reproduction with a focus on the extraordinary parental investment strategies of this secretive amphibian group. Captive breeding programs can contribute well to this approach and should be established for many more caecilian species in the future.

## **ACKNOWLEDGEMENTS**

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## APPENDIX

**Table A.** Mother/offspring data of eight *Typhlonectes natans* births. Given are: total length (TL), body width at midbody (BW) and weight of mothers and associated young, as well as coefficients of variation.

| <i>Typhlonectes natans</i> - mothers |         |            | <i>Typhlonectes natans</i> - offspring |        |         |        |            |        |
|--------------------------------------|---------|------------|--|--------|---------|--------|------------|--------|
| TL (mm)                              | BW (mm) | Weight (g) | TL (mm)                                | CV (%) | BW (mm) | CV (%) | Weight (g) | CV (%) |
| 2010                                 |         |            |  |        |         |        |            |        |
| 410                                  | 28      | 118.7      | 140                                    | 9.3    | 6       | 30.9   | 5.1        | 33.6   |
|                                      |         |            | 135                                    |        | 5       |        | 5          |        |
|                                      |         |            | 165                                    |        | 10      |        | 9.7        |        |
|                                      |         |            | 155                                    |        | 7       |        | 8.4        |        |
| 400                                  | 20      | 90.6       | 165                                    | 10.3   | 8       | 24.1   | 9.8        | 31.6   |
|                                      |         |            | 145                                    |        | 6       |        | 7          |        |
|                                      |         |            | 135                                    |        | 5       |        | 5.2        |        |
| 430                                  | 20      | 86.7       | 162                                    | 5.7    | 8       | 11.1   | 6.4        | 34.7   |
|                                      |         |            | 150                                    |        | 10      |        | 12.5       |        |
|                                      |         |            | 145                                    |        | 9       |        | 8.2        |        |
| 2011                                 |         |            |  |        |         |        |            |        |
| 454.1                                | 22.4    | 126.7      | 172.9                                  | 3.2    | 7.6     | 5.8    | 7.2        | 8.7    |
|                                      |         |            | 188.5                                  |        | 8       |        | 7.9        |        |
|                                      |         |            | 186.8                                  |        | 8.5     |        | 7.8        |        |
|                                      |         |            | 177.8                                  |        | 8.1     |        | 8.8        |        |
|                                      |         |            | 183.2                                  |        | 8.1     |        | 8.9        |        |
|                                      |         |            | 181.8                                  |        | 9       |        | 8.9        |        |
| 412                                  | 20.1    | 97.1       | 166                                    | 5.3    | 8.6     | 8.4    | 8          | 14.5   |
|                                      |         |            | 168.3                                  |        | 9.4     |        | 7.3        |        |
|                                      |         |            | 152.7                                  |        | 7.4     |        | 5.5        |        |
|                                      |         |            | 177.3                                  |        | 8.5     |        | 7.4        |        |
|                                      |         |            | 156.4                                  |        | 9.2     |        | 6.7        |        |
|                                      |         |            | 170                                    |        | 8.6     |        | 8.9        |        |
|                                      |         |            | 172.2                                  |        | 7.8     |        | 7.8        |        |
| 2012                                 |         |            |  |        |         |        |            |        |
| 461.4                                | 22.5    | 96.5       | 170.4                                  | 7.5    | 8.9     | 9.3    | 6.8        | 17.4   |
|                                      |         |            | 151.5                                  |        | 8.2     |        | 5          |        |
|                                      |         |            | 158.5                                  |        | 7.4     |        | 6.4        |        |
|                                      |         |            | 183.7                                  |        | 9.5     |        | 8.5        |        |
|                                      |         |            | 158.4                                  |        | 9.3     |        | 6.4        |        |
|                                      |         |            | 174.6                                  |        | 8.5     |        | 6.9        |        |
|                                      |         |            | 182.5                                  |        | 9.7     |        | 8.3        |        |
| 504.8                                | 27.1    | 181.1      | 168.4                                  | 3.9    | 8.2     | 13.6   | 7.8        | 8.1    |
|                                      |         |            | 176.9                                  |        | 9       |        | 7.5        |        |
|                                      |         |            | 179.1                                  |        | 9.8     |        | 8.4        |        |
|                                      |         |            | 182.5                                  |        | 9.1     |        | 7.9        |        |
|                                      |         |            | 184.8                                  |        | 10.8    |        | 9.1        |        |
|                                      |         |            | 187                                    |        | 9.4     |        | 8.5        |        |
|                                      |         |            | 167.9                                  |        | 10.5    |        | 9.1        |        |
|                                      |         |            | 177.7                                  |        | 6.9     |        | 7.4        |        |
| 2013                                 |         |            |  |        |         |        |            |        |
| 532.6                                | 26      | 171.1      | 184.8                                  | 4.9    | 7.9     | 10     | 6.3        | 20     |
|                                      |         |            | 175.2                                  |        | 7.2     |        | 5.2        |        |
|                                      |         |            | 193                                    |        | 8.5     |        | 8          |        |
|                                      |         |            | 195.2                                  |        | 9.1     |        | 8          |        |

## CHAPTER 7

### **Life history of the West African caecilian *Geotrypetes seraphini* (Amphibia: Gymnophiona: Dermophiidae)**

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## ABSTRACT

Caecilians exhibit a variety of reproductive modes, as well as different types of extensive parental care, what makes them ideal for evolutionary reproductive studies. Unfortunately, not much is known about this rather secretive living group of amphibians, making it highly important to obtain more key data about their life histories.

*Geotrypetes seraphini* is a medium-sized terrestrial caecilian amphibian, which is widely distributed in Western Africa. It is viviparous and its newborn are not precocial, as it is known from other viviparous caecilians, but altricial and they are dependent on extended parental care. We provide indirect evidence for maternal dermatotrophy accompanied by growth data of offspring during maternal care. We also document the dentitional metamorphosis of the foetal-like teeth in detail using CT-imaging and scanning electron microscopy. The altriciality of newborn young in connection with viviparity and mesolecithal (medium-yolked) eggs in *G. seraphini* characterise a previously unknown mode of caecilian reproduction.

Furthermore, we could observe communal nesting behaviour implying social behaviour patterns.

**Key words:** Skin feeding, reproduction, altriciality, maternal care, Gymnophiona, *Geotrypetes*

## INTRODUCTION

In contrast to frog and salamander biology, the third order of amphibians - caecilians - is poorly known and the majority of the 200 currently described species is considered as "data deficient" (compare IUCN 2013, see AmphibiaWeb 2014 for up-to-date species count). Caecilians are snakelike and limbless, inhabit only tropical regions and live a very secretive life, as most species are burrowing and only a few are secondarily aquatic (Himstedt 1996, Wilkinson & Nussbaum 1999, Kupfer et al. 2006b). Nevertheless, they exhibit all major reproductive modes that are known in frogs and salamanders (Duellman & Trueb 1994). This includes oviparity with free living larvae (e.g., Himstedt 1996), as it is found in Rhinatrematidae, Ichthyophiidae (e.g., Nussbaum 1977) and in some *Grandisonia*, as well as oviparity in combination with direct development, as it exists in Chikilidae, and can occur in *Grandisonia*, *Gegeneophis* (e.g., Brauer 1899, Müller et al. 2005) and in *Hypogeophis* (e.g., Wake & Hanken 1982). Furthermore, some species retain developing embryos inside their oviducts and give birth to live young (Wake 1977). Those young can be precocial, fully developed and thus able to get along by themselves. They are born relatively large (e.g., up to 43 % of the maternal length in the aquatic *Typhlonectes natans* [Reinhard & Kupfer, in prep.] or 57 % of the maternal length in the terrestrial *Schistometopum thomense* [Wake 1977]), because they had access to sufficient nutrients through feeding on hypertrophied epithelia of the maternal oviduct, using specialised foetal teeth (Parker 1956, Parker & Dunn 1964, Wake 1977, 1993). Nevertheless, the West-African Caecilian *Geotrypetes seraphini*, which is also live-bearing, has been reported to have altricial young, which are barely able to move and guarded by the female (O'Reilly et al. 1998). They also retain the foetal dentition after birth (Parker 1956, Wake 1977). *G. seraphini* is a medium-sized caecilian amphibian, widely distributed in Sierra Leone, Guinea, Liberia, Cameroon, Gabon and in the Democratic Republic of Congo (Loader et al. 2004).

Our main goal was to obtain data on the reproductive biology of *Geotrypetes seraphini*, particularly on the period of extended parental care. We also supported the characterisation of a distinct viviparous reproductive mode among the Teresomata. We carried out high-resolution  $\mu$ CT-scanning and scanning electron micrography (SEM) to obtain detailed views of the dentitional condition during ontogeny and



were able to study the reproduction of this species, since we managed to breed a colony in the laboratory.

## **MATERIAL AND METHODS**

### *Animal husbandry*

Live animals of *G. seraphini* were received through the German pet trade and kept in large, clear plastic containers (80 x 30 cm) in groups of up to ten individuals. The tanks were filled with commercial rainforest soil (JBL Terra Basis) to a filling level of about 20 cm. The soil was kept humid to wet (even with some standing water at the bottom) to create a moisture gradient in the tank, so that the caecilians were able to choose their preferred moistness. The soil was dug over at least once per month to prevent moulding and it was changed completely once or twice a year. We forewent further tank equipment, despite from a small water bowl, which was filled with fresh tap water once or twice per week, pieces of bark and some moss cover, to keep certain spots extra moist. The tanks were aerated through ventilation slits, which were carved into the sidewalls of the tanks and sealed with mosquito-screen to prevent the animals from escaping. The lids were tightly closed and secured with click-closure. The temperature of the air ranged from 21 - 28 °C and the temperature of the soil ranged from 20 - 26 °C throughout the year (highest from June to August). The air humidity of the room, where the tanks were placed, ranged from 45 - 80 % and the humidity of the tank was increased by additional water spraying for up to 3 times per week. The animals were fed ad libitum with earthworms (*Dendrobaena*) of different sizes. It proved advantageous to keep a larger earthworm colony in the tanks, so that worms of all sizes were available at any time. Small subadults and independent young were fed with freshly hatched earthworms and white worms (*Enchytraeus*).

For breeding, groups of up to ten individuals were kept in the same tank. For proper assembling of males and females, all adults were anaesthetised with Orajel/Benzocaine 20 % (1g per 1l water) and sexed through slight pressure on the cloacal region, whereby the males' phallodeum can be everted. Males also show a muscle reflex, which can be triggered through soft strokes over the cloacal region and thus helps to sex the individuals without the more invasive phallodeum-eversion-

method (see Figure 1). All animals were weighted with a Kern CM 320-IN pocket balance and sizes were measured from photos using ImageJ 1.45s. Statistical tests (linear and nonlinear regression, normality test, averages, standard deviation SD, coefficient of variation CV) were conducted using Microsoft Excel 2007 and Prism 5.01 for Windows 7. "Artificial nests", which consisted of extra, smaller plastic boxes or flower pots filled with soil and strongly rooting plants (*Epipremnum*), were put into the breeding tanks in early spring (Figure 2A). After that, disturbance was reduced to a minimum and only absolutely necessary work was done on the tanks. The light cycle was set at 11:13 daylight/night and completed by natural dusk/dawn. A subset of newborn and older young were fixed in 70 % ethanol or 4 % formaldehyde.



**Figure 1.** Tail contraction of a male *Geotrypetes seraphini*. Soft strokes over the cloacal region trigger a reflex that rolls in the posterior tip of the body. Thus, a more gentle sexing method of the specimen becomes applicable next to the phallodeum eversion method.

### *3D Synchrotron-μ-CT*

Newborn and older young (two altricial [AK01203\*, SMNS\*\* uncatalogued], one older young [AK01131]), as well as one adult specimen [SMNS uncatalogued], were prepared for SR-μ-CT, which was carried out at the synchrotron radiation facilities at DESY in Hamburg, Germany (Beamline W2, Doris III). During the scanning process, they were stored in plastic tubes filled with 70 % ethanol. The 180° scanning procedure was done with an energy of 23 keV and after reconstruction, the datasets were further processed with VG Studiomax 2.0 (Volume Graphics). The scanning focus was on the head to display the dentitional development. \*AK = Alexander Kupfer collection, \*\*SMNS = Stuttgart State Museum of Natural History.

*Scanning electron micrography (SEM)*

Tooth morphology was additionally examined with scanning electron microscopy (Philips XL30 ESEM) using a rotatable specimen holder (see Pohl 2010) and an acceleration voltage of 10kV. The examined specimen (TL = 30mm, SMNS uncatalogued) was critical-point dried and sputter-coated with gold-palladium prior to the imaging sessions.

**RESULTS**

We successfully bred *G. seraphini* in two subsequent years, which means that mating took place in captivity, *i.e.*, the females were not already pregnant when they were imported. We had six litters, of which only one could be successfully raised to adults. The females were attending their young, but shared their nests with other brooding or non-brooding females and even with males (the highest number of adults in one nest was six). They chose the provided "artificial nests" or built nesting chambers on the ground and into the corners of their tanks (Figure 2B). They seemed to prefer nests with rooting of plants. The used soil was perfect for the stability of the underground nests and passages. Breeding is not possible, if the soil is inadequate or too dry, so that the caecilians have to build their passages and nests over and over again. Any disturbance during breeding time had to be admitted, because *G. seraphini* is very sensitive and easily feels disturbed by mild vibrations or too much light. We avoided digging the soil in the tanks over during spring, when we assumed a possible breeding time. Due to this infrequent control possibility, we often observed the juveniles for the first time, when they were already older. If we dug up a brooding female with freshly newborn young, this disturbance caused a stop of the parental care behaviour of the female and thus the abandonment of its young. The newborn young are altricial and not able to move (Figure 2C). They can only lift their heads somewhat and wriggle weakly. Their heads appear very large in contrast to the rest of their bodies. The newborn were totally helpless, if their mothers abandoned them, so that they died very quickly. They were not able to ingest food, so that rearing them further was not possible (all of those abandoned juveniles were fixed).



**Figure 2.** Reproductive biology of *Geotrypetes seraphini*. (A) Breeding container with "artificial nest" and plants. (B) Nest built in the soil. Careful digging revealed a brooding female (white arrow). (C) Brooding female with a newborn (TL = 27.5 mm). (D) Two females of the same nest; note the paler skin-colour of the brooding female in contrast to the non-brooding female (the newborn is marked with a white arrow). (E) A litter of eight pigmentless juveniles - barely able to move and originally attended by two females in brooding condition. (F) Attending female with larger juveniles, already beginning to change their body pigmentation, but still closely associated with the mother. (G) Female attaining a clutch of unfertilized eggs (white arrows, n = 4), note the pale skin-colour of the female.

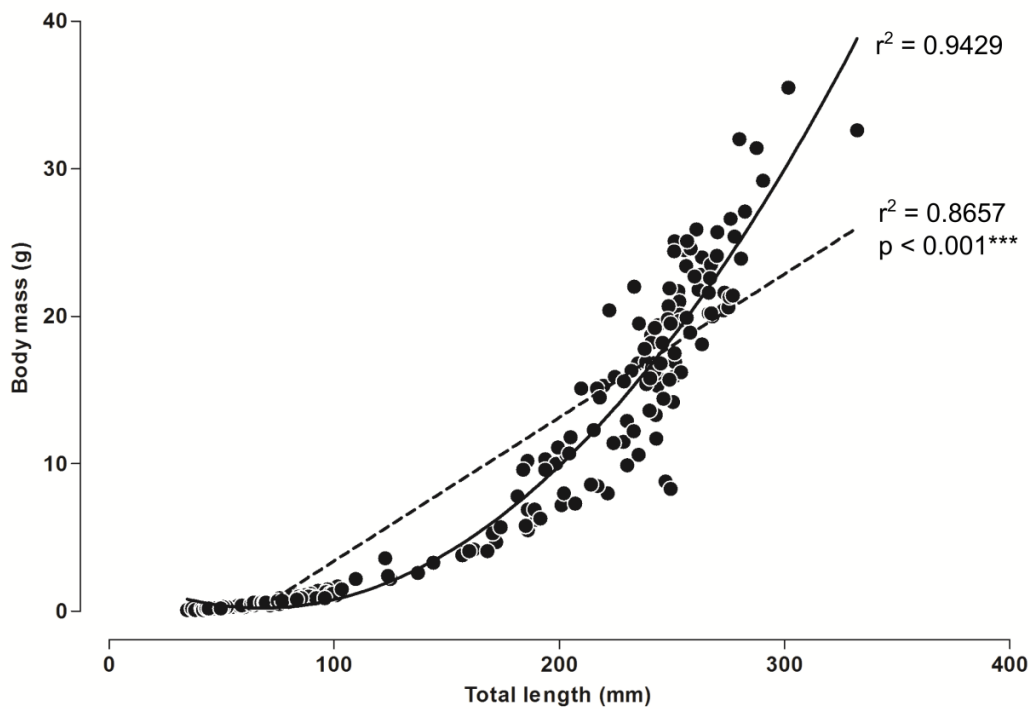
The females coiled around their offspring and appeared much paler than non-brooding adults (Figure 2D). We observed litter sizes in the nests ranging from two up to eleven juveniles. As they can be from more than one female, the maximum number of young per female could not be determined. We once found 8 young together (Figure 2E) with two brooding females and although our maximum count of eleven young was attended by only one female, the possibility that more brooding females belonged to this litter cannot be excluded. The smallest juvenile (likely just born) measured 27.5 mm.

The first litter (1/2010), which we were able to raise to adulthood, was monitored for body mass and size every once in a while (only every week in the beginning to reduce disturbance). The young were already older, when we found them, so the disturbance was no problem for the attending mother ( $n = 4$ , mean juvenile total length = 57.9 mm, SD = 1.46 mm, CV = 2.52 %; mean juvenile body mass = 0.36 g, SD = 0.05 g, CV = 13.61 %; maternal total length = 211.65 mm, maternal body mass = 10.7 g). The young grew about 30 % during the first six weeks after we found them ( $n = 4$ , mean juvenile total length = 75.56 mm, SD = 8.43 mm, CV = 11.16 %; mean juvenile body mass = 0.7g, SD = 0.12 g, CV = 16.5 %). At that time, the tentacle became clearly visible, their colour became darker, they likely started feeding on their own (white worms and smallest earthworms were supplied sufficiently) and they already moved around on their own inside the tank, whilst they stayed close to their mother in the weeks before (see a picture of a mother with already darker coloured juveniles in Figure 2F). The mother was able to keep up her body mass during the prolonged care (no lack of food in captivity, even when not leaving the nest), but after the young fed by themselves, the female gained more mass, likely by preying more actively.

After seven weeks, the young had grown about 43 % and definitely fed independently ( $n = 4$ , mean juvenile total length = 82.8 mm, SD = 9.38 mm, CV = 11.3%; mean juvenile body mass = 0.95 g, SD = 0.27 g, CV = 27.9 %). The general mean growth rate was 5.7 % during the first three months after finding the litter. After 35 weeks, their size had more than doubled ( $n = 4$ , mean subadult total length = 201 mm, SD = 6.8 mm, CV = 3.4 %; mean subadult body mass = 11.5 g, SD = 2.41 g, CV = 20.98 %). The four subadults were growing more slowly afterwards and reached a mean total length of 231 mm (SD = 8.28 mm, CV = 3.6 %) and a mean

body mass of 16.3 g (SD = 0.73 g, CV = 4.5 %) after one year. As they already reached the size of their mother, it could be concluded that *G. seraphini* can become sexually mature after one year (at least in captivity without food limitation). See a general growth curve of *G. seraphini* (with all animals and offspring included) in Figure 3 and all litter data (at the time of discovery) in Table 1.

We also observed once, that a female laid her unfertilized eggs and guarded them. The behavioural program of parental care proceeded normally, *i.e.*, the female coiled around the eggs and its skin appeared paler (Figure 2G).



**Figure 3.** Growth estimation curve including total length (in mm) and body mass (in g) of *Geotrypetes seraphini* (n = 238 measurements). Given are the results for both linear and nonlinear regression.



**Table 1.** Litter data of *Geotrypetes seraphini* from successful breeding in 2010/2011; TL = total length. Sizes and litter mass do not refer to newborn data, but to the date of discovery. The young of litter 2/2011 were born with an interval of 1 week (2 first, 3 one week later). \*Recently newborn were not measured directly, because we hoped that the females continue maternal care despite disturbance; one newborn could be measured from photographs.

|                   | Litter<br>1/2010 | Litter<br>2/2010 | Litter<br>3/2010 | Litter<br>1/2011 | Litter<br>2/2011 | Litter<br>3/2011 |
|-------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| litter size       | 4                | 2                | 11               | 4                | 5                | 8                |
| Ø litter TL       | 57.9 mm          | 27.5 mm*         | -                | 76.8 mm          | 36.7 mm          | 45 mm            |
| litter mass (Σ)   | 1.44 g           | -                | 2 g              | 3.4 g            | 0.6 g            | 1.8 g            |
| n adults in nest  | 1                | 2                | 1                | 1                | 6                | 4                |
| brooding females  | 1                | 1                | 1                | 1                | 1                | 2                |
| date of discovery | April<br>(end)   | May<br>(end)     | June<br>(end)    | June<br>(early)  | June<br>(early)  | June<br>(early)  |

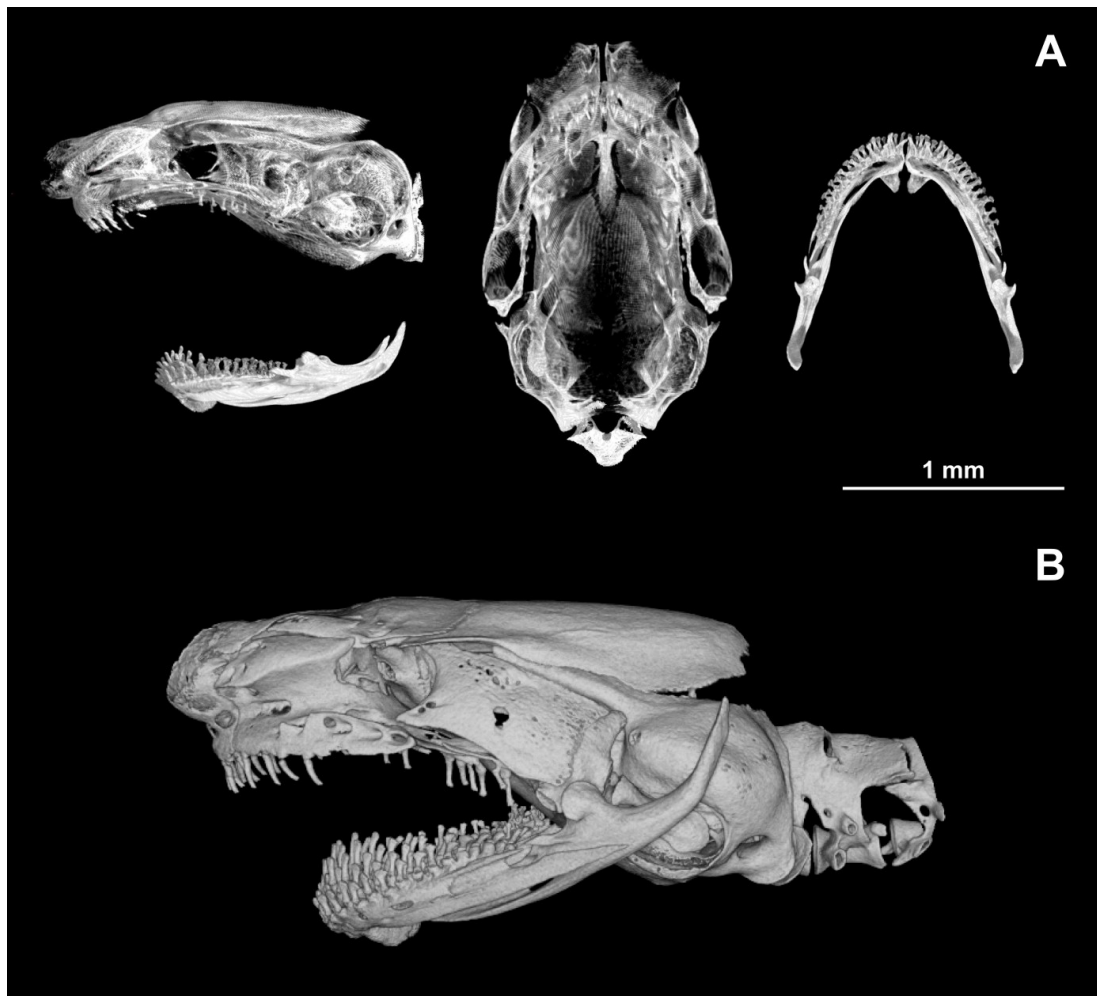
### *Tooth morphology*

The examination of the SRμCT scan of the youngest, altricial specimen (AK01203, TL = 36 mm, see Figure 4A) showed only weak ossification, what made the exact total tooth count difficult. Only the premaxillary-maxillary tooth row of the upper jaw had teeth, whereas no teeth had broken through on the vomero-palatium. In contrast, the teeth of the lower jaw were numerous (41 to 43 dentary teeth). The teeth of the upper jaw appeared simply bicuspid, whereby the dentary teeth were broader, shovel-like and had multiple cusps. Another CT-scan provided similar results (see Figure 4B).

SEM photography of another altricial young (TL = 37 mm) revealed a more details of the tooth morphology. The cusps of the bicuspid upper jaw teeth are small and uniform (Figure 5), whereas the dentition of the lower jaw is more variable (Figure 6). The tooth necks of all teeth are narrower than the tooth crowns and the crowns are shovel-like, but either rounded with four or five moderate cusps (anterior dentary teeth), or sharply blade-like with longer, more numerous cusps (five to seven cusps, posterior dentary teeth).

The dentition already started to change in another young, as revealed by SRμCT (AK01131, TL = 68 mm, see Figure 7A). We counted four premaxillary teeth on each side, eight maxillary teeth on each side, three (left) to four (right) vomerine and up to eight palatine teeth on each side of this specimen. The lower jaw was in between the foetal and the adult dentition with ten to eleven adult dentary teeth

already broken through on each side, but still several multicuspid, shovel-like dentary teeth present. Splenial teeth were already broken through (nine to ten on each side) as well. All adult-shaped mono- or bicuspid, predatory teeth are pointed and slightly curved backwards. The size of the specimen corresponds approximately to the sizes of juveniles when they become independent. They still remain with their mother during this phase, but already access additional food sources at the onset of dentitional metamorphosis.

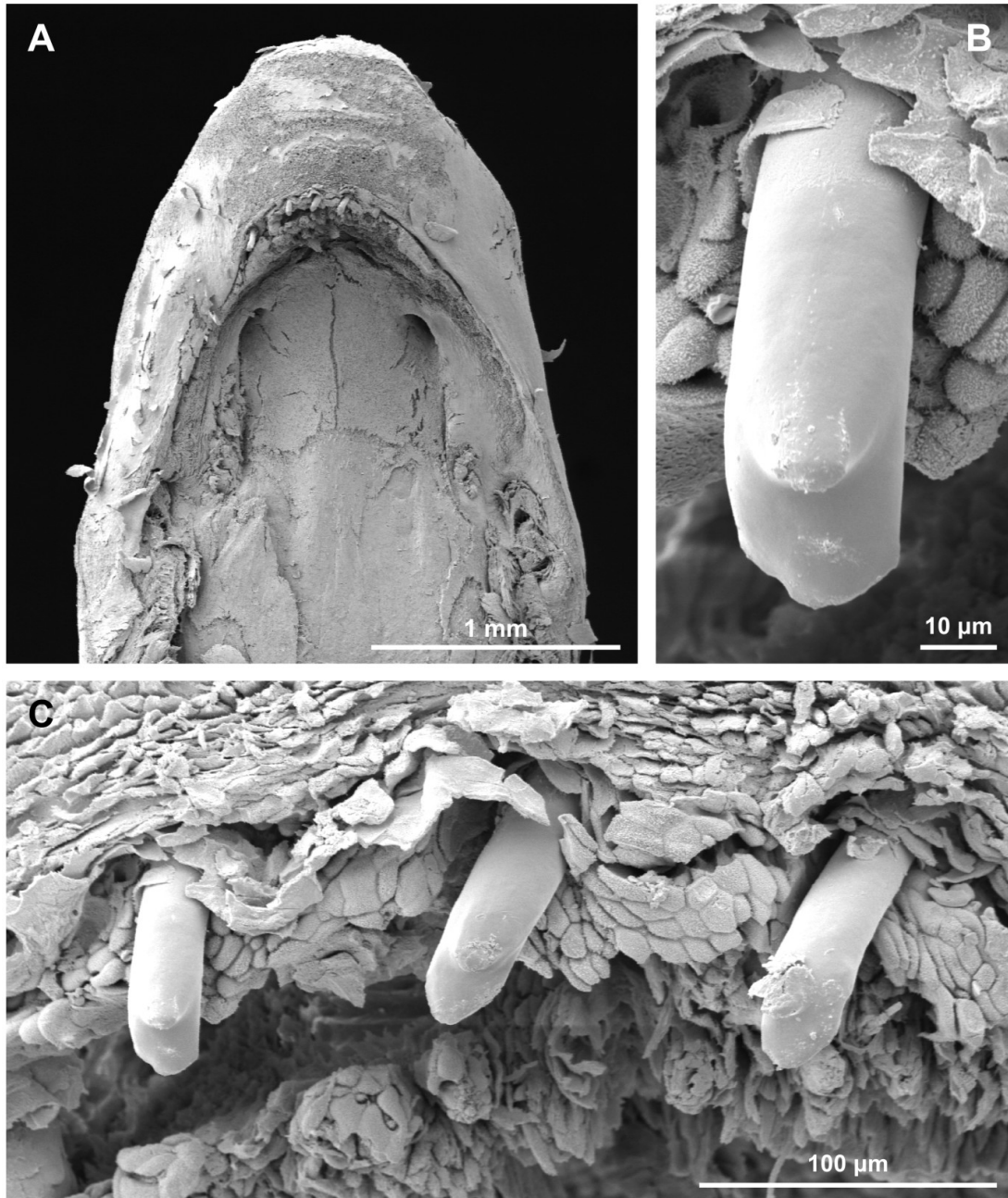


**Figure 4.** Dentition of altricial *Geotrypetes seraphini*. (A) SRμCT scan of an altricial young (AK01203; TL = 36 mm). (B) CT-scan of another altricial young (SMNS uncatalogued, TL = 54 mm).

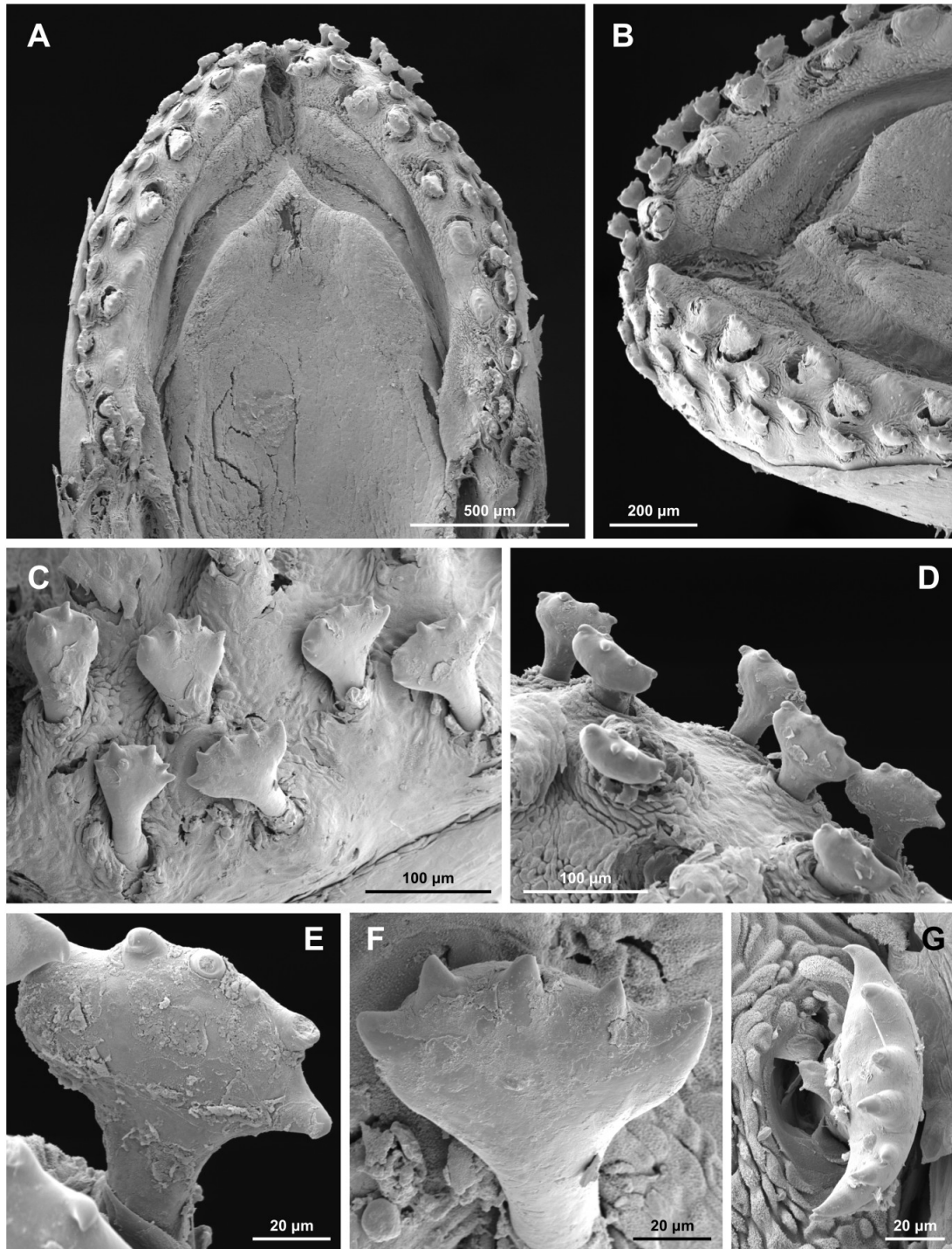
The adult dentition appears more uniformly monocuspid or bicuspid and is sharply pointed. SRμCT scan of an adult female revealed the following tooth count on each side (Figure 7B): four premaxillary teeth, ten to eleven maxillary teeth, four to five



vomerine teeth, 14 to 15 palatine teeth in the upper jaw and 18 dentary, as well as 14 splenial teeth in the lower jaw. Most teeth of the lower jaw, especially the anterior dentary and splenial teeth, are large, heavily pointed backwards and clearly bicuspid.



**Figure 5.** Retained foetal dentition (upper jaw) of altricial *Geotrypetes seraphini*, scanning electron microscopy (SEM). (A) Overview of the upper jaw - only few teeth are broken through. (B/C) Details of the upper jaw bicuspid teeth.



**Figure 6.** “Foetal dentition” (lower jaw) of altricial *Geotrypetes seraphini*, scanning electron microscopy (SEM). Overview of the lower jaw: (A) dorsal and (B) dorsolateral view. (C/D/E) The anteriormost teeth are rounded with four to five cusps, the teeth in posterior direction (F) and posteriormost (G) gradually increase in sharpness and have up to seven cusps.

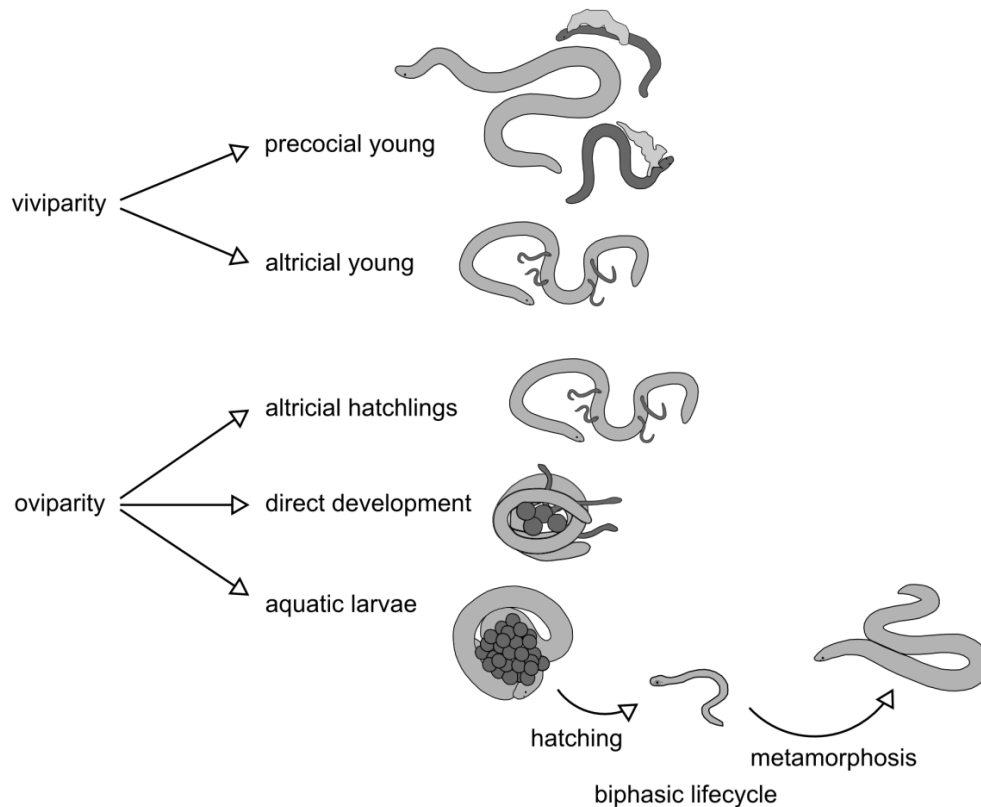


**Figure 7.** Dentition of *Geotrypetes seraphini*. (A) Dentition of a juvenile (AK01131, TL = 68 mm), which already entered dentitional metamorphosis. The foetal teeth are replaced by adult teeth, but the transition is gradual, so that dermatotrophy and solitary feeding are possible at the same time. The young still stay with their mothers during this transitional phase, but start wandering farther off from time to time. (B) Dentition of an adult female (TL = 220 mm), photo© T. Kleinteich. The adult dentition is uniformly pointed and mono- or bicuspid.

## DISCUSSION

Although the number of currently described caecilian amphibians is considerably low in contrast to frogs or salamanders, they are in no way inferior with respect to the variety of their reproductive modes (*e.g.*, San Mauro et al. 2014, but see a summary of the caecilian main reproductive modes in Figure 8). Oviparous caecilian species either have a typical amphibian biphasic life cycle with indirect development and an aquatic larval stage, undergo direct development or have altricial young. All of these oviparous modes are combined with parental care such as nest building, egg

attendance up to the provision of nutrients via maternal dermatotrophy (e.g., Kupfer et al. 2006a, 2008, Kouete et al. 2012, San Mauro et al. 2014). Most viviparous caecilian species nourish their offspring via uterotrophy inside the oviducts and thus have large, precocial juveniles at the time of birth (Wake 1977, 1993).



**Figure 8.** Summary of the different reproductive modes of caecilian amphibians. Oviparity occurs in association with aquatic larvae (e.g., *Ichthyophis*), direct development (e.g., *Chikila*) or altriciality (e.g., *Boulengerula*). Viviparous species have either precocial (e.g., *Typhlonectes*) or altricial young (*Geotrypetes*).

#### *Altriciality of newborn young*

O'Reilly et al. (1998) reported the viviparous *Geotrypetes seraphini* to have altricial instead of precocial juveniles. They briefly described that the newborn are helpless and that females guard their offspring during an extended phase of parental care. They also concluded that females likely provide nutrients via maternal dermatotrophy, like *Boulengerula taitanus* (Kupfer et al. 2006a), does. We successfully kept and bred *G. seraphini* in the laboratory and came to the same

result. Viviparity in combination with altriciality of young and maternal dermatotrophy was never been observed in any other caecilian species besides *Geotrypetes*. Loader et al. (2003) found a remarkably small, weakly ossified young of *Scolecophorus vittatus* with a foetal-like dentition and they concluded, that *S. vittatus* might have a similar reproductive mode as *G. seraphini*. However, a striking difference between these two species is the amount of yolk provided. Many phylogenetically derived viviparous caecilians (Teresomata) reduced their yolk amount to meso- or microlecithal, whilst they increase offspring quality by providing alternative parental investment strategies. *Geotrypetes seraphini* is exceptional, as it has an egg volume equivalent to many dermatotrophic oviparous species, such as *Boulengerula* ssp. In terms of yolk investment it thus differs from viviparous scolecophorid caecilians, such as *S. vittatus*, which have microlecithal eggs (Kupfer et al., in review).

#### *Embryo development, dentition and maternal dermatophagy*

Newborns of our litters were totally dependent on the care of their mothers and they also had a specialised, foetal-like dentition, as it is found in oviparous species with maternal dermatotrophy (Kupfer et al. 2006a, Wilkinson et al. 2008, Koeute et al. 2012, Wilkinson et al. 2013). Parker & Dunn (1964) examined the embryos and foetuses inside the oviducts and found embryos with lengths of 17 mm and triradiate gills still coiled around their yolk sac inside the egg membrane (approx. 4 mm) and at this time no teeth were erupted. At lengths of 25 mm, the first teeth began to erupt and most (but not all) embryos were still coiled inside the eggs with only rests of yolk present (Parker & Dunn 1964). The smallest newborn we discovered measured only 27.5 mm. Parker & Dunn (1964) reported that the first embryos emerge from the egg membrane at about 25 mm with an erupted dentition. It is likely that young of *Geotrypetes* are born not long after hatching (lecithotrophic viviparity) and that the egg volume determines the intrauterine hatchling size.

From the time of birth, the young are still dependent on their mothers, which differ from non-brooding, dark blue coloured adults in having much paler skin, a difference also noted by O'Reilly et al. (1998). The histological and histochemical examination of the skin of brooding *B. taitanus* revealed that the cells of the stratum corneum are more voluminous, the epidermis is thicker, the epidermal cytoplasm is full of lipid

inclusions and the skin, thus, also much paler than in non-brooding females to provide sufficient nutrients for the young during the extended maternal care (Kupfer et al. 2006a). Unfortunately, due to the disturbance sensitivity of the brooding mothers we were not able to directly observe 'skin-feeding'-behaviour in vivo, but the indirect evidence for maternal dermatotrophy in *Geotrypetes* is very strong. Parker & Dunn (1964) found that free-living young with stomachs full of skin material, but they concluded that they had eaten their own sloughed skin. Autodermatophagy is a common behaviour in amphibians (Weldon et al. 1993) and it cannot be excluded that the young also eat their own skin, but nevertheless they need a reliable, permanent food source. Stomach contents of some of our young also contained only skin material.

Sanderson (1937) also reported the stomachs of the young *Geotrypetes* to be filled with skin. We did not supply any small food for the young until we found the nests, so that only too large earthworms were present as food for the adults. Despite this, we found litters with already larger and well-developed young, which therefore had no other food source available, except the skin provided by the mother.

We also examined the dentition of several specimens of different ages or sizes respectively, which gave further indirect indication for maternal dermatotrophy. The smallest young (TL 36 mm) showed a typical, foetal-like dentition, which is to some degree similar in crown shape to that of *Siphonops annulatus* (compare Wilkinson et al. 2008). The shovel-like teeth are numerous in the lower jaw, so that the main rasping movement functions through the lower jaw and the fewer upper jaw teeth, which resemble a juvenile form of the adult teeth with less sharp cusps, may only give support in holding the pieces of skin. The heads of the newborn appear much too large in comparison to the rest of their bodies, what could be ascribed to likely well-developed jaw muscles to ensure proper feeding.

These teeth were also already described by Sanderson (1937), who found several nests of brooding *G. seraphini* in Assumbo/Mamfé, Cameroon.

Parker & Dunn (1964) described newborn with lengths of 73 - 77 mm, what seems too large in contrast to our results. They also specified that those newborn had adult-like, bicuspid teeth in between the multicuspid teeth of the lower jaw, what corresponds to our findings of the young in dentitional metamorphosis (TL = 68

mm). Likely, Parker & Dunn (1964) were mistaken with the time of birth. At this state of dentitional development and total length, our juveniles already started to feed independently and moved away from their mothers. Adult teeth are relatively uniform and either bi- or monocuspid.

Interestingly, Sanderson (1937) reported a brooding female, which coiled around its young in a chamber half-filled with water and interestingly, the young "all with their heads pointing towards her tail". This might indicate that female *G. seraphini* also produce a liquid, which is released from the cloaca, similar to *S. annulatus* (see Wilkinson et al. 2008). The young aggregate around the female cloaca and imbibe liquids, whereas possible functions could be nutrition, microbial transfer, water balance or communication (Wilkinson et al. 2008). We did not observe such a behaviour in *G. seraphini*, but further breeding and using non-stop video recording in the breeding containers should give insight into this matter.

#### *Communal nesting behaviour*

We found some nests inhabited by more than one brooding female and even non-brooding adults, whether males or females. Generally, communal nesting is taxonomically widespread among animals and much more common in reptiles and amphibians than recognised so far (Doody et al. 2009). Doody et al. (2009) reviewed a broad data basis about communal nesting in reptiles and amphibians and they found 83 frog and 53 salamander species (4 % of 3303 oviparous amphibian species) to nest communally, but the situation is different in *G. seraphini*, as the species is viviparous, guards altricial young and additionally has to provide nutrients without which the offspring would not be able to survive. Within caecilians, communal nesting has been reported by Kupfer et al. (2008), who found mothers of *Boulengerula taitanus* with their eggs or young near conspecific brooding females. Furthermore, non-attending females and males could also be associated in close proximity to a nest. Interestingly, young could be adopted by other attending females and also fed on their skin (Kupfer et al. 2008). We found our *Geotrypetes* to be very social and they commonly use the same hiding spots, sometimes in close body contact. Almost nothing is known about caecilian social behaviour and kin recognition and the only detailed study has been carried out on aquatic *Typhlonectes natans*, which mark shelters via chemical cues (Warbeck 2002). Shelters, which are

already inhabited by conspecifics, are favoured above unmarked shelters. The animals are even able to discriminate between related and unrelated conspecifics, as well as between males and females depending on which they prefer at a certain reproductive period (e.g., females prefer male chemical cues during the reproductive phase) (Warbeck 2002). If individual *Geotrypetes* are separated and placed in fresh soil without any species-specific chemical cues, they do not take very long to find each other and stay together (SR, pers. obs.). Although many hypotheses about the benefits of communal nesting in amphibians exist (see Doody et al. 2009), the pronounced social behaviour of *Geotrypetes* seems to be a likely precursor for the communal nesting behaviour, but might have the additional benefit that young could be attended and even nourished by other brooding females (see also Kupfer et al. 2008). But how far a cooperative brooding exists needs to be studied further. Otherwise, several adults in one nest likely reduce the risk of predation. That even males are tolerated in the nests speaks for a distinct social behaviour similarly reported for *T. natans* (Warbeck 2002). Although male *T. natans* are aggressive to some degree during the breeding season in captivity, they are not necessarily territorial (Warbeck 2002, Wells 2007, Reinhard & Kupfer, in prep.). We were not able to detect any aggressive behaviour between the individual *Geotrypetes*.

#### *The evolution of viviparity in caecilians*

Maternal dermatotrophy has been considered a precursor for the evolution of viviparity in the Gymnophiona. Usually, oviparous species only provide yolk to nourish their offspring (lecithotrophy) and thus have macrolecithal or at least mesolecithal eggs (Gomes et al. 2012, Kupfer et al., in review). Within Teresomata, oviparity is either linked to direct development (and to macrolecithal eggs) or to the hatching of altricial young from mesolecithal eggs and maternal dermatotrophy. The usage of skin as a food source is not unusual, as autodermatotrophy is common in amphibians and a useful way to recycle nutrients (Weldon et al. 1993). The specialised dentition of the altricial young is likely a preadaptation for the intraoviductal feeding of the young of viviparous caecilians (see Kupfer et al. 2006a), which have similar teeth for scraping oviductal epithelia. The fecundity of viviparous species is lower than in oviparous species and the amount of yolk provided also decreased to microlecithal eggs, because the investment is shifted



towards alternative strategies of nutrient provisioning. Viviparity has evolved several times within derived caecilians, their young are larger and have an increased fitness, whether they are already born precocial or altricial and receive further care, which lets them grow to a precocial state via maternal dermatotrophy. Nevertheless, viviparity and precocial-born young seem to be the safest mode supporting the offspring and parental fitness, because *e.g.*, the risk of predation or exposure to pathogens is excluded, if the young are retained inside the female body until they are large and strong enough to survive on their own. The altriciality in combination with larger eggs, viviparity and maternal dermatotrophy in *G. seraphini* is exceptional and comprises a new reproductive mode of caecilians. Further studies, especially concerning the majority of caecilians, which still comprise a mystery in terms of their reproductive biology, are urgently needed to fully understand the evolution of caecilian life history.

## **ACKNOWLEDGEMENTS**

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## SYNOPSIS

*Life history strategies and sexual dimorphism of the Amphibia with special emphasis on the Urodela (Newts and Salamanders) and the Gymnophiona (Caecilians)*

Among vertebrates, the nearly 7300 species of amphibians (see AmphibiaWeb 2014 for an up-to-date species count) show the highest variability of reproductive biologies. Unfortunately, many amphibians are threatened by habitat loss, pollution or diseases (*e.g.*, Stuart et al. 2004, Martel et al. 2013). Additionally, a significant amount of species are poorly studied and this is especially true for caecilian amphibians. Thus, more comparative and fundamental baseline studies are needed to further understand the extraordinary diversity of amphibian biology and life cycles. Urodeles and caecilians are highly suitable study organisms, as they exhibit a very large diversity of life history strategies (Duellman & Trueb 1994) despite their relatively small species numbers in contrast to anurans.

This dissertation was prepared to elucidate some important fundamental aspects of amphibian reproductive strategies, especially focusing on salamanders and caecilians. The evolution of parental care strategies and sexual dimorphism are crucial to understand the evolutionary framework of life histories and mating systems. I presented a survey on different amphibian species, which were readily accessible, to show how divergent selective pressures and behavioural strategies can influence the phenotype of the genders in connection to their reproductive roles and within different habitats, as well as evolutionary changes and adaptations of different reproductive modes.

The individual studies were based on morphological and morphometrical analyses or on direct observations of breeding behaviours in salamander and caecilian species in the laboratory. Three species (one salamander species, two caecilians species) were successfully bred in the course of this study and revealed new and unexpected insights into their life history strategies (chapters 5-7). Three more amphibian species (salamanders) were chosen for studying sexual size dimorphism in connection with age and terrestrial versus aquatic habitats (chapters 2-4). The selection of the species regarded herein was mainly influenced by the accessibility of specimens, which represent a certain variety of life history strategies (including different habitats, *i.e.*, terrestrial versus aquatic), to provide an exemplary presentation of the factors that

influence the morphology as well as the behaviour of amphibians. Selecting species with accessible living specimens was even more difficult, as many amphibian species are never or only rarely available in captivity (especially caecilians).

The first two species, which were chosen for the SSD study, were *Salamandra algira* and *Mertensiella caucasica* - representatives of the phylogenetically basal 'True Salamander'-clade of the Salamandridae (chapter 2). These salamanders were accessible in large specimen series at different natural history collections (Munich, Bonn, Hamburg) and were also cleared for the skeletochronological analysis, what is not always possible under the curatorial protocol in such collections.

Both salamander taxa were monomorphic in body size, but they showed prominent male-biased dimorphism of the limbs, which is clearly related to their mating system. A crucial trait for salamander reproduction is the invention of the spermatophore, which occurs in the seven families of the Salamandroidea, allows internal fertilization and is produced by a distinct set of cloacal glands (Sever 2003b). For a proper transfer of sperm, many terrestrial salamanders mate by guiding the female over the spermatophore (*e.g.*, Plethodontidae) or by applying an amplexus (Houck & Arnold 2003). Salamandrids exhibit different types of amplexus (head-to-head, ventral, dorsal and restraint via tail, jaw or limbs), with *Salamandra* and *Mertensiella* using a ventral amplexus (Arnold 1986, Houck & Arnold 2003). The selection pressure for strong limbs ensuring a proper amplexus thus seems very high and accounts for the sexual dimorphism in both species. The consistency of these traits throughout ontogeny also supports their significance.

By dissection of the *Salamandra algira* specimens, it was possible to study fecundity traits of both males and females in relation to the age data (presented in chapter 3). The results represent baseline data for *S. algira*, which is currently recognised as vulnerable in the IUCN red list of threatened species (see IUCN 2013). The fecundity of both sexes increases with age and size. Large females are often associated with an increased fecundity (*e.g.*, Kupfer 2007) and the connection between fecundity traits and age has previously been shown in other species (*e.g.*, Misawa & Matsui 1999). The interrelationship of age, size and fecundity should favour a delayed female maturity, so that females can grow longer before the growth-reproduction trade-off arises and the available energy is divided. Sexual bimaturity is

common in amphibians and females often mature later than males (Wells 2007). Chapter 2 already showed that males grow faster than females until the age of approximately five years and after that females can catch up, an indicator for sexual bimaturity in *S. algira*. As mentioned before, the sexes of this species lack a sexual dimorphism of body size, since selection favours not only female size through fecundity selection, but also male size due to their engagement in rivalry fights. Thus, the sizes approximate mutually, finally leading to the lack of SSD.

In chapter 4, the focus of the sexual dimorphism study was on a species, which mates in fully aquatic habitats. *Triturus marmoratus* males enter the water earlier than females after both sexes have ended their terrestrial hibernation phase, or they stay in the water throughout the year (Nöllert & Nöllert 1992). The place of courtship and mating is completely different in this newt species than it was in the terrestrial salamanders examined before. Courtship and mating sites are poor predictors of the courtship behaviour itself (Houck & Arnold 2003), but they can influence body adaptations and sexual dimorphism linked to the behaviour. In general, sexual dimorphism of the phylogenetically derived *T. marmoratus* is more complex than in *S. algira* and in *M. caucasica*. I was also able to demonstrate this trend already in my diploma thesis, which included a larger literature review on salamandrid sexual dimorphism (Reinhard 2010). Phylogenetically basal taxa are lacking sexual size dimorphism, but have dimorphic body proportions, such as limb dimorphism. The members of the European-Newt-clade (*Lissotriton*, *Mesotriton*, *Calotriton*, *Triturus*, *Ommatotriton*, *Neurergus*) firstly display totally divergent courtship behaviours in contrast to the more basal relatives among the Salamandridae. They use a tail-fanning movement (except *Calotriton*) to attract females via chemical cues and afterwards they turn and lead the receptive female over the deposited spermatophore. Because they have no physical contact as known from species with an amplexus mating, sexual dimorphism is most significant in body traits that attract females to males (Reinhard 2010). At this point, all phylogenetically derived salamandrids show a female-biased SSD again in concordance with an increased fecundity (except species with male rivalry).

All of my previous sexual dimorphism studies vividly demonstrate that it is necessary to use large specimen sample series and to analyse the data with

comparative methods, *i.e.*, adjusted to a general body measure such as snout-vent-length in salamanders. Otherwise, cryptic dimorphism in particular is covered especially in large-bodied species with obviously size-divergent genders.

The evolution of sexual dimorphism can also be favoured by other behavioural strategies, such as male territoriality or parental care. The phylogenetically basal aquatic salamander *Siren intermedia* (Sirenidae) (see chapter 5) exhibits a pronounced male-biased size dimorphism and furthermore, males have significantly larger heads and masseter muscles (see also Petranks 1998). Larger head sizes are often reported in connection with ecological niche divergence (*e.g.*, Shine 1989, Vincent & Herrell 2007), courtship biting (*e.g.*, *Euproctus*, see Rimpp & Thiesmeier 1999a/b) or male competition (*e.g.*, Hasumi 1994). I noticed a considerable aggression increase in males and territorial behaviour (occupying shelters and 'nest-building') in late winter and early spring. Shortly after that, I was also able to observe the mating behaviour of this species and thus it was possible to obtain direct evidence for external fertilization in *Siren* (to the best of my knowledge, *Siren intermedia* was bred only twice in captivity before, but breeding behaviour was not reported). External versus internal fertilization has been a long debated enigma in sirenids, although external fertilization was already expected due to the lack of cloacal glands for spermatophore production in males and the lack of spermathecae in females (Sever 2003b). After the mating process was over, it was surprising that the males stayed with the eggs and aggressively guarded them. This fact, the biting throughout the courtship and the increased territoriality were likely leading to the pronounced sexual dimorphism in this species. Paternal care was extensive and included not only guarding, but also active cleaning of the nest site. Surprisingly, the males continue parental care even after hatching of the larvae. Post-hatching parental care is highly uncommon in salamanders and has been described only for the terrestrial plethodontid *Hydromantes strinatii* (Oneto et al. 2010). The parental care behaviour in *Siren* might have evolved due to the large population densities in the natural habitats and hence egg predation is common (Scroggins & Davis 1956, Hampton 2009), making a parental protection of eggs necessary. This in turn should make females choose very large males, also with prominent masseter muscles, but this needs to be further accessed via experimental female choice tests in the future. Furthermore, the eggs of *Siren intermedia* are relatively large and require an



increased investment of the female. It could be assumed that females might care in this case, but the external fertilization mode predicts male care according to the association hypotheses (see Ridley 1978, Gross & Sargent 1985).

Guarding (and associated behaviours, *e.g.*, nest-cleaning) is the only parental care behaviour exhibited by salamanders (Wells 2007), whereas caecilian parental care is more diverse and can also include feeding of the young (*e.g.*, Kupfer et al. 2006b). The foetuses of some oviparous caecilian species and altricial hatchlings were reported to have a specialised dentition, which is used to scrape off the maternal skin during a prolonged phase of parental care after hatching (*e.g.*, Kupfer et al. 2006b). A similar dentition is found in the foetuses of viviparous species – the eggs are retained inside the oviduct and the specialised teeth are used for scraping and feeding on the oviductal epithelium (Wake 1977b). Thus, the young are large enough at birth to live independent from their mothers. Maternal dermatophagy ("skin-feeding") has been considered a preadaptation for the feeding inside the oviduct. I had the chance to directly observe the reproductive biology of two viviparous caecilian species, one with precocial (*Typhlonectes natans*, see chapter 6) and one having altricial young (*Geotrypetes seraphini*, see chapter 7). *Geotrypetes* shows a new reproductive mode of caecilian amphibians, as it is viviparous, has altricial young and mesolecithal eggs (similar to direct-developing, oviparous, skin-feeding species). The existence of maternal dermatophagy in *G. seraphini* has been proposed since O'Reilly et al. (1998). The captive breeding project of *G. seraphini* turned out difficult because of the sensitivity to disturbance of the adults and the long initial phase until the most suitable captive conditions were established, but nevertheless, I was able to induce breeding behaviour in the laboratory. Although I could only rear one litter to adulthood, it gave interesting insights into the growth of *G. seraphini* until maturity. The study also provided further strong evidence for maternal dermatotrophy although the active behaviour could not be observed. The CT-imaging of newborn young, young in dentitional metamorphosis and an adult female documented the foetal-like dentition, which is used for "skin-feeding" in dependent young, and its transition towards the adult condition. Interestingly, the social system (and likely communication) of the adults is pronounced in *G. seraphini*, since I observed communal nesting behaviour with nests containing more than one brooding female as well as even brooding or non-brooding females and males together in the nest.

Whereas it is likely that *G. seraphini* is lecithotrophic viviparous, with its young hatching only shortly before birth and receiving further nourishment in a post-birth care phase including maternal dermatophagy, the aquatic *Typhlonectes natans* is matrotrophic viviparous and provides nutrients for its young via uterotrophy. I monitored a breeding population during several subsequent years and recorded baseline data about the courtship behaviour as well as mother-offspring body trait relationships (chapter 6). This data revealed a high maternal investment, as the young were born with up to 43 % of the maternal length. Similar results are already known for the sister species of *T. natans*, *T. compressicauda* (see Wake 1977b, Billo et al. 1985). The fecundity is reduced in these species, but vitally compensated through a higher offspring quality.

Both studies of the chapters six and seven revealed new and partly unexpected findings and contribute to fill in some missing data, which is still prominent for caecilian amphibians. Gaining data using laboratory populations is challenging, especially if the study species were barely kept or bred before, but it is also crucial for documenting unknown reproductive behaviours.

### *Methods*

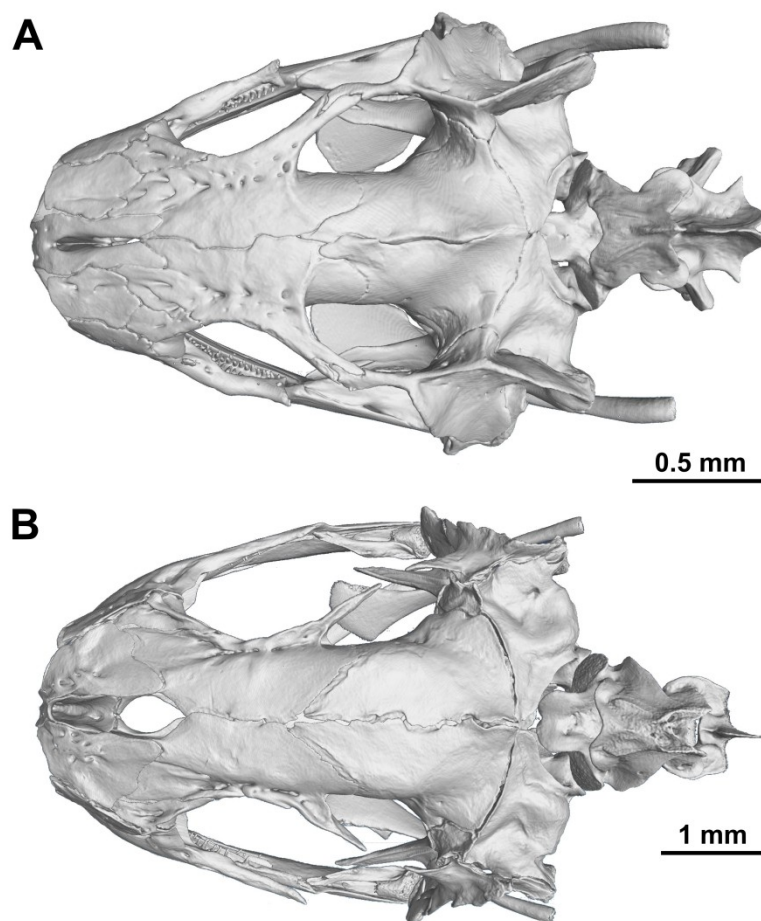
I employed several methodologies in the course of my doctoral studies in pursuit of the research objectives.

Firstly, traditional examination of morphological traits was carried out on preserved specimens housed at the collections of natural history museums. Morphometric measurements constituted the data source of the chapters 2 to 4. Only larger sample series were used, to obtain the best possible statistical support for the results. By employing numerous descriptive and analytic statistics, cryptic sexual dimorphism patterns were found. Some specimens were cleared for dissection, so that situs preparation could be performed.

Later in the course of my dissertation, I had the chance to learn additional techniques for assessing sexual dimorphism using geometric morphometrics, since I participated in a training course of the University of Manchester (Analysis of Organismal Form - course code BIOL51111) supervised by Dr. Christian Peter Klingenberg. Unfortunately, I had no chance to include this method into this doctoral thesis, but I will extensively use this new skill for my future studies.

Age data best supports the understanding of the ontogeny of sexual size dimorphism (chapters 2 and 3). Therefore the widespread histological technique of skeletochronology was used.

Furthermore, modern techniques, such as scanning electron microscopy (SEM) and Synchrotron- $\mu$ -CT, were applied. The detailed SEM improved the visualisation of the dentition of *Geotrypetes* specimens. Although the  $\mu$ -CT works at high-resolution, minute details of single teeth are better depicted using the aid of SEM., what requires critical-point drying of the samples prior to microscopy. The Synchrotron- $\mu$ -CT, which was performed at the Deutsches Elektronen-Synchrotron (DESY) in Hamburg, is a state of art and now frequently used scientific research method. It includes the major benefit, that it can portray internal structures despite being non-invasive, what is of particular interest when dealing with rare museum specimen. The best results were achieved for total skull scans, but scans of soft tissue are also possible, when applying staining methods prior to the scanning procedure (see Kühnel 2013). The SR $\mu$ CT provides three dimensionality of scans when processed with dedicated software (VG Studiomax 2.0) and allows virtual cutting through or separation of bone structures, an important detail to visualize the separation of upper and lower jaw and thus dentitional metamorphosis in the caecilian *G. seraphini* (chapter 7). As I have scanned the skulls myself (and a lot more samples of various species as assistance for colleagues) at the DESY (Beamline W2) during several visits since 2009, I extensively learned to apply this method (see also Figure 1).



**Figure 1.** SRμCT scans of two newt species (Urodela: Salamandridae). (A) *Calotriton asper* (adult male, NKM EF - A875/01) and (B) *Ommatotriton ophryticus* (adult female, SMNS uncatalogued). CT scans provide a state of the art method to obtain morphological data of rare museum specimens without tissue damage or dissection. Combined with analytics, such as geometric morphometrics, studies of sexual dimorphism can be carried out with high accuracy in future studies.

A fundamental step for successful reproductive biology research is to directly study behaviours of target species in vivo either in the field or if not possible in the laboratory. I was able to establish scientifically-based breeding programs for three amphibian species (*Siren intermedia*, chapter 5; *Typhlonectes natans*, chapter 6 and *Geotrypetes seraphini*, chapter 7), which all lacked basic reproductive data, such as the mode of fertilization, courtship and breeding behaviour or information on the mode of parental care. All species were rarely bred in captivity.

My extensive animal husbandry programs were rewarded with the first report of courtship, mating and extended paternal care in *S. intermedia*, the first successful breeding of *G. seraphini* after the O'Reilly et al. (1998) original brief note and a new

caecilian reproductive mode, as well as insights into the extensive maternal investment of *T. natans*.

Finally, by performing extensive literature reviews employing all available search methods and databases new hypotheses were generated (all chapters).

### *Conclusions and perspectives*

This dissertation provides baseline data on the reproduction and sexual dimorphism of salamanders and caecilians. It contributes to our overall understanding of the evolution of amphibian life history strategies. As data deficiency affects many caecilian amphibian species, new information is highly important, especially for evaluating the vulnerability of species having specialized mating systems to the growing variety of extinction risks.

The extensive use of natural history collections can provide valuable insights, particularly when technically advanced methods, such non-invasive 3D- $\mu$ CT imaging is employed. The combination of these methods with further computer-based analytics, such as geometric morphometrics, are powerful tools to assess sexual dimorphism and thus the evolution of mating systems with the highest possible accuracy. The non-invasiveness of this approach provides access to even rare museum specimens of natural history collections all over the world.

The individual chapters presented herein also highlight the significance for additional studies on the evolutionary reproductive biology of amphibians, *i.e.*, by installing further scientifically managed captive breeding programs at university level, as well as in zoo and museum collections. They would serve as the key data on amphibian reproduction. I also gathered more data, which is not fully included herein, but it will be processed and prepared for publication in the future. I am highly motivated to extend my studies of amphibian life history in the future and hope to contribute with different approaches, supported by the skills I gathered while researching for and writing this doctoral thesis.

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## **EHRENWÖRTLICHE ERKLÄRUNG**

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe. Sämtliche Stellen, die anderen Quellen entnommen wurden sowie persönliche Mitteilungen sind unter deren Angabe kenntlich gemacht. An der Erstellung der beinhalteten Manuskripte haben Co-Autoren mitgewirkt, mein Eigenanteil ist jedoch in der Arbeit ausgewiesen (siehe 'Overview of manuscripts', S. 24).

Die Hilfe eines Promotionsberaters wurde nicht in Anspruch genommen. Dritte haben weder unmittelbar noch mittelbar geldwerte Leistung für Arbeiten, die mit dem Inhalt dieser Dissertation zusammenhängen, von mir erhalten.

Ich versichere weiterhin, dass ich die vorliegende Arbeit nicht für eine staatliche oder andere wissenschaftliche Prüfung als Prüfungsarbeit eingereicht habe und an keiner anderen Hochschule das Verfahren zum Erwerb des akademischen Grades Dr. rer. nat. beantragt habe.

Die Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena ist mir bekannt.

Ort, Datum

Sandy Reinhard

## LEBENS LAUF

### PERSÖNLICHES

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Name Sandy Reinhard  
Geburtsdatum/-ort 10.05.1986 in Rudolstadt, Thüringen

### SCHULISCHE AUSBILDUNG

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2004 Allgemeine Hochschulreife  
1996 – 2004 Staatliches Gymnasium Fridericianum, Rudolstadt

### UNIVERSITÄRE AUSBILDUNG

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2011 – 2014 Promotion zum Thema "*Life history strategies and sexual dimorphism of the Amphibia with special emphasis on the Urodela (Newts and Salamanders) and the Gymnophiona (Caecilians)*" am Institut für Spezielle Zoologie und Evolutionsbiologie der Friedrich-Schiller Universität Jena  
2004 – 2010 Studium der Biologie (Diplom) an der Friedrich-Schiller Universität Jena, Hauptfach: Spezielle Zoologie, Nebenfächer: Anthropologie, Verhaltensphysiologie (Universität Leipzig), Titel der Diplomarbeit: "*Die Evolution des Sexualdimorphismus und der Lebensstrategien der Echten Salamander und Molche (Amphibia: Urodela: Salamandridae.*"

### AKADEMISCHE BESCHÄFTIGUNG

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2010 – 2013 Wissenschaftliche Hilfskraft am Institut für Spezielle Zoologie und Evolutionsbiologie, FSU Jena  
2008 – 2010 Studentische Hilfskraft am Institut für Spezielle Zoologie und Evolutionsbiologie, FSU Jena

### FÖRDERUNGEN

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2012 Sachmittelbeihilfe zur Teilnahme an einem Online-Short-Course der University of Manchester durch die Graduierten-Akademie der FSU-Jena  
2011 Landesgraduiertenstipendium der Graduierten-Akademie der FSU Jena für das Promotionsprojekt  
2011 Sachmittelbeihilfe durch das ProChance Programm, Förderlinie A2 der FSU Jena für die Teilnahme an Kongressen in Saarbrücken und Trier/Luxembourg  
2009 Wilhelm-Peters Fond der Deutschen Gesellschaft für Herpetologie und Terrarienkunde als Reisekostenzuschuss für die Diplomarbeit

Sandy Reinhard