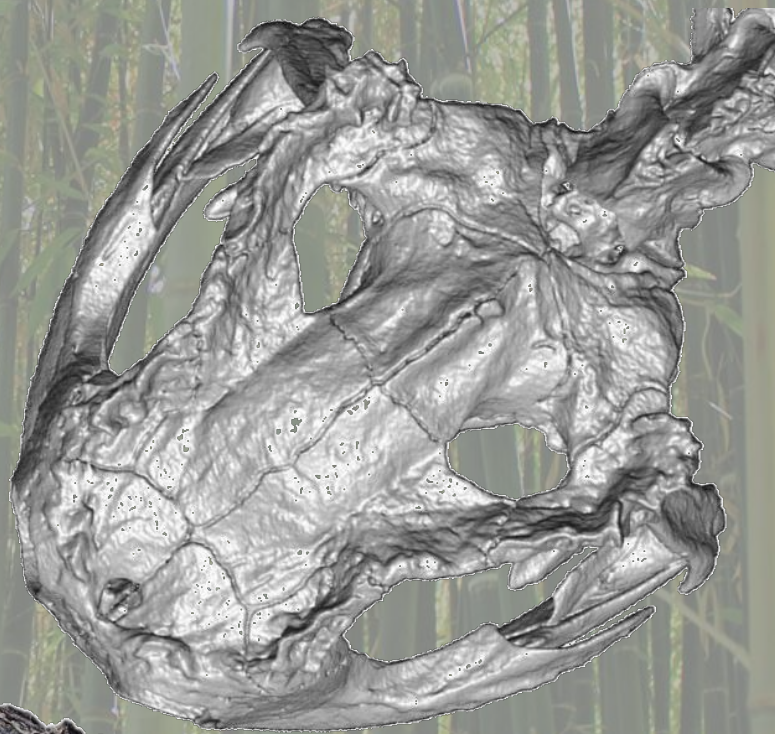


**Doktorarbeit**

**Evolution and paleobiology  
of salamanders (Caudata:  
Salamandridae) with a special  
emphasis on sexual dimorphism**

von

**Peter Pogoda**





**Evolution and paleobiology  
of salamanders (Caudata:  
Salamandridae) with a special  
emphasis on sexual dimorphism**

**Dissertation**

der Mathematisch-Naturwissenschaftlichen Fakultät  
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*in tribute to my dad*

*all I am has only been possible thanks to him*





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## Abbreviations

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2D	two-dimensional
3D	three-dimensional
CS	centroid size (a measure of size in geometric morphometrics)
CT	computed tomography or computer tomography
$\mu$ CT	micro computed tomography
GM	geometric morphometrics
PCA	principal component analysis
SD	sexual dimorphism
SSD	sexual size dimorphism
SShD	sexual shape dimorphism
KIT	Karlsruhe Institute of Technology
SMNS	State Museum of Natural History Stuttgart



## Zusammenfassung

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Im Tierreich unterscheiden sich bei zahlreichen Arten die Weibchen und Männchen in ihrer äußeren Gestalt. Dies kann sich in unterschiedlichen Ausprägungen widerspiegeln wie der Größe, Farbe oder Körperschmuck und -anhänge. Während endotherme Wirbeltiere in dieser Hinsicht schon lange erforscht werden, besteht bei den ektothermen Wirbeltieren noch Forschungsbedarf. Vermehrt werden Unterschiede in der Form von Körper oder Körperteilen untersucht. Diese sind meist recht schwach ausgeprägt und daher nicht sofort ersichtlich und auch mit traditionellen Methoden nur bedingt zu erfassen. Die genauen evolutiven Mechanismen hinter diesen sexuellen Größen- und Formendimorphismen beginnen wir gerade erst in den Blickpunkt zu rücken und zu verstehen. Ein integrativer Ansatz, der verschiedene Ausprägungen von Sexualdimorphismen mit der Ökologie der Arten vereinbart, kann dabei helfen einzelne Mechanismen der Evolution aufzulösen. Derartige Korrelationen zwischen der Morphologie und der Lebensweise rezenter Arten sind ein Schlüsselfaktor für die Rekonstruktion von ausgestorbenen Arten. Bei Wirbeltieren sind meist nur die Knochen fossil überliefert. Daher wird mehr Wissen über die Osteologie der rezenten Arten benötigt, um diese auf die fossilen Vorfahren zu übertragen.

Die sogenannten Echten Salamander der Familie Salamandridae stellen ein ideales Modellsystem für die Untersuchung von Sexualdimorphismen im Zusammenhang mit der Ökologie dar, da die Arten dieser Gruppe in vielerlei Hinsicht hier Unterschiede aufweisen und so ein Vergleich möglich ist. Im Rahmen dieser Arbeit sollte intensiv untersucht werden, welche weiteren bisher unentdeckten Größen- und im speziellen Formdimorphismen bei basalen echten Salamandern auftreten, wie diese sich in der Osteologie widerspiegeln und wie die Osteologie mit der Ökologie korreliert um daraus Rückschlüsse auf ausgestorbenes Leben schließen zu können.

Das erste Kapitel behandelt das Ausmaß von Sexualdimorphismen am Nördlichen Brillensalamander *Salamandrina perspicillata* (SAVI, 1821), welcher dem phylogenetisch ursprünglichsten Abstammungszweig der Salamandridae angehört. Die ursprünglichen Brillensalamander dienen dazu auch die Evolutionsgeschichte des Sexualdimorphismus innerhalb der Salamandriden zu verstehen. Es wurden Messstrecken der äußeren Morphologie sowie der Osteologie genutzt und mittels eines neuen statistischen Ansatzes wurde explizit auf sexuelle Formdimorphismen hin getestet. Obwohl vergleichbare Messstrecken der äußeren und inneren Morphologie/Osteologie ein gleiches Bild ergaben, kamen im Knochenbau weitere,



bisher unbekannte, dimorphe Merkmale zum Vorschein. Das Ausmaß des Formdimorphismus stellte sich als weitaus größer dar als bisher bekannt war.

Im zweiten Kapitel wird die Osteologie des Brillensalamanders an den gleichen Individuen mittels Geometrischer Morphometrie (GM) untersucht, um den Formendimorphismus genauer aufzulösen und diese neue Methode mit der traditionelleren Methode aus dem ersten Kapitel zu vergleichen. Besonders im Beckengürtel konnten größenunabhängige Geschlechtsunterschiede nachgewiesen werden, die wahrscheinlich auf die unterschiedliche Rolle der Geschlechter während der Reproduktion zurück zu führen sind.

Im dritten Kapitel wurde die Schädelmorphologie der ebenfalls phylogenetisch ursprünglichen Rippen- und Krokodilmolche (Pleurodelini) untersucht und in den Zusammenhang zu ausgewählten Reproduktionsmerkmalen gesetzt. Rippen- und Krokodilmolche besitzen einen guten Fossilbericht, welcher vom Eozän bis in das Pliozän hineinreicht. Folglich schloss die Analyse die fossile Gattung *Chelotriton* ein, um weitere Rückschlüsse auf die verwandtschaftlichen Beziehungen, die Evolutionsgeschichte aber auch auf die Ökologie ziehen zu können. Die europäischen Rippenmolche waren weit von den asiatischen Krokodilmolchen differenziert. Ebenfalls konnten die zwei Untergattungen der Krokodilmolche (im engeren Sinne) – *Tylotriton* und *Yaotriton* – erstmals morphologisch getrennt werden. Das Paarungsverhalten korrelierte mit den unterschiedlichen Schädelmorphologien. Fossile *Chelotriton* repräsentiert mehrere Arten und wies eine eher terrestrisch orientierte Lebensweise und Reproduktion auf.

Im vierten Kapitel wurde die Ausprägung von Geschlechtsunterschieden bei verschiedenen Arten der Krokodilmolche untersucht und diese in Relation zum Paarungsmodus, der bei den Krokodilmolchen variiert, gesetzt. Die Ausprägungen zwischen den Arten unterscheiden sich dabei deutlich. Der Paarungsmodus konnte jedoch nur in der Schädelmorphologie als erklärender Faktor bestätigt werden, während dies für den Oberarmknochen nicht zutraf. Da Letzterer dennoch Unterschiede aufweist, müssen hier andere selektive Faktoren einwirken.



## Summary

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Sexual dimorphism (SD) i.e., the difference in morphology between males and females, is a common phenomenon in the animal kingdom. SD can be observed in various forms such as gender-dependent differences in size, colouration or the presence of ornaments. While warm-blooded vertebrates are well investigated in the context of SD, cold-blooded vertebrates i.e., amphibians and reptiles are still neglected. Often sexes are differentiated in terms of size, termed sexual size dimorphism (SSD) or even in terms of shape differences, so termed sexual shape dimorphism (SShD). Sexual size and shape dimorphisms are often rather subtle and hard to capture when employing traditional analysing methodology. The evolutionary mechanism underlying those features are still under debate. An integrative approach in a phylogenetic context including different patterns of SD connected to the ecology of a respective species can help to reconstruct and understand the evolutionary historical patterns leading to differences between males and females. This is of significant interest for the reconstruction of ancient life in a paleontological sense. The most prominent remains of fossil vertebrates are hard tissues e.g., bones and teeth. Hence, more knowledge on the osteology of extant taxa is needed to allow conclusions on their fossil relatives.

True salamanders of the family Salamandridae are a perfectly suited model system for the investigation of SD and its evolutionary history, because this group exhibits diverse reproductive modes and life history strategies linked to different patterns of SD. In this thesis, I investigated extensively patterns of sexual size and shape dimorphism in basal salamandrid salamanders. Further, I wanted to set morphology into context with a species' ecology to allow conclusions on the ecology of extinct salamander taxa.

In the first chapter, I used the spectacled salamander *Salamandrina perspicillata* (SAVI, 1821), the phylogenetically most basal genus of the Salamandridae, to extensively investigate patterns of sexual size and shape dimorphism in the external morphology and osteology by linear measurements. Therefore, I employed a novel integrative statistical approach. The results showed that comparable characters in the soft and hard tissue revealed similar patterns of SD. The osteology harbours also so far unknown pattern of SD.

In the second chapter, I used geometric morphometrics (GM), which represents a novel upcoming technique in morphology research, to reveal even more subtle shape differences. To this end, I used the same set of specimens as in chapter one. This enabled me to compare GM



with the traditional approach of capturing morphological differences. Generally, the outcome of both methods is similar if appropriate data is gathered, but via GM, smaller shape differences were revealed. Especially, the pelvic girdle harboured size independent shape differences likely corresponding to the different roles of males and females during reproduction.

In the third chapter, I investigated the cranial morphology of the ribbed and crocodile newts (Pleurodelini) and set it into context to selected reproductive traits. Ribbed and crocodile newts bear an extensive fossil record spanning from the Eocene towards the Pliocene. Consequently I included the closely related but extinct genus *Chelotriton* into my analyses to obtain further hints on the relationship of extinct and extant taxa and to draw conclusions on the ecology of *Chelotriton* based on morphology-ecology correlations. European ribbed newts were well separated from the Asian crocodile newts. For the first time it was possible to define clear-cut morphological differences for the two subgenera of crocodile newts i.e., *Tylotriton* and *Yaotriton*. Regarding the selected reproductive traits, the mating mode affected the cranial shape evolution. Fossil *Chelotriton* likely represents several species exhibiting a rather terrestrial lifestyle and reproduction.

In the fourth chapter, I investigated patterns of SSD and SShD in several species of crocodile newts and in relation with their mating mode, which variably includes an amplexus or mating dances. Trajectories of shape changes from males to females differed interspecifically. The mating mode could explain interspecific SD trajectory differences in cranial but not humerus morphology. Nevertheless, humerus morphology differed also in shape among species exhibiting different mating modes, indicating other selective forces are acting on limb morphology. An interaction of allometric trajectories different between species but similar between the sexes are likely responsible for the variable SD patterns among polymorphic crocodile newts.

## List of publications

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### Published manuscripts

**POGODA, P., M. ZUBER, T. BAUMBACH, R.R. SCHOCH & A. KUPFER (2020):** Cranial shape evolution of extant and fossil crocodile newts and its relation to reproduction and ecology. – *Journal of Anatomy*, 237: 285–300. <https://doi.org/10.1111/joa.13201>.

**POGODA, P. & A. KUPFER (2020):** Sexual shape dimorphism in the cranium and pelvic girdle of Northern spectacled salamanders, *Salamandrina perspicillata*, investigated via 3D geometric morphometrics. – *Salamandra*, 56: 113–122

**POGODA, P. & A. KUPFER (2018):** Flesh and bone: An integrative approach towards sexual size dimorphism of a terrestrial salamander (genus *Salamandrina*). – *Journal of Morphology*, 279: 1468–1479. <https://doi.org/10.1002/jmor.20883>

### Manuscripts in preparation

**POGODA, P., M. ZUBER, T. BAUMBACH & A. KUPFER:** Clasp and dance: Mating mode promotes variable sexual size and shape dimorphism trajectories in crocodile newts (Caudata: Salamandridae). Under revision in *Ecology & Evolution*, published as preprint in AUTHOREA. July 26, 2021. <http://dx.doi.org/10.22541/au.162731360.07245217/v1>



## Manuscripts not included in thesis

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LOREDANA, M., A. VILLA, G. PITRUZELLA, L. ROOK, **P. POGODA**, A. KUPFER & M. DELFINO: Osteology of the Italian endemic spectacled salamanders, *Salamandrina* spp. (Amphibia, Caudata, Salamandridae): selected skeletal elements for palaeontological investigations. – *Journal of Morphology* 281, 1391-1410. <https://doi.org/10.1002/jmor.21254>

SCHOCH, R.R., **P. POGODA** & A. KUPFER (2019): The impact of metamorphosis on the cranial osteology of giant salamanders of the genus *Dicamptodon*. – *Acta Zoologica*, 102, 88-104. <https://doi.org/10.1111/azo.12318>.

**POGODA, P.** & A. KUPFER. (2019): High osteological variation in a terrestrial salamander (genus *Salamandrina*). – *Zoologischer Anzeiger*, 281: 39-43. <https://doi.org/10.1016/j.jcz.2019.06.004>

**POGODA, P.** (2019): Reproduction and paternal care in the Asian bird poop frog, *Theloderma albopunctatum* (Liu and Hu, 1962). – *Herpetology Notes*, 12: 439-441.

MAERKER, M., S. RHEINHARD, **P. POGODA** & A. KUPFER (2016): Sexual size dimorphism in the viviparous caecilian amphibian *Geotrypetes seraphini seraphini* (Gymnophiona: Dermophiidae) including an updated overview of sexual dimorphism in caecilian amphibians. – *Amphibia-Reptilia*. 37: 291-299. <https://www.doi.org/10.1163/15685381-00003057>





## Author contributions

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after § 6 Abs. 2 Satz 3 of the doctoral regulations (Promotionsordnung)

### **Peter Pogoda**

Developed and conceptualised research ideas for all chapters. Organised loans of crocodile newt specimens from natural history collections in Germany (ZFMK, ZMB, ZSM), France (MNHN), Canada (ROM) and U.S.A. (CAS, USNM, NCSM) and fossil specimens from the Generaldirektion Kulturelles Erbe Rheinland-Pfalz, Mainz. Applied for funding for workshop costs, travel grants and  $\mu$ CT costs. Generated great majority of the  $\mu$ CT scans. Conducted all experimental set ups, collected all data (measurements and landmark digitization) and analysed the data used in all publications. Contributed as first author to all chapters in this thesis. Drafted all manuscripts and improved these during the peer-review process.

### **Alexander Kupfer**

Supervised all experiments, provided scientific input, and helped to conceptualise research ideas. Organised loans of *Salamandrina* and of *Tylotriton* specimens from SMF, Frankfurt a. M., MTKD, Dresden, NMB, Basel (Switzerland) and NHMW, Vienna (Austria). All loans from natural history collections were taken under his care. Contributed as senior author to all chapters. Helped to improve all manuscript drafts during peer-review process.

### **Rainer R. Schoch**

Provided expertise in handling and data generation from fossil specimens. Provided funding for the CT scanning of one fossil specimen. Loans of fossil *Chelotriton* from natural history collections were taken under his care. Contributed to chapter III.

### **Marcus Zuber**

Provided access to the CT-lab at the KIT, Campus Nord and helped to generate approximately a third of the  $\mu$ CT scans of newts used in chapter III and IV. Contributed to chapter III and IV.

### **Tilo Baumbach**

Provided access to the CT-lab at the KIT, Campus Nord.



## Introduction

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Animals have developed a marked variation of body plans. Although, during evolution the general animal bauplan possessed such a high flexibility, the possibilities of a single species to evolve further are highly restricted by its phylogenetic background. Nevertheless, in every species there is some degree of variability in their morphology allowing adaptations to future requirements (e.g., MATHER 1966). The study of morphological variation across taxonomic levels helps to understand how selection promotes species differentiation and e.g., how a species can adapt to changing environmental features (for urodeles e.g., JOCKUSCH 1997, ROMANO & FICETOLA 2010, BONETT & BLAIR 2017). Maybe one of the strongest effects on species evolution is their own life history (BONETT et al. 2018). Life histories do even vary within a species especially among sexually reproducing species where males and females invest differently in their offspring, leading to different reproduction strategies and ultimately to different morphologies.

## Sexual dimorphism

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Such a different appearance of females and males is referred to as sexual dimorphism (SD). It is quite common in the animal kingdom and found in all taxa and even in plants (WILLSON 1991, FAIRBAIRN et al. 2007, BARRETT & HOUGH 2013). Charles Darwin already summarised the different selection forces acting on males and females leading to their different morphologies (DARWIN 1871). Sexual dimorphism can be expressed in various forms either in e.g., body size, colouration, ornamentation like dewlaps, large feathers, etc. or by weaponry (Fig. 1). In some animal taxa regularly similar patterns among species are found. In mammals often males are larger than females (e.g., Lindenfors et al. 2007). In some cases, such as in e.g., elephant seals SD reaches an enormous scale where males are up to four-times heavier than females (SCHEFFER 1958). In many ruminant taxa males exhibit antlers or horns while these are absent or way smaller in females. The common case in birds is that males are the sex often exhibiting decorative plumage (HILL 2006). In general the assumption is that the same trait in females and males is genetically correlated and the genetic basis for SD is polygenic (LANDE 1980, LANDE & ARNOLD 1985). In order to evolve intersexual different morphologies, the genetic correlation of a trait between the sexes must be lower than one and males and females have to possess different evolutionary optima for a trait on which selection is acting.





Figure 1: Examples for sexually dimorphic traits in the animal kingdom. A: decorative plumage in peacock (*Pavo cristatus*) male, B: vocal sacs in male European tree frog (*Hyla arborea*), C and D: different body colouration in male (C) and female (D) sand lizard (*Lacerta agilis*), E and F: differences in cranial proportions in Cayman blue iguana (*Cyclura lewisi*) (E: female, F: male). © PETER POGODA

Various selective forces are under debate to promote the evolution of SD in animals. (1) The oldest one, which already DARWIN (1871) addressed is the **sexual selection theory**. As already mentioned, males and females invest differently in their offspring. While females produce large eggs or must bear their offspring until birth and often care for them afterwards, males contribute only small and presumably cheap sperm (BATEMAN 1948, NAKATSURU & KRAMER 1982). Thus, males can produce a large number of offspring while females are much more limited in their capacity. In such species, males have to compete against each other in one or the other way to get access to receptive females and achieve the maximum reproductive output (CAMPBELL 1972, ANDERSSON 1994, GAGE & BARNARD 1996, ABLE 1999). Larger individuals or those with larger weaponry have an advantage in agonistic interactions and can access more females. Such strong male-male competition for females is commonplace in mammals (e.g., CLUTTON-BROCK et al. 1977, CLUTTON-BROCK et al. 1982). Sexual selection could also act by mate choice. In species where female mate choice is strong, preferred male traits are positively selected. Beside body size this can especially include traits such as ornamentation (LANDE & ARNOLD 1985). Over generations these traits are maintained in the population leading to larger (SHINE 1979, ANDERSSON 1994) or more colourful males (HOUDE & ENDLER 1990), even this might be disadvantageous or dangerous for the males. As an example, females of the túngara frog prefer the mating call of larger males over the call of smaller ones (e.g., RYAN 1985) and in the widowbird, females prefer males with more elaborated decorative plumage although this handicaps males in their flying abilities (ANDERSSON 1982). The general assumption is that



large or more ornamented males exhibit with more reliability a better health condition and a better genetic background resulting in a higher fitness of their offspring and/or a better ability of the male to care for the mother and the young, being the reason why females prefer those males (e.g., HALLIDAY 1983, HALLIDAY & VERRELL 1986, HALLIDAY & VERRELL 1988, HILL 2006).

(2) The **female's fecundity theory** is centred on the idea that females are the limiting factor for the reproductive output of a population, and thus, for males. It hypothesizes that females are selected for larger size to gain selective advantage by the production of more or larger offspring (DARWIN 1871, HEDRICK & TEMELES 1989). In various taxa it has been shown that female size can correlate with their reproductive output e.g., in butterflies (WIKLUND & KARLSSON 1988), moose (SAND 1996), sand lizards (OLSSON 1993) and various amphibians (SALTHE 1969, KUPFER 2009). Higher fecundity is the most likely cause why males prefer larger females as mates (VERRELL 1985b, SARGENT et al. 1986, OLSSON 1993). In anurans it is the regular case that females are the larger sex (SHINE 1979, KUPFER 2007). In recent years, it was demonstrated that females can signal higher fecundity also by other traits leading to male mate choice on those traits and thus, to sexually dimorphic characters other than size (e.g., LAPLANTE 2015, BELLIURE et al. 2018, LÜDTKE & FOERSTER 2018, LÜDTKE & FOERSTER 2019).

(3) Probably the theory hardest to test and to prove is the **ecological niche theory** (SELANDER 1966, SLATKIN 1984, TEMELES 1986). It predicts that males and females occupy different niches to reduce competition among each other, leading to sex-specific morphological adaptations to the respective niche. By far the most studies focus on different food niches either by increasing body size (PETERS & GRUBB JR 1983, PRICE 1984, TEMELES 1986) or adapting cranial morphology in one sex (SELANDER 1966, TEMELES & ROBERTS 1993, HERREL et al. 1999, LUISELLI et al. 2002, SHETTY & SHINE 2002, IVANOVIĆ & KALEZIĆ 2012). However, even though intersexual differences in head morphology are often found it does however not automatically imply dietary divergence (HERREL et al. 1999, COSTA et al. 2015). Also, other selective forces can act differently on the cranial morphologies of the sexes in those species. So far only rarely other ecological parameters than food niche have been considered as driving force for SD. BLEIWEISS (1999) investigated patterns of SD in context with the breeding behaviour of hummingbirds. He could show correlations among SD and breeding behaviour in this bird group, but it was also highly correlated to the respective feeding behaviour of the different species and of males and females, highlighting the complex underlying mechanisms.



The latter example shows that SD unlikely arises only due to one selective force, but rather due to the combined action of several selection mechanisms. A vivid example for this is the European common toad, *Bufo bufo* (HALLIDAY & VERRELL 1986). This species has a very short reproductive period of only one to two weeks where all males and females of a population approach their breeding ponds. As females do not breed every year, the number of males is always higher than that of receptive females. Hence, males show a marked scramble competition for those females (Fig. 2 A, SINSCH et al. 2009). It has been clearly demonstrated that larger males have an advantage over smaller males in displacing those from females and defending themselves against being displaced from females' backs (DAVIES & HALLIDAY 1979). A larger body size confers a high advantage for the males and selection should actively favour those. Nevertheless, the females are the larger sex (SINSCH et al. 2009) assuming that selection on female fecundity is stronger than sexual selection in males in common toads (Fig.2 B).

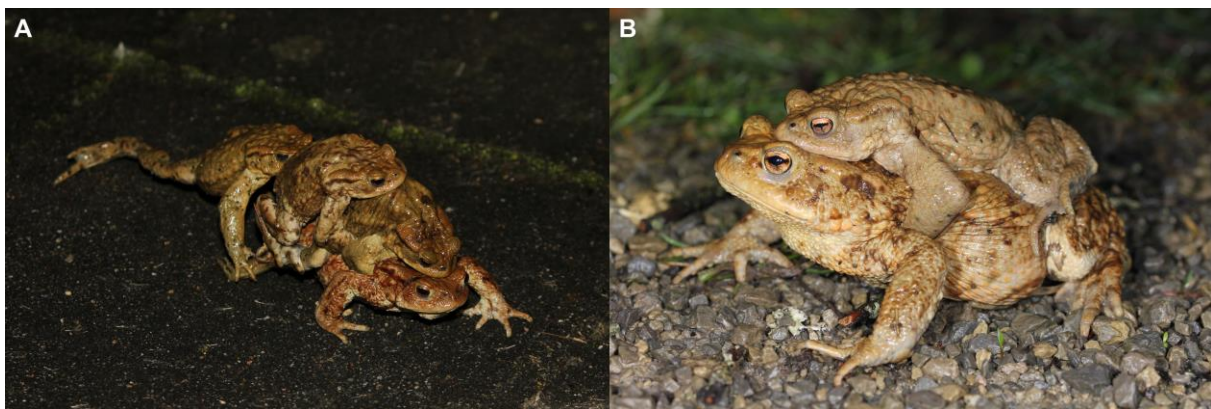


Figure 2: A sexually dimorphic species is the common toad, *Bufo bufo*. A: The males exhibit strong scramble competition for receptive females, B: females are the larger sex in this species and carry a male to the reproduction ponds on their backs.  
© PETER POGODA

As already addressed above, SD can be expressed in various forms. Beside colouration (dichromatism) and ornamentation, sexual size dimorphism (SSD) is one of the most studied manifestations of SD and is known from many animal groups (FAIRBAIRN 2007, FAIRBAIRN et al. 2007). Sexual size dimorphism is not only related to overall body size but also to size differences in single body parts e.g., male wood frogs *Lithobates sylvaticus* possess longer fore limbs to better hold females during amplexus (HOWARD & KLUGE 1985), and the smaller sex of snakes exhibits relative larger eyes to compensate loss of visual capacity due to their smaller cranial size (FAIMAN et al. 2005, FAIMAN et al. 2018). Morphological differences in body shape also occur irrespective of size meaning that proportions of body measurements are different. Such morphological divergence of shape and body form are referred to as sexual shape



dimorphism (SShD). As SShD is often rather subtle, research and understanding of its evolutionary origin has just begun to rise (e.g., SHETTY & SHINE 2002, SCHWARZKOPF 2005, IVANOVIĆ et al. 2008, GIDASZEWSKI et al. 2009, ALARCÓN-RÍOS et al. 2017). Allometry i.e., the change of shape during ontogenetic growth, occurs in most taxa. Individuals of different body size possess also different body shapes. Thus, shape dimorphism could be simply a side effect of selection on size in one sex (GOULD 1975, SCHWARZKOPF 2005). And of course, it is often found that patterns of SShD follow the patterns of allometric shape changes such as in mammals and lizards (e.g., BRUNER et al. 2005, CARDINI & ELTON 2008, KALIONTZOPOULOU et al. 2008, SCHUTZ et al. 2009). Size-unrelated components of shape differences were also detected indicating differential selective forces (e.g., BUTLER & LOSOS 2002, BRUNER et al. 2005, VIDAL et al. 2005, IVANOVIĆ & KALEZIĆ 2012). An alternative explanation for the development of SShD is different phenotypic trait variation in males and females (BUTLER & LOSOS 2002). The sex with the greater phenotypic variance may respond differentially, although the same selective pressure is acting on both sexes, resulting in shape dimorphism until a selective equilibrium is reached. Due to its often-low expression quantifying SShD requires a denser and more accurate data collection and multivariate statistics (e.g., MALMGREN & THOLLESSON 1999, BUTLER & LOSOS 2002, ROMANO et al. 2009a, REINHARD et al. 2015, HERREL et al. 2017). Traditionally the size and shape of life was gathered via linear measurements which possess limited capability. In the recent two decades geometric morphometrics (GM) has arisen as a new and valuable technique for capturing animal shape, receiving much attention by researchers (ADAMS et al. 2004, KALIONTZOPOULOU 2011, ZELDITCH et al. 2012). Compared to traditional methods it has been shown that GM is more capable in assessing even small shape differences (BLANCO & GODFREY 2006, ARENDT 2010, BRENO et al. 2011, SCHMIEDER et al. 2015, ILIĆ et al. 2019). This fact makes this method especially rewarding for quantifying SShD (ABDEL-RAHMAN et al. 2009, GÓMEZ-VALDÉS et al. 2012, BERNS & ADAMS 2013).

### **The model system: Salamandridae**

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Within the urodeles, “true salamanders” of the family Salamandridae represent an eligible model system for investigating patterns and evolution of SSD and SShD. Currently the Salamandridae comprise 127 extant species in 21 genera (Fig. 3, FROST 2018, VEITH et al. 2018). Within Caudata the Salamandridae represent the second most speciose family behind the lungless salamanders, Plethodontidae (FROST 2018). True salamanders have their main distribution area in Eurasia (SPARREBOOM 2014), but two genera including seven species were



able to colonize North America as well (PETRANKA 1998). The large majority of species inhabit the temperate climate zone, but a few do even reach the tropics in Southeast Asia (SPARREBOOM 2014, WANG et al. 2018). What makes this group so interesting for research on SD is their immense variability in lifestyles, reproductive modes and their diverse life history strategies (e.g., KIEREN et al. 2018). The Salamandridae comprise species living totally terrestrial even circumventing aquatic larvae e.g., *Salamandra atra*, to almost fully aquatic species such as the Asian genera *Cynops* and *Paramesotriton* (GROSSENBACHER & THIESMEIER 2003, SPARREBOOM 2014). True salamanders include species with high variation in their reproductive output. While some genera and species deposit up to several hundred eggs, other give birth to a small number of larvae or even only a couple of fully metamorphosed young (GROSSENBACHER & THIESMEIER 1999, GROSSENBACHER & THIESMEIER 2003).

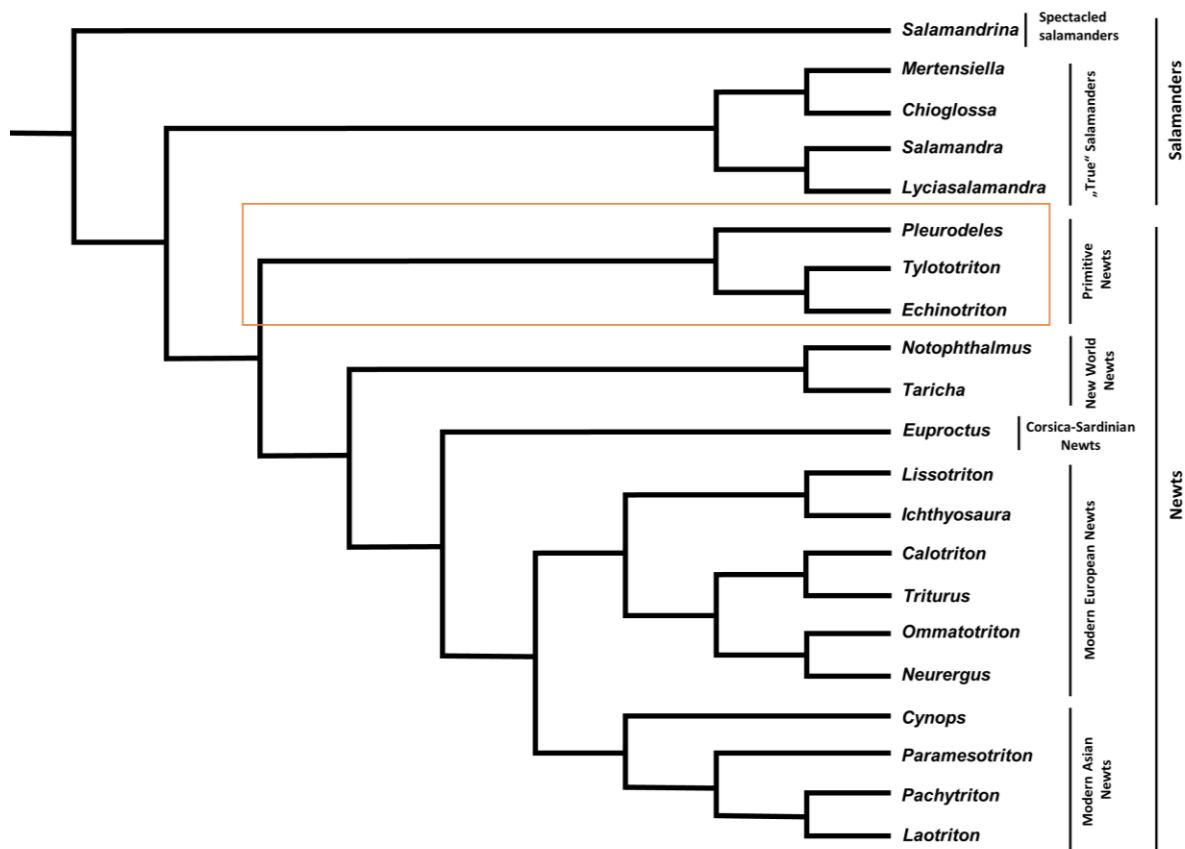


Figure 3: Phylogeny of the Salamandridae modified after ZHANG et al. 2008. The Pleurodelini are highlighted by an orange box.

In addition, a large variety of mating behaviours can be observed. In terrestrial “true” salamanders such as *Salamandra* and *Lyciasalamadra*, mating takes place on land via a ventral amplexus i.e., the male grabs the female from below with its fore limbs (e.g., HIMSTEDT 1965) whereas in *Mertensiella* clasping takes place in shallow streams (STEINFARTZ & MUTZ 1998,



FRANZEN 1999). Whereas newts such as *Triturus*, *Lissotriton* and *Cynops* mate mainly in water, some other newts mate on land, as some species of *Tylototriton* and *Echinotriton* (SPARREBOOM 2014, HERNANDEZ 2016). Males of e.g., European newts (*Triturus*, *Lissotriton*, *Ichthyosaura*) perform an elaborate, stereotypic mating dance to convince females to take up their spermatophores (e.g., THIESMEIER & SCHULTE 2010). A highly specialised mating behaviour has evolved among European brook newts of the genera *Calotriton* and *Euproctus*. Males grasp the females with their mouth in order to carry those away to avoid interference by other males. For copulation they embrace the female pelvic region with their tail and directly transfer their spermatophore directly into the cloaca of the female (CLERGUE-GAZEAU 1999, RIMPP & THIESMEIER 1999a, RIMPP & THIESMEIER 1999b).

This large disparity in the ecology of the Salamandridae can be assumed to have triggered the evolution of different SD patterns. The direction of SSD in body size is often estimated via the SSD-index or SDi after LOVICH & GIBBONS (1992) which is calculated as (mean SVL of larger sex/mean SVL of smaller sex)-1. Positive values indicate a female-biased SSD and negative values a male-biased SSD. AMAT (2019) investigated SSD in body size in 86 species of the family. He found that in 55 species (64%), males and females differed significantly in size. Compared to all caudates, which show only in 55.5% of the species a significant SSD, this value is rather high (AMAT 2019). Of those 55 species, 34 (61,8%) exhibited a female-biased and 21 (38,2%) species a male-biased SSD. The mean SSD-index was 0.087, meaning a size disparity of about 8.7%, showing that in most species the body size divergency was relatively small but substantial. The range of the SSD-index reached from -0.16 (*Ommatotriton*) to 0.28 (*Cynops*) indicating a high diversity in SSD patterns. The larger body size in females is mainly attributable to their elongated trunk (e.g., MALMGREN & THOLLESSON 1999, ROMANO et al. 2009a, LABUS et al. 2013, REINHARD et al. 2015). Beside body size dimorphism, a variety of other dimorphic characters have been found in salamandrids. Males of various taxa regularly exhibit larger cloacae, longer limbs and heads (e.g., MALMGREN & THOLLESSON 1999, BOVERO et al. 2003, AMAT et al. 2015, REINHARD & KUPFER 2015, REINHARD et al. 2015). Some occasionally dimorphic traits are tail length (MALMGREN & THOLLESSON 1999, LABUS et al. 2013), digit ratios (KACZMARSKI et al. 2015) and various cranial measurements (e.g., REINHARD & KUPFER 2015, KHOSHNAMVAND et al. 2018).

An important group for the understanding of evolutionary mechanisms are the spectacled salamanders, the phylogenetically most basal group of true salamanders represented only by one genus – *Salamandrina* – including two species: *S. tertigitata* (BONNATERRE, 1789) and *S.*





*perspicillata*, (SAVI, 1821). Spectacled salamanders are endemic to the Italian peninsula and exceeding only rarely ten centimetres in total length (ROMANO et al. 2009b). Gravid females visit small, slow floating creeks and streams for egg deposition, while males spend their entire life in terrestrial habitats after metamorphosis (ZUFFI 1999). In the mating season, males become more active and are seeking for receptive females. If found, they are courting the females and transfer their spermatophores (ZUFFI 1999). Such substantial differences in life history traits between the sexes likely induce different selection on morphological traits among the sexes. For *Salamandrina perspicillata* some sexually dimorphic characters such as longer trunk length in females and wider head in males are known (ROMANO et al. 2009a). Nevertheless, more intense research on SD would be required to shed more light on the complex process of SD–evolution.

While for a large proportion of species from the tree of true salamanders, data on SD are available, there are still some gaps. Closing these is mandatory to achieve a full picture and draw respective conclusions on the mechanisms leading to morphological dimorphic sexes. Especially Asian newts need more attention by researchers, because still less is known on morphological variation between and within species and sexes in those. Among them, a highly diverse group, so far barely studied, are the ribbed and crocodile newts, often also named “primitive newts”, which represent a monophyletic lineage referred to as Pleurodelini (TSCHUDI 1838, DUBOIS & RAFFAELLI 2009, VEITH et al. 2018). Pleurodeline “salamanders” represent the basal group of the newts – Pleurodelinae (Fig. 3) and thus, they constitute an important clade for the reconstruction and understanding of evolutionary mechanisms forming SD in the Salamandridae as a whole. The Pleurodelini currently consist of three extant genera – *Pleurodeles* MICHAELLES, 1830, *Echinotriton* NUSSBAUM & BRODIE, 1982 and *Tylototriton* ANDERSON, 1871 (Fig. 4). Although the latter represent the most diverse genus within the Salamandridae, currently including 30 species (FROST 2018), there is only one targeted study on SD (SEGLIE et al. 2010). The other two genera comprise only three species each. The ribbed newts – *Pleurodeles* – are distributed in the Western Mediterranean region (SALVADOR & GARCÍA-PARÍS 1999, CARRANZA & WADE 2004) while crocodile newts – *Echinotriton* and *Tylototriton* – are found in East and South-East Asia from temperate to tropical regions (WANG et al. 2018). Pleurodeline newts harbour high potential for the understanding of the evolution of SD. Even within this small group, many different ecologies have evolved. In crocodile newts different patterns of mating modes were described (ROY & MUSHAHIDUNNABI 2001, UTSUNOMIYA & MATSUI 2002, ZIEGLER et al. 2008, FLECK 2010a, FLECK 2010b, NI et al. 2015,



HERNANDEZ 2016, HERNANDEZ 2017, WANG et al. 2017, HERNANDEZ 2018, RAUHAUS & ZIEGLER 2019). Mating can take place either with a ventral amplexus or by a stereotypic circle dance in terrestrial or limnic habitats. The subsequent clutch deposition taking place also either on land or in water. Further, these newts inhabit various habitats from tropical rainforests to montane grassy landscapes (e.g., BERNARDES et al. 2013, QIAN et al. 2017). These ecological differences in closely related species may allow to trace ecological parameters which are responsible for morphological divergence in crocodile newts.

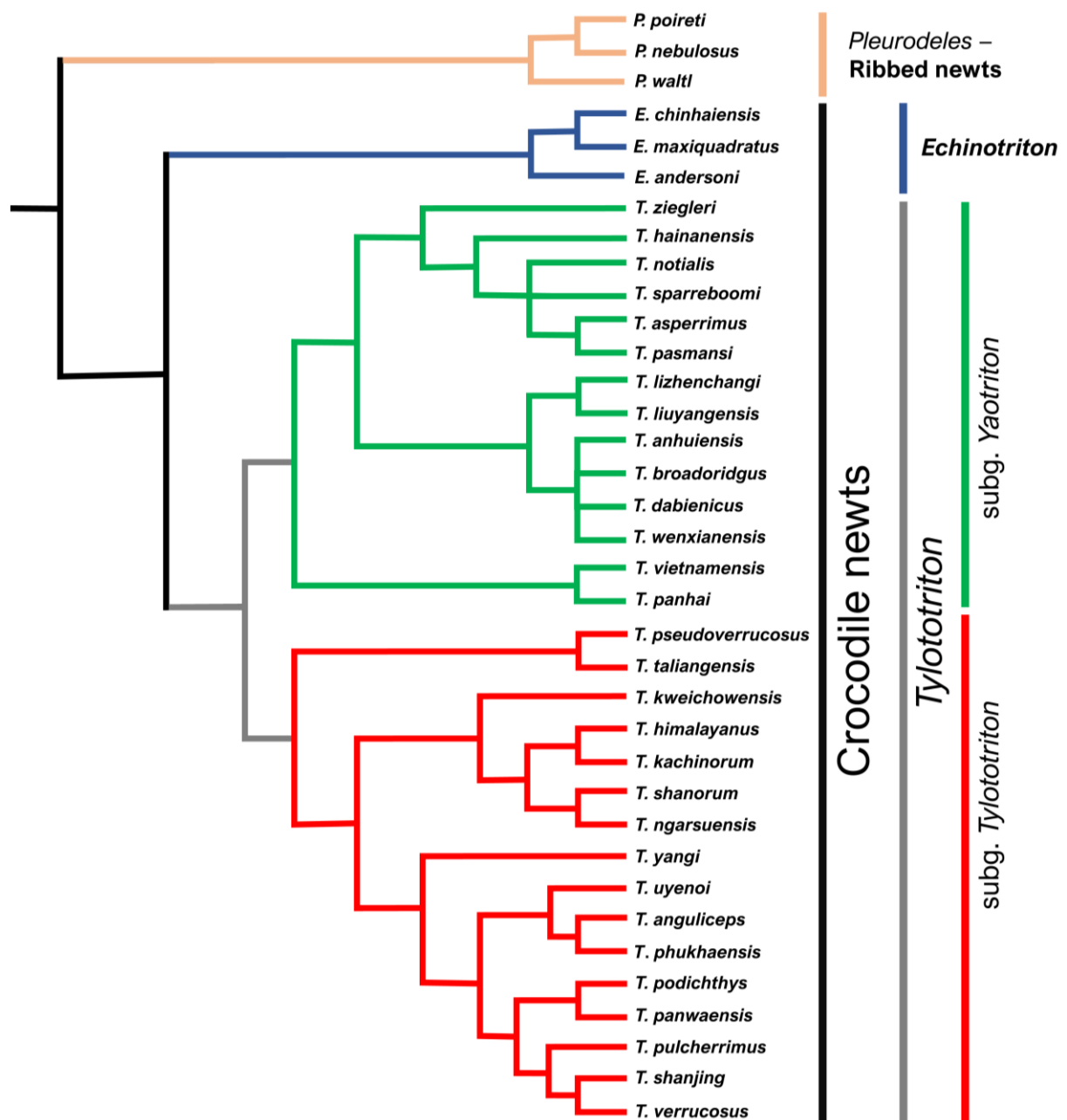


Figure 4: Phylogeny of the Pleurodelini including 36 species of *Pleurodeles*, *Echinotriton* and *Tylostotriton* modified after CARRANZA & WADE 2004, WANG et al. 2018, GRISMER et al. 2019, ZAW et al. 2019, BERNARDES et al. 2020 and POMCHOTE et al. 2020.



## Reconstructing ancient life histories

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To understand and reconstruct ancient life and their environments, we depend on what we know about extant taxa and how they interact with biotic and abiotic features of their habitat. With the knowledge of extant ecological communities, it is possible to get an imagination how former life assemblages have looked like (e.g., BROWN et al. 2017, SMITHWICK et al. 2017). The accuracy depends highly on the quantity and quality of preserved specimens from all components of the habitat i.e., from plants to the top-predators but also the paleoclimate.

Although tetrapods share a common ancestral bauplan, they evolved a huge variety of morphologies, adapted to almost every habitat found on earth. Despite this fact, we can find in phylogenetically non-related taxa the evolution of analogous body shapes when equal niches are occupied as a result of convergent selection. For example, one of the most studied characters is the dentition and its relation to diet (e.g., HOTTON III 1955, STRAIT 1993, DEAN et al. 2007, HERREL et al. 2008). Based on tooth shape food niches can be predicted and reconstructed. In vertebrates the main fossil remains represent hard tissues i.e., bones and teeth. Thus, conclusions on the ecology of extinct vertebrate species depend on the knowledge of the functional and anatomical features of such structures in extant taxa (MACFADDEN 2000, BRUSATTE et al. 2010, SHARP 2014, MOTANI et al. 2015, BROWN et al. 2017).

Primates are well investigated in their osteology, while most other vertebrates were neglected by this discipline so far (e.g., WOOD 1976, DIERBACH 1986, PLAVCAN 2001). Most studies on SD in urodeles take only the external morphology into account. The consideration of the osteology will be an important goal in the future, providing new insights into the divergent morphology of males and females. In true salamanders some evolutionary aspects of morphology were already investigated, especially of the cranial skeleton (IVANOVIĆ et al. 2009, CVIJANOVIĆ et al. 2014, IVANOVIĆ & ARNTZEN 2014, IVANOVIĆ & ARNTZEN 2017), but also of the postcranium (ARNTZEN et al. 2015, GOVEDARICA et al. 2017). The evolution of skeletal elements such as the cranium is very complex in salamandrids and while the general shape strongly depends on the phylogenetic history, the unique frontosquamosal arch was reduced several times independently in this family of urodeles (IVANOVIĆ & ARNTZEN 2017). Studies investigating the SD of the osteology of salamandrids are scarce (IVANOVIĆ & KALEZIĆ 2012). Externally dimorphic traits such as longer limbs in male salamanders likely affect also the osteology of those body parts. Further, the differences in size might also affect biomechanical features leading to rather subtle adaptations in osteology shape e.g., due to different muscle attachments. Thus, osteology likely reveals unknown patterns of SD. If it will be possible to



link different patterns of SD in extant taxa to defined ecological traits and life histories such as specific mating systems and mating behaviours, we will be able to draw even more detailed conclusions on how ancient life might have looked like. In different Mesozoic marine reptiles successful tracing of SD was already demonstrated (MOTANI et al. 2015, MOTANI et al. 2018). As size differences between fossil specimens cannot be used as a-priori character to distinguish the sexes, tracing first SShD is the way of choice to determine the sex. MOTANI and colleagues provided a new method that enables the sex identification in all size categories based on shape differences. These findings allow then conclusions on other characteristics of a population such as sex ratios and potential selection pressures.

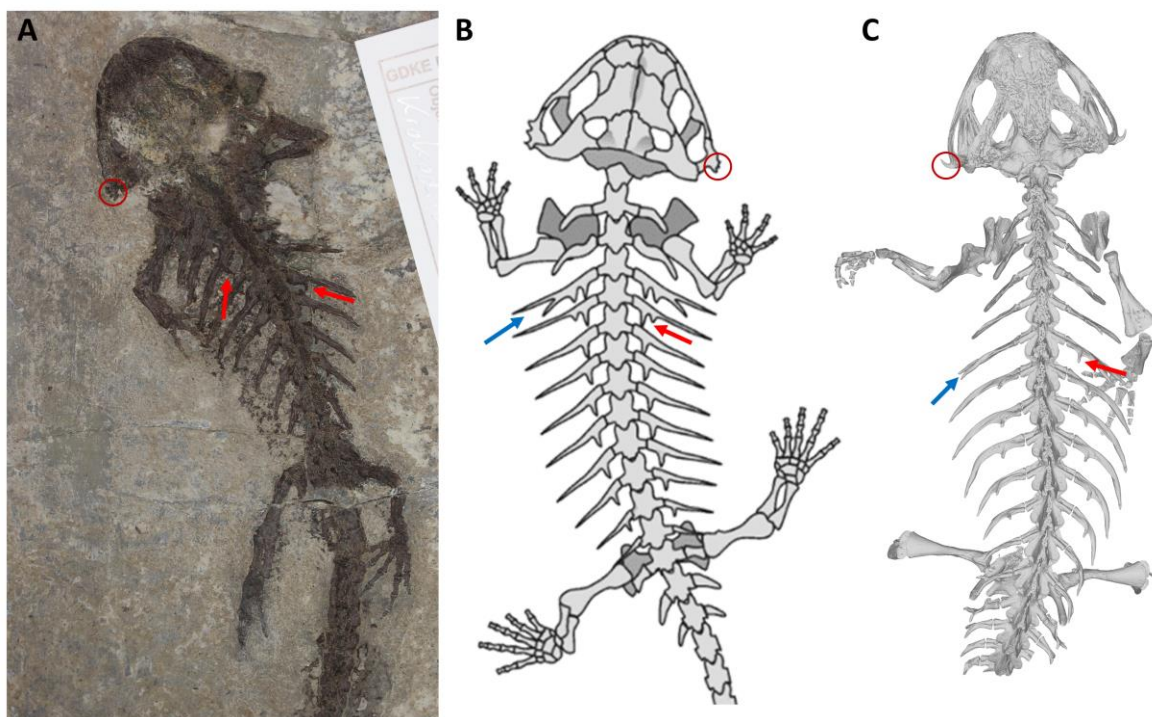


Figure 5: Comparison of the skeleton of *Chelotriton* † and *Echinotriton andersoni*. A: *Chelotriton*-specimen PW-2015-5163 from Enspel deposit, Rhineland-Palatine, B: Schematic drawing of *Chelotriton* osteology after SCHOCH et al. 2015, C:  $\mu$ CT reconstruction of the skeleton of *Echinotriton andersoni* from Okinawa Island (CAS 22304). Red circles indicate appendages on the quadrate bone, red arrows indicate epipleural processes on ribs, blue arrows indicate bifurcated ribs. (B) is used by permission of R. SCHOCH.

Beside living members, the family Salamandridae includes an uncertain number of extinct genera and species (ESTES 1981, MARJANOVIĆ & WITZMANN 2015). The fossil genus *Chelotriton* POMEL, 1853 is to date classified as a pleurodeline newt (MARJANOVIĆ & WITZMANN 2015, SCHOCH et al. 2015). Known specimens exhibit characters typical for *Echinotriton* and *Tylototriton* (Fig. 5), to which they are likely closer related than to *Pleurodeles*, the third extant genus of pleurodeline newts (SCHOCH et al. 2015). Currently there are four species of *Chelotriton* described (GOLDFUSS 1831, POMEL 1853, WESTPHAL 1980,



BAILON 1989), but the genus awaits general revision in order to clarify taxonomic affinity of known specimens (SCHOCH et al. 2015). Remains of *Chelotriton* are about 50-11 mya old, spanning from the Eocene to Miocene and were found from Spain to East Europe and the Middle East (WESTPHAL 1977, WESTPHAL 1980, HELLMUND & BÖHME 1987, ROCEK 1988, RAGE & BAILON 2005, ROCEK 2005, MCNAMARA et al. 2012, VASILYAN et al. 2017). From southwest Germany several well preserved specimens of *Chelotriton* were excavated in recent years (Fig. 6, ROCEK & WUTTKE 2010, SCHOCH et al. 2015). These specimens provide new important data on the evolutionary history of the morphology of pleurodeline newts and may allow new insights into the evolution of ecological and reproductive traits in this polymorphic salamander group.

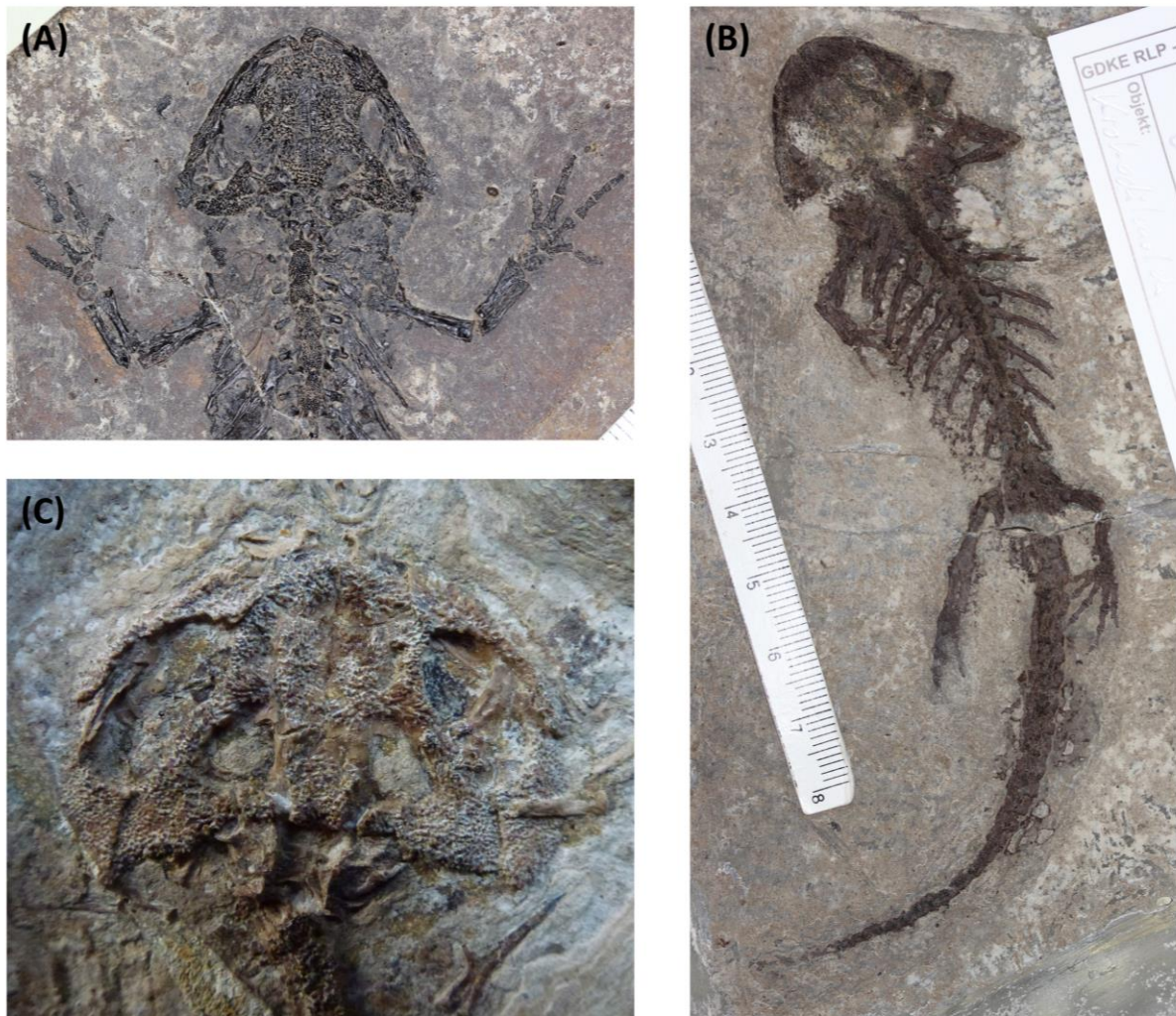


Figure 6: Well preserved specimens of *Chelotriton* sp. excavated in southwest Germany provided new data for the understanding of the evolution of basal salamandrid newts. A and B from Enspel, Rhineland-Palatine, C from Randeck Maar, Baden-Wuerttemberg. A: PW-1988-5058, B: PW-2015-5163, C: SMNS 80672 © PETER POGODA



## Study aims

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The aim of my PhD thesis was manifold. It should provide a basis for future directions in research on correlations of ecology and morphology, using salamanders as model system. A focus was set on SD, which can be divided into SSD and SShD. While research on SSD is well established in many different taxa (FAIRBAIRN et al. 2007), recognition and investigation of SShD just arose recently. Patterns of SSD and SShD can provide valuable insights into the different evolutionary forces forming the body shapes of females and males. Hereby, divergent patterns of morphology correlating with ecology in extant taxa are of key interest. Detecting and realizing general patterns is mandatory to help reconstruct ancient life from which mostly only hard tissues are preserved nowadays in the fossil record. Hence, more data on these tissues from extant species are needed in order to be able to reconstruct ancient life, which will help us to understand even better how life formed on a larger timescale.

First, I wanted to investigate extensively potential patterns of SSD and SShD in salamandrid salamanders, with a special focus on the phylogenetically most basal lineages i.e., *Salamandrina* and Pleurodelini. This was achieved in chapters I, II and IV.

Second, I aimed at providing a detailed comparison of traditional, linear morphometrics with geometric morphometrics for the first time in adult amphibians and elucidate what the different outcomes concerning SD are. This was laid out in chapter II.

Third, the integration and comparison of morphology from soft and hard tissues should provide data on how patterns of SD in these tissues are comparable and allow conclusions on the expression of SD from one tissue to the other. This was demonstrated in chapters I and III.

Fourth, I planned to reconstruct ancient salamander life history based on the morphology and ecology of extant relatives. This was done in chapter III.



## Results and Discussion

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### Chapter I: Flesh and bone and sexual dimorphism

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published as:

**POGODA, P. & A. KUPFER** (2018): Flesh and bone: An integrative approach towards sexual size dimorphism of a terrestrial salamander (genus *Salamandrina*). – Journal of Morphology, 279: 1468–1479. <https://doi.org/10.1002/jmor.20883>

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### Summary

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Differences between males and females in body size and selective linear measurements are referred to as SSD. Sexual size dimorphism is regularly studied in urodeles (see KUPFER 2007, AMAT 2019). Divergence other than size is also possible and quite common in the animal kingdom, but much less studied so far. Sexual shape dimorphism is often more subtle than other dimorphic traits like ornamentation and body size and requires a larger amount of data and advanced multivariate statistics (e.g., MALMGREN & THOLLESSON 1999, SCHWARZKOPF 2005, GIDASZEWSKI et al. 2009, ALARCÓN-RÍOS et al. 2017). Shape dimorphism is not as obvious to quantify and can be expressed in various forms depending on how measurements are related to size. E.g., a single cranial measurement can be set in relation to the entire body size or only to the size of the cranium.

Urodeles are an interesting model system for investigating the different patterns of SD. Although they exhibit a relatively constant body plan, a huge diversity in body size evolved in this group (RAFFAELLI 2013, SPARREBOOM 2014). Even within single families like the true salamanders of the family Salamandridae, observed morphological variations are significant.

The great majority of studies on SD in urodeles focused on the external morphology (e.g., MALMGREN & THOLLESSON 1999, BOVERO et al. 2003, BAKKEGARD & GUYER 2004, FONTENOT JR & SEIGEL 2008, IVANOVIĆ et al. 2008, SEGLIE et al. 2010, ZHANG et al. 2014, AMAT et al. 2015, REINHARD & KUPFER 2015, REINHARD et al. 2015, XIONG et al. 2017) hence, less is known about patterns of SD in the osteology of salamanders and newts (IVANOVIĆ et al.



2009, IVANOVIĆ & KALEZIĆ 2012). Several reasons occur why research on osteology is of interest in this field. First, soft tissues might cover differentially shaped bones and hide completely new dimorphic traits between sexes. The knowledge about these hidden dimorphic characters could lead to new and different conclusions regarding function of the respective body part (e.g., LYNCH 1971, HOWARD & KLUGE 1985, NAVAS & JAMES 2007, IVANOVIĆ & KALEZIĆ 2012). Second, bones are less prone to artefactual alterations due to water loss during preservation (LEE 1982, VERRELL 1985a) and third, osteology is of major importance to allow comparison with extinct, fossilized species. To allow comparisons with extant members we need to understand how patterns that we see in the external morphology are manifested in their osteology.

Spectacled salamanders of the genus *Salamandrina* are the most basal lineage within the family Salamandridae (ZHANG et al. 2008, PYRON & WIENS 2011). Hence, they likely play a key role in understanding the evolutionary processes of SD. Here I studied specimens of the Northern spectacled salamander - *S. perspicillata*. I used the largest amount of linear



Figure 7: Comparison of a male and female cranium of the same length of *Salamandrina perspicillata* to demonstrate sexual shape dimorphism in cranial morphology. Scale bar = 2mm





Table 1: Results of analysis of covariance (ANCOVA) of 45 external characters in *Salamandrina perspicillata* to assess SSD. The first PC of a PCA including representative characters of the entire body architecture was used as a covariate. For character abbreviations see original paper in the appendix.

<b>Character</b>	<b>F</b>	<b>P</b>	<b>Sex bias</b>
<b>Head</b>			
HL	6.61	<.05	M
HW	0.08	n.s.	–
HH	0.05	n.s.	–
ML	1.73	n.s.	–
IND	1.31	n.s.	–
EL	1.36	n.s.	–
IOD	0.24	n.s.	–
END	0.08	n.s.	–
SEL	2.29	n.s.	–
<b>Trunk</b>			
SVL	14.68	<.001	F
TLL	0.58	n.s.	–
AGD	68.94	<.0001	F
SHW	0.99	n.s.	–
CHW	3.11	n.s.	–
GG	7.22	<.01	F
CLL	33.04	<.0001	M
CLW	24.93	<.0001	M
FSL	31.64	<.0001	M
TLHB	0.42	n.s.	–
TLHM	0.14	n.s.	–
TLWB	0.75	n.s.	–
TLWM	0.23	n.s.	–
<b>Forelimb</b>			
ARM	9.34	<.01	M
UAL	1.87	n.s.	–
LAL	4.89	<.05	M
HAL	7.49	<.01	M
UAD	5.16	<.05	M
LAD	11.14	<.01	M
1FI	3.58	n.s.	–
2FI	17.43	<.0001	M
3FI	10.7	<.01	M
4FI	2.18	n.s.	–
<b>Hind limb</b>			
LEG	11.07	<.01	M
ULL	3.13	n.s.	–
LLL	1.86	n.s.	–
FOL	19.18	<.0001	M
ULD	7.3	<.001	M
LLD	6.97	<.05	M
1TO	8.86	<.01	M
2TO	18.64	<.0001	M
3TO	14.2	<.001	M
4TO	3.05	n.s.	–



measurements so far used in tailed amphibians to quantify SSD and SShD. In total I measured 45 external characters and 36 characters in the osteology using  $\mu$ CT scans and digital landmarks. This allowed me to evaluate how comparable patterns of SD in soft and hard tissues are. Osteology, so far relatively little investigated in salamanders in context of SD, also likely harbours still undisclosed patterns of SD. Further, the comparison of data from the external

Table 2: Results of analysis of covariance (ANCOVA) in 36 osteological characters of *Salamandrina perspicillata* to assess SSD. The first PC of a PCA including representative characters of the entire body architecture was used as a covariate. For character abbreviations see original paper in the appendix.

Character	F	P	Sex bias
Cranium			
SL	16.78	<.001	M
SW	0.07	n.s.	–
MXL	1.54	n.s.	–
PMW	1.63	n.s.	–
UND	0.67	n.s.	–
ND	0.22	n.s.	–
NCL	8.58	<.01	M
NCW	0.05	n.s.	–
OL	6.18	<.05	M
Forelimb			
HUL	11.54	<.01	M
HUW	0.11	n.s.	–
UL	14.81	<.001	M
CdhH	3.91	n.s.	–
CdhL	2.63	n.s.	–
1FPh	2.21	n.s.	–
2FPh	4.37	<.05	M
3FPh	6.43	<.05	M
4FPh	10.4	<.01	M
Hind limb			
FML	1.79	n.s.	–
THL	0.12	n.s.	–
FIBL	14.3	<.001	M
TSI	0.39	n.s.	–
TSB	0.16	n.s.	–
2TPh	4.74	<.05	M
3TPh	14.85	<.001	M
4TPh	20.48	<.0001	M
5TPh	9.13	<.01	M
Pelvis			
PVL	0.12	n.s.	–
aPVW	1.24	n.s.	–
pPVW	0.92	n.s.	–
mPVW	0.49	n.s.	–
ISCHW	1.85	n.s.	–
PBW	7.46	<.05	M
ILL	1.05	n.s.	–
ILW	32.04	<.0001	F



morphology and osteology should provide substantial information on how comparable the different approaches are regarding the interpretation of SSD and SShD. I used traditional statistics as well as advanced multivariate approaches to determine patterns of SSD and SShD including PCA, (multivariate) analysis of covariance (ANCOVA; MANCOVA).

External measurements revealed similar sexually dimorphic traits as already found in other salamanders and newts within the family and other taxa (e.g., MALMGREN & THOLLESSON 1999, BOVERO et al. 2003, BAKKEGARD & GUYER 2004, MARZONA et al. 2004, LABUS et al. 2013, ZHANG et al. 2014, REINHARD & KUPFER 2015, REINHARD et al. 2015, XIONG et al. 2019). This included a larger cloaca, longer limbs and head in males, while females exhibited a longer trunk and overall body size. Comparable osteological characters revealed to a large extent the same patterns of dimorphisms, but not in all traits, e.g., externally thicker limbs in males did not correspond to any kind of size and shape differences in long bones (Tab. 1, 2).

Analysis of osteology revealed dimorphic characters previously unknown in salamanders otherwise being covered by soft tissues (Tab. 2). The most prominent was the longer nasal cavity in male spectacled salamanders (Fig. 7) which might correlated with an increased olfactory performance enabling males to find receptive females more easily (CEDRINI & FASOLO 1971, DAWLEY 1984, DAWLEY 1992, DAWLEY & CROWDER 1995, SCHUBERT et al. 2008). Further, also the pelvic girdle showed novel dimorphic characters in males and females, requiring further research. This study revealed that SSD and SShD does occur in all body parts and is very complex. Depending on whether measurements are set in relation to the body size or only to the size of the specific body part, interpretation could vary. In females, likely selection for fecundity enlarges their trunk to increase number or size of eggs, whereas male traits are likely selected for multiple mates, male-male competition and to increase successful spermatophore transfer. Results from other taxa and phylogeny showed that some traits e.g., female-biased body size and male-biased cloaca and limb size evolved very early in salamandrids (Fig. 8), whereas cranial shape is more variable and likely responds differentially in respect to environmental and ecological parameters. To fully reconstruct evolutionary history of SD in true salamanders, more research is needed in some groups, especially Asian salamandrid salamanders.



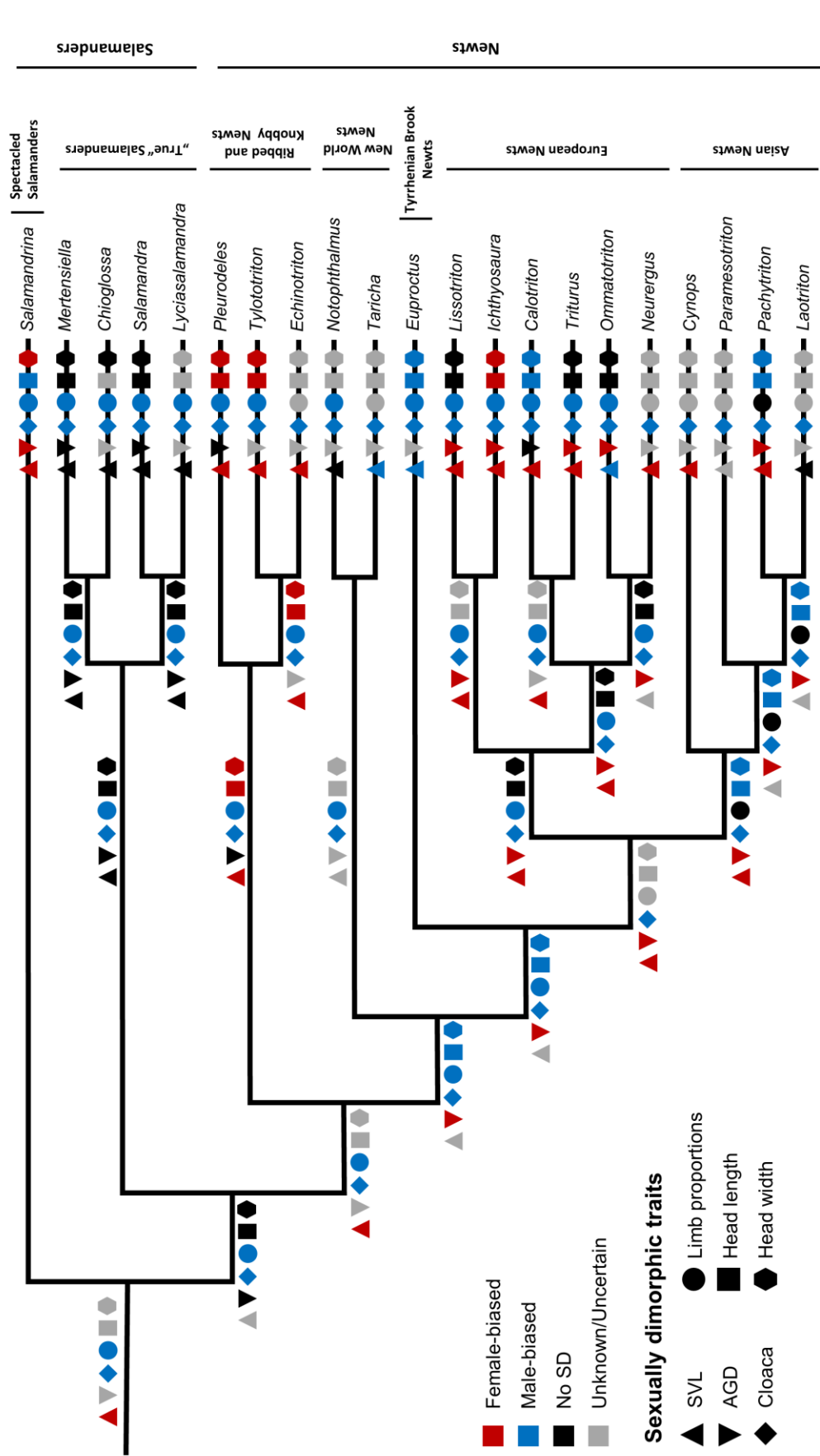


Figure 8: Phylogenetic relationships of Salamandridae and the distribution of SD of six, widely investigated characters. Characters are displayed for each genus and at internal nodes of the phylogeny. Grey symbols indicate noninvestigated characters or that a species of a given genus express both directions of SSD preventing conclusions on the ancestral state. Internal node characters were plotted after a > 50% majority rule, while known character states were given priority over "unknown/uncertain" states. Equivocal characters received an "unknown/uncertain"—grey marking. The phylogeny was modified from ZHANG et al. (2008). References are listed in the supporting information

## Chapter II: Shape dimorphism – yesterday and today

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published as:

**POGODA, P. & A. KUPFER (2020):** Sexual shape dimorphism in the cranium and pelvic girdle of Northern spectacled salamanders, *Salamandrina perspicillata*, investigated via 3D geometric morphometrics – *Salamandra*, 56 (2): 113–122.

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### Summary

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As illustrated above, SShD is sometimes hard to quantify. This requires a denser data collection, larger sample size and more advanced analytical approaches to detect those accurately. Geometric morphometrics is a relatively new technique for the investigation of shape in both, 2D and 3D space and gained much attention by researches in the last decades (ADAMS et al. 2004, KALIONTZOPOULOU 2011). In GM the shape of an object is gathered by points, so called landmarks, that are set on homologous morphological structures (ZELDITCH et al. 2012). Several studies (BLANCO & GODFREY 2006, ABDEL-RAHMAN et al. 2009, ARENDT 2010, BRENO et al. 2011, SCHMIEDER et al. 2015, ILIĆ et al. 2019) showed that GM is superior in detecting especially subtle shape differences compared to linear measurements, making this method a valuable approach in accurately quantifying SShD.

In this study, I used 3D GM data on the osteology of the selected body parts, the cranium and pelvic girdle, of spectacled salamanders to investigate SShD in more detail. The cranium and pelvis exhibited enough well-suited structures to place homologous landmarks and limited kinesis. In chapter one, I was able to reveal new patterns of SSD in these two body parts. The pelvic girdle plays an important role for reproduction and locomotion. While females deposit many eggs, males deposit spermatophores only occasionally. Thus, it is curious that this osteological structure has never been investigated in detail in tailed amphibians in context to SD. Applying GM should allow to access subtle shape differences between males and females in the investigated body parts. Second, I wanted to compare the outcome of traditional and geometric morphometrics, providing the first comparison of these methods in adult urodeles. Therefore, I used the same set of  $\mu$ CT scans from the Northern spectacled salamander, *S. perspicillata*, as in chapter I.



Table 3: Results of multivariate regression of shape data on size (as logCS) and sex to test for static and unique allometry in three-dimensional shape data of cranial and pelvic girdle morphology of *Salamandrina perspicillata*. Significant p-values are given in bold.

	Cranium			Pelvic girdle		
	DF	F	P	DF	F	P
Static Allometry: shape ~ logCS	1	2.52	<b>0.0003</b>	1	5	<b>&lt;0.0001</b>
Unique Allometry: shape ~ sex*logCS	1	0.87	0.64	1	1.86	0.054

On the cranium, 43 and on the pelvic girdle 20 3D landmarks were digitized. Analyses were performed in the statistical software R (R DEVELOPMENT CORE TEAM 2019) using the packages ‘geomorph’, ‘RRPP’ and ‘Morpho’ (SCHLAGER 2017, COLLYER & ADAMS 2018, ADAMS et al. 2019). Shape data was first tested for allometry in general and for males and females separately. Via a Procrustes ANOVA shape data and centroid size (CS), the measure of size in GM (BOOKSTEIN 1997), was tested for differences between the sexes.

Both osteological structures showed common allometric shape changes between males and females (Tab. 3). The cranium and the pelvis turned out to be morphologically dissimilar in shape between the sexes but not in CS. In males, the cranium was slender with a longer snout and occipital region and shorter maxillary bones (Fig. 9). In the pelvic girdle, the ischiopubis was broader in males than in females. Further, the two halves of the ischiopubis were arranged

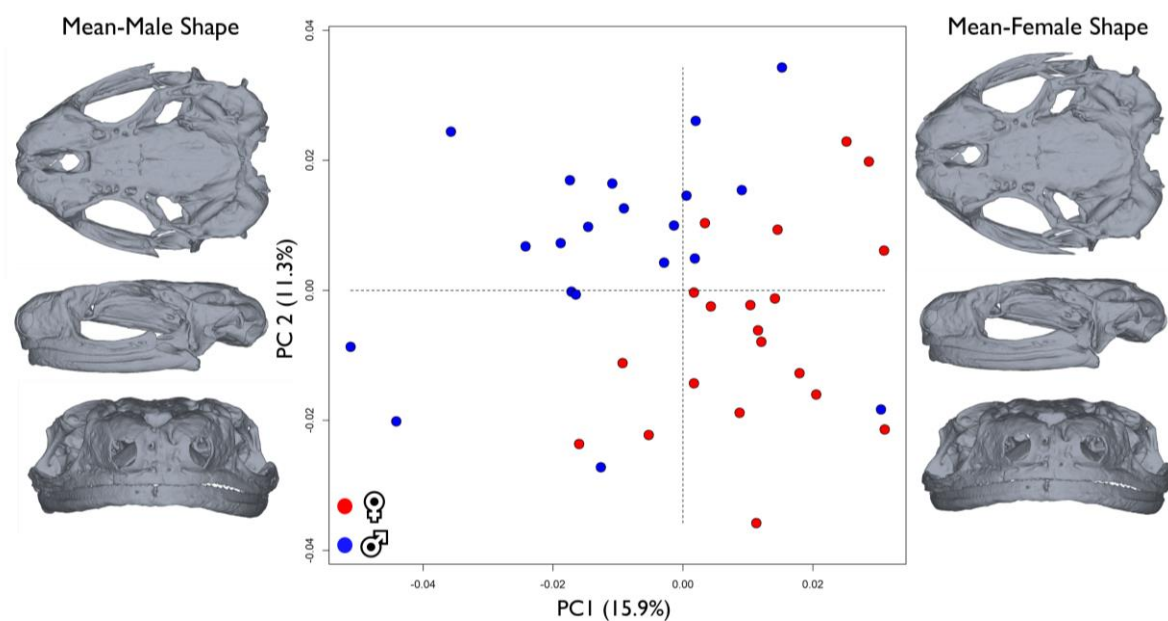


Figure 9: Morphological variation of the cranium of *Salamandrina perspicillata* displayed by the first and second principal component of a PCA including 43 3D-landmarks. The mean shapes of male and female salamanders are displayed as warped meshes from the overall mean shape. The shape changes are magnified by the factor of three.



in a flat V in males and the ilia exhibited a stronger torsion in males, while in females the ilia were wider (Fig. 10).

The shape changes between males and females followed the same direction as the allometry does. This was also shown in European newts (IVANOVIĆ & KALEZIĆ 2012). As males exhibited relative to body size longer crania, SShD might be at least partly due to selection on size in one sex. Differentially shaped crania in males might be associated with agonistic behaviour among males, larger or longer crania generally being associated with higher competitiveness in salamanders (BAKKEGARD & GUYER 2004, MARVIN 2009). Such behaviour has been observed in *Salamandrina* occasionally (ZUFFI 1999, UTZERI et al. 2005). Another explanation could be associated with the olfactory sense. The nasal cavity was elongated in male individuals and a longer snout may be associated to that. A larger nasal cavity, which might correlate with an increase in the vomeronasal organ, increases the olfactory performance of males, enabling those to find receptive females more easily (DAWLEY 1992, SCHUBERT et al. 2008). For the ecological niche divergence theory there is actually no evidence in spectacled salamanders (COSTA et al. 2015) as well in other family members, as no differences in food items between males and females were found (GRIFFITHS 1986, GRIFFITHS 1987, IVANOVIĆ & KALEZIĆ 2012). Pelvis shape differences likely are attributable to the different reproductive

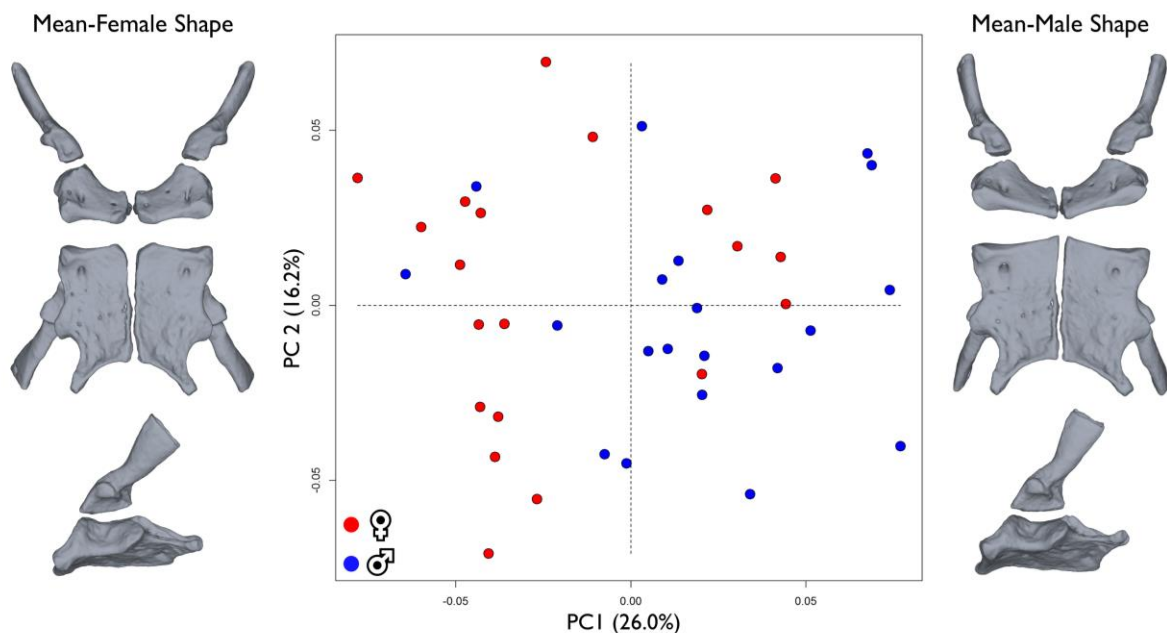


Figure 10: Morphological variation of the pelvic girdle of *Salamandrina perspicillata* displayed by the first and second principal component of a PCA including 20 3D-landmarks. The mean shapes of male and female salamanders are displayed as warped meshes from the overall mean shape. The shape changes are magnified by the factor of two.



requirements. While males deposit spermatophores on land, females lay larger eggs under water under stones and other structures, which requires different locomotion.

In general, both methods can yield similar results in context of SD, if appropriate data is used. In traditional morphometrics many measurements and appropriate statistics are needed. Applying GM, shape differences were revealed in a broader extent. Regarding SSD, GM exhibit minor weaknesses, as only the current landmark configuration is used for calculations, not considering the body size. Once a specimen is digitalised, either by photographs or CT scans, including larger sample size and large number of landmarks is less time consuming than taking additional linear measurements. Further, a digitalised specimen cannot be altered anymore in its morphology by the researcher during handling underlining the superiority of GM in gathering subtle shape differences (e.g., ADAMS & ROHLF 2000, GABELAIA et al. 2018). Geometric morphometrics is the method of choice to study morphological variation nowadays. Nevertheless, there is always the potential to combine both, GM and traditional morphometrics.





## Chapter III: How ecology shapes morphology

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published as:

**POGODA, P., M. ZUBER, T. BAUMBACH, R.R. SCHOCH & A. KUPFER:** Cranial shape evolution of extant and fossil crocodile newts and its relation to reproduction and ecology. – *Journal of Anatomy*, 237: 285–300. <https://doi.org/10.1111/joa.13201>.

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### Summary

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The different classes and groups of animals evolved very distinct body shapes. Nevertheless, due to evolutionary forces acting in similar ways in non-related taxa occupying similar niches, conclusions on their ecology from their morphology can be drawn (HERREL et al. 2004, DEAN et al. 2007, HERREL et al. 2008). E.g., the dentition of an animal is closely related to its diet and feeding behaviour across taxa (e.g., HOTTON III 1955, STRAIT 1993). This is especially of interest for the reconstruction of ancient life, as we have to conclude from life of extant to extinct species (e.g., BROWN et al. 2017, SMITHWICK et al. 2017). In vertebrates, this is mainly restricted to the osteology, as bones are the most and best-preserved body parts in the fossil record. The cranium is a well-suited structure to do so. It is composed of various bones fusing during ontogeny to a functional unit. This evolutionary background provides high flexibility for cranial shape evolution. The cranium includes most of the sensory organs and is essential for e.g., the food intake and perception of the environment. These features allowed and forced vertebrates to evolve a huge variety in head morphologies. The cranial morphology does respond even to little niche shifts (DEAN et al. 2007, IVANOVIĆ & ARNTZEN 2017, BERTRAND et al. 2019).

While true salamanders evolved distinct cranial morphologies (IVANOVIĆ & ARNTZEN 2017), their basal group of ribbed and crocodile newts (Pleurodelini) is rather conservative in that context (HERNANDEZ et al. 2018). This group of newts consists of three extant genera namely *Pleurodeles*, *Echinotriton* and *Tylototriton* (Fig. 4). Generally accepted is the differentiation of *Tylototriton* into the two subgenera *Tylototriton* and *Yaotriton* (DUBOIS & RAFFAELLI 2009). What makes this group interesting for research is their polymorphic reproduction (SPARREBOOM 2014, KIEREN et al. 2018) including different modes and strategies (e.g., KUZMIN et al. 1994, SPARREBOOM 1999, SPARREBOOM et al. 2001, BERNARDES et al. 2017, PASMANS et al. 2017). Additionally, with *Chelotriton* exists another extinct genus of



pleurodeline newts closely resembling crocodile newts (MARJANOVIĆ & WITZMANN 2015, SCHOCH et al. 2015). Several well-preserved specimens of *Chelotriton* became available from several deposits in southwest Germany (Fig. 6) (ROCEK & WUTTKE 2010, SCHOCH et al. 2015). This allows to investigate the evolutionary history of this group on a larger timescale.

In this chapter, I investigated the cranial morphology of pleurodeline newts interspecifically and set it in relation to selected ecological traits. This should provide new insights into the evolution of cranial shape in this conservative group and into ecology-morphology correlations. The latter should provide new data to enable draw conclusions on the reproductive ecology in extinct relatives. Further, I wanted to add new data which provide further hints on the relationship of extinct and extant taxa.

I used 2D GM of the external and osteological cranial morphology in lateral and dorsal view. In total, 157 newt specimens from natural history collections plus additional suited

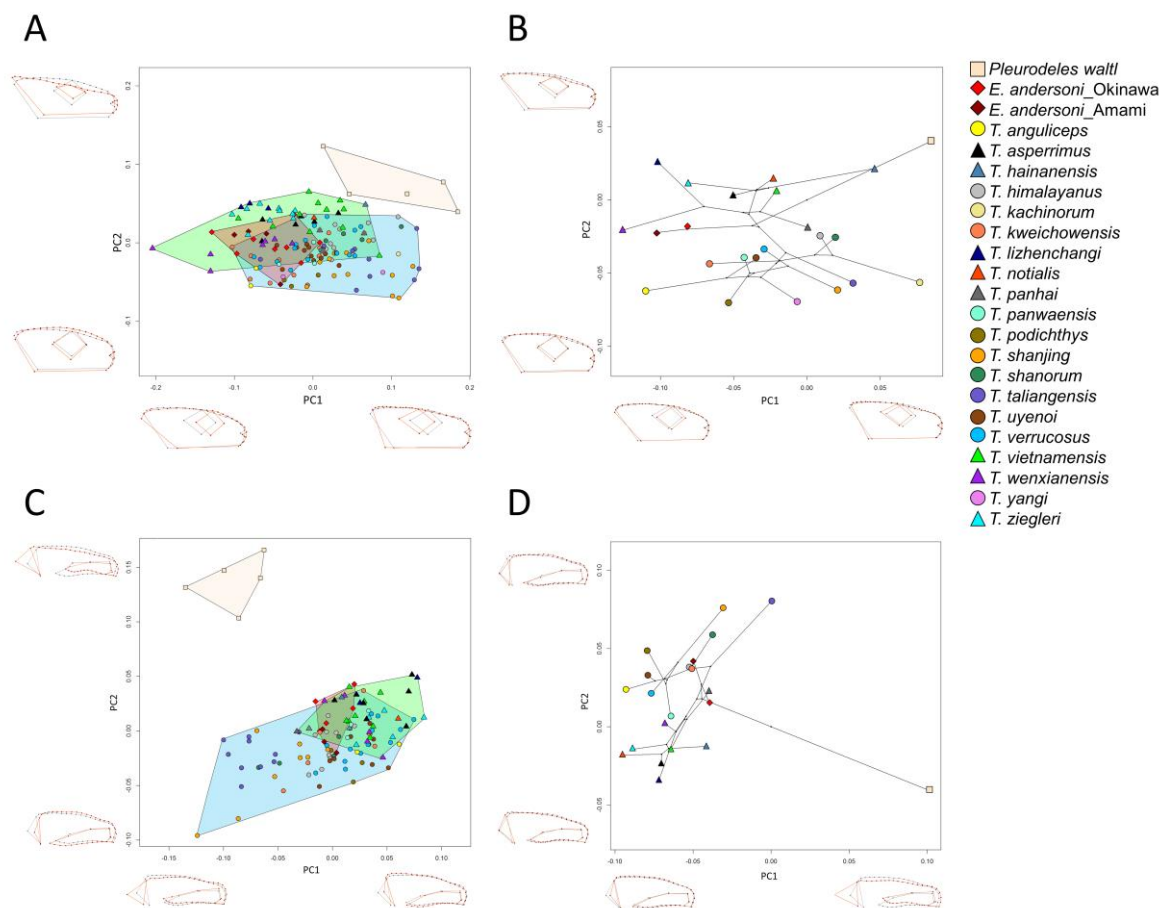


Figure 11: Morphospaces of GPA aligned, allometry-free shapes built by the first two principal components of the lateral, cranial morphology of pleurodeline newts. Square: *Pleurodeles*, diamond: *Echinotriton*, circle: subgenus *Tylotriton*, triangle: subgenus *Yaotriton*. Black wireframe corresponds to the mean shape, red wireframe represents the shape at the extreme value of the respective PC axes. A: external morphology of all specimens, B: external morphology of species mean-shapes, C: osteology of all specimens, D: osteology of species mean-shapes.



photographs from literature were used to analyse the external morphology. From those, 121 specimens including eight fossil specimens of *Chelotriton* from two deposits were CT-scanned to access the cranial osteology. *Chelotriton* specimens were retrodeformed prior to landmark acquisition to reduce artefacts due to taphonomy (TALLMAN et al. 2014). As SD would add additional variation into the data, I used only male specimens. A PCA was conducted to explore the morphospace of the different genera and species. Via Procrustes ANOVA I tested whether cranial shape and CS differed between genera and species. For further analyses, for each species a mean shape was calculated and the phylogenetic signal estimated. Finally, I applied phylogenetic ANOVA to test whether cranial shape correlated with ecological and reproductive traits.

Ribbed newts, genus *Pleurodeles*, turned out being morphologically distinct from all crocodile newts (*Echinotriton* & *Tylototriton*). Crocodile newts themselves showed a large overlap in their morphospace. The subgenus *Tylototriton* occupied the largest one. A more differentiated picture is found when the mean shapes of each species is analysed. In all datasets, a good morphological distinction of the two subgenera of *Tylototriton* was achieved (Fig. 11). The genus *Echinotriton* overlapped in its morphospace closer with the subgenus *Yaotriton* than subgenus *Tylototriton*. The phylogenetic signal was strong in the entire group and the genus *Tylototriton*, but weak or absent in its subgenera (Tab. 4). Post-hoc testing of Procrustes

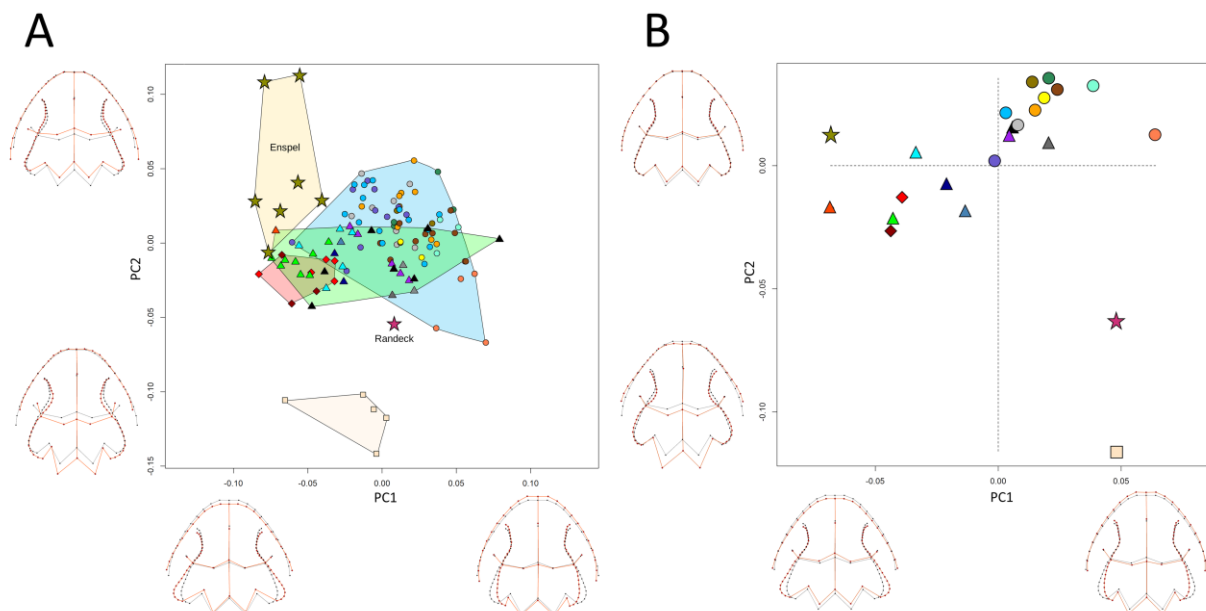


Figure 12: Morphospaces of GPA aligned, allometry-free shapes built by the first two principal components of the dorsal, cranial morphology of pleurodeline newts including fossil *Chelotriton*. Square: *Pleurodeles*, diamond: *Echinotriton*, circle: subgenus *Tylototriton*, triangle: subgenus *Yaotriton*, star: *Chelotriton*. Black wireframe corresponds to the mean shape, red wireframe represents the shape at the extreme value of the respective PC axes. A: PCA plot of all specimens, B: PCA plot of species mean-shapes. For colour coding see figure 11.



Table 4: Test for phylogenetic signal in two-dimensional morphometrics cranial datasets of pleurodeline newts, genus *Tylotriton* and the two subgenera *Yaotriton* and *Tylotriton*. Significant p-values are given in bold.

<b>Pleurodelini</b>	<b>K</b>	<b>p</b>
Head lateral	0.363	<b>0.0039</b>
Head dorsal	0.428	<b>0.0048</b>
Skull lateral	0.51	<b>0.0035</b>
Skull dorsal	0.52	<b>&lt;0.0001</b>
<b>Genus <i>Tylotriton</i></b>		
Head lateral	0.55	<b>0.035</b>
Head dorsal	0.58	<b>0.0073</b>
Skull lateral	0.46	0.2
Skull dorsal	0.69	<b>&lt;0.0001</b>
<b>Subgenus <i>Tylotriton</i></b>		
Head lateral	0.59	0.026
Head dorsal	0.73	<b>0.025</b>
Skull lateral	0.48	0.38
Skull dorsal	0.69	<b>0.013</b>
<b>Subgenus <i>Yaotriton</i></b>		
Head lateral	0.68	0.5
Head dorsal	0.64	0.63
Skull lateral	0.68	0.37
Skull dorsal	0.7	0.36

ANOVA revealed that all pleurodeline newt genera were morphologically distinct in their cranial morphology. Centroid size showed only little difference between genera, but some species showed consistent size differences. In the analysis including fossil *Chelotriton*, morphological shape changes along PCs were consistent to prior analysis. Fossil newts from the two deposits turned out to be morphologically different (Fig. 12). Further, they did not overlap in their morphospace with extant members. *Chelotriton* represented the largest group of pleurodeline newts. Distribution of species along the climatic gradient, from tropical forests to temperate grasslands, did not correlate with morphology. Available data on reproductive traits showed that the oviposition site, either terrestrial or aquatic, correlated with phylogeny while mating mode (amplexus or mating dance) was associated with the cranial shape corrected for phylogeny (Tab. 5).

Here, I could demonstrate that external morphology and osteology provide similar results regarding cranial shape variation. However, as the osteology of the cranium provides better and more suitable landmarks, it likely represents a better basis for evolutionary research. The results



Table 5: Procrustes ANOVA's in a phylogenetic framework of two-dimensional morphometric shape datasets of crania of pleurodeline newts tested for ecological traits: Significant p-values are given in bold. \* Note that *Chelotriton*-shape per se is not included in the models of LH-traits as no information on those are available for this genus. They rather affect data processing (GPA alignment) of the remaining shapes prior analysis.

<b>Head lateral</b>	<b>Model</b>	<b>DF</b>	<b>F</b>	<b>p</b>
	shape ~ mating mode	1	3.61	<b>0.044</b>
	shape ~ mating habitat	1	1.61	0.199
	shape ~ oviposition site	1	1.74	0.17
	shape ~ climate	3	0.65	0.67
<b>Head dorsal</b>				
	shape ~ mating mode	1	0.96	0.39
	shape ~ mating habitat	1	0.46	0.76
	shape ~ oviposition site	1	1.22	0.27
	shape ~ climate	3	1.48	0.012
<b>Skull lateral</b>				
	shape ~ mating mode	1	8.41	<b>0.011</b>
	shape ~ mating habitat	1	0.34	0.075
	shape ~ oviposition site	1	1.12	0.3
	shape ~ climate	3	0.92	0.44
<b>Skull dorsal</b>				
	shape ~ mating mode	1	3.58	<b>0.014</b>
	shape ~ mating habitat	1	0.61	0.72
	shape ~ oviposition site	1	1.3	0.25
	shape ~ climate	3	1.03	0.38
<b>Skull dorsal incl. <i>Chelotriton</i>*</b>				
	shape ~ mating mode	1	2.67	<b>0.041</b>
	shape ~ mating habitat	1	0.41	0.88
	shape ~ oviposition site	1	1.29	0.26
	shape ~ climate	3	0.96	0.5



confirm the assumption of a conservative morphological differentiation within crocodile newts, while ribbed newts are well separated (see also IVANOVIĆ & ARNTZEN 2017). This coincides with the spatial distribution of the genera, *Pleurodeles* being restricted to the Western Mediterranean, whereas *Echinotriton* and *Tylototriton* occur in East Asia, partly overlapping in their distribution areas (GROSSENBACHER & THIESMEIER 1999, HOU et al. 2014, WANG et al. 2018). Although crocodile newts overlap in their morphospace to a large extent, analysis of the species' mean shapes provided the first comprehensive support for a morphological differentiation of the two subgenera within *Tylototriton*, including a strong phylogenetic signal in pleurodeline newts. *Echinotriton* showed morphological similarities to the subgenus *Yaotriton* also sharing a similar ecology e.g., terrestrial oviposition. In this case it is unapparent whether cranial shape represents an adaptation to ecology or is rather constraint by phylogeny. Corrected for phylogeny, only the mating mode significantly affected cranial shape. Morphological disparity of *Chelotriton* to extant crocodile newts might underpin their separate position in the evolutionary history of pleurodeline newts. Further, the specimens of the different deposits also correspond to slightly different geological periods, likely representing distinct species. Cranial morphology of *Chelotriton* from one deposit resembles closer the morphology of *Yaotriton* and *Echinotriton*, which might be interpreted as hinting at a more terrestrial lifestyle.



## Chapter IV: Does ecology shape sexual dimorphism?

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In preparation:

**POGODA, P., M. ZUBER, T. BAUMBACH & A. KUPFER:** Clasp and dance: Mating mode promotes variable sexual size and shape dimorphism trajectories in crocodile newts (Caudata: Salamandridae). Under revision in *Ecology & Evolution*, published as preprint in AUTHOREA. July 26, 2021. <http://dx.doi.org/10.22541/au.162731360.07245217/v1>

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### Summary

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To better understand how different patterns of SD evolved, a comparative approach is needed in which at best closely related taxa with divergent ecological traits are compared to reveal which selective forces might shape SD in different directions. Crocodile newts of the genera *Echinotriton* and *Tylototriton* are a well suitable model system to answer this question as they are the most diverse group within Salamandridae and possesses polymorphic reproduction systems. For spermatophore transfer, these newts perform either a stereotypic circle dance at which the females cloaca is guided over the deposited spermatophore or the male applies a ventral amplexus and then let the females pelvic region drop down on the placed spermatophore (HERNANDEZ 2016). These different behaviours require different moving patterns which may lead also to differentiation of morphology between species.

In this chapter, I investigated patterns of sexual size and shape dimorphisms in several species of crocodile newts differing in their mating mode. The selected species include each major clade of these enigmatic newts. This chapter should provide new insights into the selective forces forming different SD trajectories linked to a species ecology, providing a new basis for the understanding of the evolutionary mechanisms forming different morphologies in males and females.

The head morphology in salamandrids is known to differ between males and females in various forms in size and shape (e.g., MALMGREN & THOLLESSON 1999, IVANOVIĆ & KALEZIĆ 2012, AMAT et al. 2015, ALARCÓN-RÍOS et al. 2017). As shown in chapter III, cranial shape differs between the different mating modes. Knowledge on SD in crocodile newts is so far very limited (SEGLIE et al. 2010, PHIMMACHAK et al. 2015). The forelimbs play a major role when comparing amplexant and circle dancing species. Hence, the forelimb and cranial morphology



Table 6: Results of a Procrustes ANOVA on 3D morphometric shape data of the humerus (a) and cranium (b) of crocodile newts (*Echinotriton* and *Tylotriton*) to test for size-shape allometric relationship and shape differences between species and sex. Significant p-values are given in bold.

	Df	SS	F	Z	P
<b>(a) Humerus shape</b>					
Size	1	0.052	12.2422	5.1659	<b>&lt;0.0001</b>
Species	8	0.52131	15.3404	13.493	<b>&lt;0.0001</b>
Sex	1	0.052	12.2419	5.9927	<b>&lt;0.0001</b>
Size × Species	8	0.06435	1.8935	3.6992	<b>0.0002</b>
Size × Sex	1	0.0054	1.2712	0.7827	0.2173
Species × Sex	8	0.0515	1.5155	2.6089	<b>0.0058</b>
Size × Species × Sex	8	0.04296	1.2642	1.3734	0.0835
Residuals	190	0.80709			
<b>(b) Cranium shape</b>					
Size	1	0.07857	24.2181	8.9468	<b>&lt;0.0001</b>
Species	8	0.47169	18.1735	21.2585	<b>&lt;0.0001</b>
Sex	1	0.00659	2.0323	2.6806	<b>0.0037</b>
Size × Species	8	0.04496	1.7323	5.0003	<b>&lt;0.0001</b>
Size × Sex	1	0.00314	0.9666	0.0356	0.4806
Species × Sex	8	0.03238	1.2476	2.0453	<b>0.0226</b>
Size × Species × Sex	8	0.03127	1.2049	1.5578	0.0603
Residuals	191	0.61967			

were set in focus in this chapter. To do so, I applied shape analyses by 3D GM of cranial and humerus morphology and applied linear models (Procrustes ANOVA) to reveal potential selective forces leading to different shape differences between males and females. CT-scans of 227 specimens covering nine species of crocodile newts were performed. To gather shape variation 45 landmarks and six landmarks plus 50 semi-landmarks, respectively, were digitized on the cranium and humerus. A PCA was conducted to explore the overall shape variation in both body parts. A full factorial model design was precluded by the model system. Thus, several models had to be run to test for the interested potential sources of morphological variation. Allometric shape changes are a main source of variation in salamanders (IVANOVIĆ & ARNTZEN 2014, IVANOVIĆ & ARNTZEN 2017) and was explicitly explored. A trajectory analysis was performed in order to test and visualize different patterns of shape changes between males and females among species. Mating mode was tested whether it explains considerable shape variation and SD trajectories.





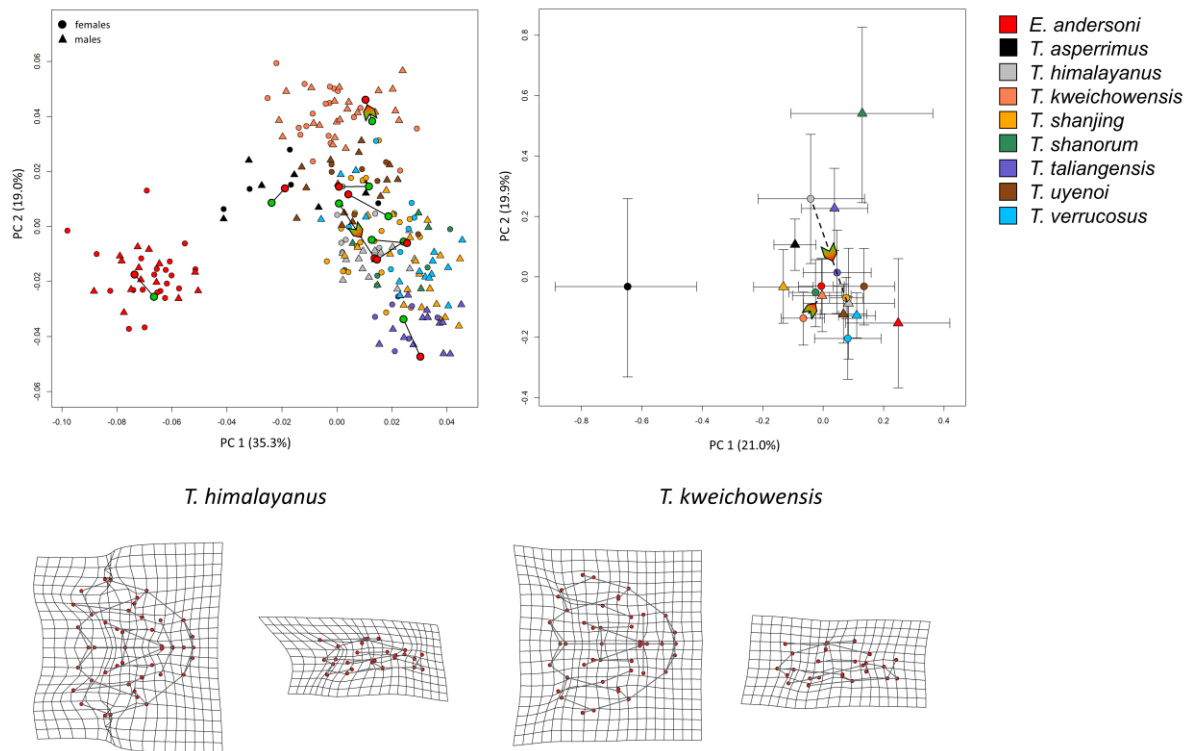


Figure 13: Trajectory analysis demonstrating cranial shape changes by sexual dimorphism in crocodile newts – *Tylotriton* spp. and *Echinotriton andersoni*. Analysis was performed with specimen data set (upper left) and species mean predictions (upper right). Green dots and circles, respectively represent males and red dots and triangles represent females. In the lower row TPS-deformation grids of two exemplary species, which exhibit different trajectories are illustrated and marked by an arrow in the upper graphs. Shapes changes were visualized from male to female shapes and are magnified by the factor of three.

*Echinotriton andersoni* occupied a distinct morphospace in both, humerus and cranial shape and generally appeared to exhibit a more robust morphology i.e., thicker humeri and a stronger ossified cranium than *Tylotriton*. In humerus and cranium, allometric shape changes were strongly developed (Tab. 6). Allometric trajectories differed between species but not the sexes. With increasing size, the humerus became relative thinner and in the cranium squamosal and dorso-lateral bony ridges became elaborated and the connection of the maxilla with the pterygoid and quadratum were more pronounced than in smaller crania. Analysis revealed different trajectories of SD patterns between species in cranial (Fig. 13) and humerus morphology (Fig. 14, Tab. 6). Mating mode described significant variation in SShD-patterns when size as covariate was included in cranial shape, but not in humerus shape (Tab. 7). Nevertheless, shape between mating modes differed also in the humerus. Humerus shape changes from the overall mean shape to either male or female shape was similar but differed in their magnitude. In cranial shape in amplexing species, males and females showed different shape changes to the overall mean whereas dancing species showed similar shape changes.



Analyses of SSD patterns revealed differences between species and mating modes. In both body parts, amplexing species showed SSD whereas dancing species did not (Tab. 8).

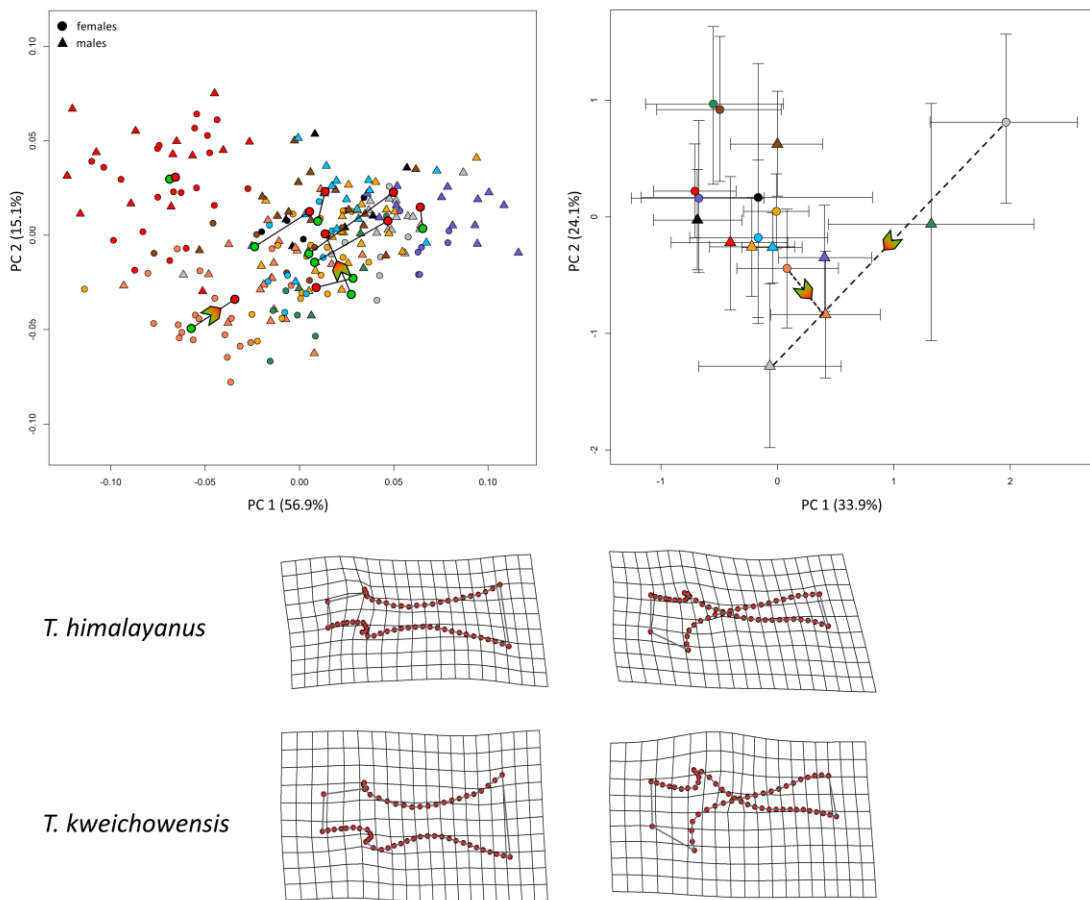


Figure 14: Trajectory analysis demonstrating humerus shape changes by sexual dimorphism in crocodile newts – *Tylototriton* spp. and *Echinotriton andersoni*. Analysis was performed with specimen data set (upper left) and species mean predictions (upper right). Green dots and circles, respectively represent males and red dots and triangles represent females. In the lower row TPS-deformation grids of two exemplary species which exhibit different trajectories are illustrated and marked by an arrow in the upper graphs. Shapes changes were visualized from male to female shapes and are magnified by the factor of three.

This study provides a significant contribution on the knowledge of SD in pleurodeline newts so far rarely investigated and often limited to few linear characters (SEGLIE et al. 2010, PHIMMACHAK et al. 2015, FEI & YE 2016) and fills a gap in this research field in salamandrid salamanders. Most of the observed cranial shape differences between males and females are similar to other members of this family (IVANOVIĆ et al. 2008, IVANOVIĆ & KALEZIĆ 2012, ALARCÓN-RÍOS et al. 2017). Different allometries between species but not the sexes indicated high flexibility of allometric trajectories. This was already shown for the entire family and selected species groups (IVANOVIĆ & KALEZIĆ 2012, IVANOVIĆ & ARNTZEN 2017). Crocodile newts have a wide distribution area including many different macrohabitats from the tropics to the temperate climate zone providing the basis for ecological adaptations whereas sex roles and



Table 7: Results of Procrustes ANOVA on 3D morphometric shape data of the humerus (a,b) and cranium (c,d) of crocodile newts (*Echinotriton* and *Tylotriton*) to test mating mode as a source of different SShD-patterns between species. Significant p-values are given in bold.

Humerus shape				Cranium shape						
	Df	SS	F	Z	P	Df	SS	F	Z	P
<b>(a) Shape</b>										
Sex	1	0.07468	12.0665	5.1554	<0.0001	1	0.02077	3.9271	4.0012	<b>0.0002</b>
Mating mode	1	0.1398	22.5875	6.4138	<0.0001	1	0.08331	15.7536	7.8498	<0.0001
Sex × Mode	1	0.00809	1.3074	0.802	0.2134	1	0.00494	0.9348	-0.0401	0.5012
Residuals	222	1.37403				223	1.17926			
<b>(b) Size as covariate</b>										
Size	1	0.052	8.8207	4.527	<0.0001	1	0.07857	16.1129	7.8370	<0.0001
Sex	1	0.08462	14.3539	5.5015	<0.0001	1	0.01238	2.5378	2.855	<b>0.0035</b>
Mating mode	1	0.13052	22.1391	6.4716	<0.0001	1	0.06952	14.2569	7.7197	<0.0001
Sex × Mode	1	0.00851	1.4431	1.0257	0.1541	1	0.00901	1.8474	1.9898	<b>0.0292</b>
Size × Mode	1	0.02488	4.2202	3.2723	<b>0.0003</b>	1	0.03158	6.4757	5.6303	<0.0001
Size × Sex	1	0.00603	1.0235	0.2734	0.3943	1	0.00819	1.6791	1.6685	0.0531
Size × Sex × Mode	1	0.00481	0.8154	-0.2323	0.5914	1	0.01111	2.2793	2.556	<b>0.0083</b>
Residuals	218	1.28523				219	1.06792			
<b>(c) Shape</b>										
Sex	1	0.02077	3.9271	4.0012	<b>0.0002</b>	1	0.02077	3.9271	4.0012	<b>0.0002</b>
Mating mode	1	0.08331	15.7536	7.8498	<0.0001	1	0.08331	15.7536	7.8498	<0.0001
Sex × Mode	1	0.00494	0.9348	-0.0401	0.5012	1	0.00494	0.9348	-0.0401	0.5012
Residuals	223	1.17926				223	1.17926			
<b>(d) Size as covariate</b>										
Size	1	0.07857	16.1129	7.8370	<0.0001	1	0.07857	16.1129	7.8370	<0.0001
Sex	1	0.01238	2.5378	2.855	<b>0.0035</b>	1	0.01238	2.5378	2.855	<b>0.0035</b>
Mating mode	1	0.06952	14.2569	7.7197	<0.0001	1	0.06952	14.2569	7.7197	<0.0001
Sex × Mode	1	0.00901	1.8474	1.9898	<b>0.0292</b>	1	0.00901	1.8474	1.9898	<b>0.0292</b>
Size × Mode	1	0.03158	6.4757	5.6303	<0.0001	1	0.03158	6.4757	5.6303	<0.0001
Size × Sex	1	0.00819	1.6791	1.6685	0.0531	1	0.00819	1.6791	1.6685	0.0531
Size × Sex × Mode	1	0.01111	2.2793	2.556	<b>0.0083</b>	1	0.01111	2.2793	2.556	<b>0.0083</b>
Residuals	219	1.06792				219	1.06792			



investment into reproduction presumably stayed constant during species differentiation. Size differences and interaction of different allometric trajectories between species and the sexes, respectively, likely represent one of the main sources for the observed divergent patterns of SShD in this group. Further, I could show that the differentiation of SD-trajectories in the cranium can be partly attributed to the different mating modes found in *Tylotriton*, whereas in the humerus other selective forces must have influenced patterns of SDs. To fully understand and illustrate the evolution of morphological trajectories, more information on the species' ecologies are necessary for future studies.

Table 8: Results of a Procrustes ANOVA on 3D morphometric shape data of the humerus (a) and cranium (b) of crocodile newts (*Echinotriton* and *Tylotriton*) to test for SSD and mating mode as a potential selection force for different SSD-patterns between species (c, d). Significant p-values are given in bold.

	Df	SS	F	Z	P
<b>(a) Humerus size</b>					
Species	8	2.0433	38.2325	6.8966	<b>&lt;0.0001</b>
Sex	1	0.193	28.8894	2.0828	<b>&lt;0.0001</b>
Species × Sex	8	0.1218	2.2799	1.7555	<b>0.0228</b>
Residuals	208	1.3895			
<b>(b) Cranium size</b>					
Species	8	2805.8	38.9619	6.9389	<b>&lt;0.0001</b>
Sex	1	813.7	90.3948	2.5681	<b>&lt;0.0001</b>
Species × Sex	8	164.1	2.2787	1.7348	<b>0.0249</b>
Residuals	209	1881.3			
<b>(c) Humerus size with mating mode</b>					
Sex	1	0.1617	10.2769	1.62461	<b>0.0018</b>
Mating mode	1	0.0325	2.0677	0.91544	0.1491
Sex × Mating mode	1	0.0608	3.8643	1.18851	<b>0.053</b>
Residuals	222	3.4927			
<b>(d) Cranium size with mating mode</b>					
Sex	1	1045.9	55.5155	2.3744	<b>&lt;0.0001</b>
Mating mode	1	275.3	14.6103	1.7744	<b>&lt;0.0001</b>
Sex × Mating mode	1	142.5	7.5664	1.4634	<b>0.0064</b>
Residuals	223	4201.2			



## Conclusions

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In this thesis I investigated morphological disparity in basal salamandrids with a focus on sexual dimorphisms. Especially, I aimed to reveal rather subtle size and shape differences between males and females, which so far received little interest in morphology and evolutionary research. Therefore, I applied a comparative approach testing how external morphology and osteology coincide in patterns of SD, how capable the different methods are in revealing subtle shape differences, and I tested potential ecological traits as selective force leading to divergent morphologies in the sexes.

Sexual dimorphism is very complex in urodeles and multivariate statistics and approaches have to be applied to fully uncover morphological disparities (HERREL et al. 1999, BUTLER & LOSOS 2002, IVANOVIĆ & KALEZIĆ 2012, HERREL et al. 2017). Size and shape differences can be detected in different body parts of the urodelean bauplan like in limbs and the cranium (MALMGREN & THOLLESSON 1999, MARVIN 2009, REINHARD & KUPFER 2015, REINHARD et al. 2015). Especially, SShD is often rather subtle and not obvious for the observer at first sight (SHETTY & SHINE 2002, SCHMIEDER et al. 2015, ILIĆ et al. 2019, this study). As shown in chapter I, analyses of the external morphology and comparable osteological measurements generally disclose similar patterns of SD allowing generally to confer results from one to the other. Removing the influence of soft tissues from samples e.g., by  $\mu$ CT scans, provides access to many morphological structures in the osteology otherwise not accessible for morphometrics (IVANOVIĆ & KALEZIĆ 2012, this study). Our morphometric analyses of the osteology disclosed so far unknown sexually dimorphic characters in the investigated body parts. Especially interesting were sexually dimorphic measurements and overall shape of the pelvic girdle in the spectacled salamander disclosed in chapter I and II. Although the pelvic girdle plays an important role during reproduction it was never before investigated in detail in terms of SD in urodeles. Traditional morphometrics via linear measurements is also able to uncover shape differences (MALMGREN & THOLLESSON 1999, REINHARD & KUPFER 2015, this study), but GM is the method of choice in this field of research nowadays as the latter method has its special strength in capturing subtle shape differences and visualizing those (BLANCO & GODFREY 2006, ABDEL-RAHMAN et al. 2009, ARENDT 2010, BRENO et al. 2011). The findings of chapter II demonstrated the superiority of GM in capturing shape differences also in adult urodeles for the first time. Different theories try to explain evolutionary mechanisms of SD (FAIRBAIRN 2007). Especially the underlying mechanisms leading to subtle shape differences are still often



unclear but also hard to pinpoint, as advantages for one of the sexes are not easy to prove (e.g., HERREL et al. 1999, SHETTY & SHINE 2002, IVANOVIĆ & KALEZIĆ 2012, HERREL et al. 2017). Some dimorphic characters like larger trunks in females and divergent shapes of the pelvis suggest that the different reproductive roles of males and females play a major role in forming shapes at least in specific body parts. Other dimorphic body parts are not directly linked to reproduction as e.g., the cranium, and potentially other, additional selective forces are acting indirectly. An important source of SShD are allometric shape changes during growth as sexually dimorphic shape changes follow also allometric shape changes (IVANOVIĆ & KALEZIĆ 2012, this study). In combination with SSD, i.e., when one sex is selected for a larger size, this yields simultaneously to shape differences between the larger and the smaller sex. This pattern can be observed also in single body parts like in the cranium, which was demonstrated in chapter II. Selection mechanism leading to size differences as food niche partitioning or agonistic competition would have to be tested in more robust experimental approaches in salamanders. Here I used the monophyletic and, in terms of reproduction, polymorphic pleurodeline newts to test whether ecological and reproductive traits can explain morphological disparity between the sexes. Although crocodile newts exhibited an extraordinary conservative morphology (HERNANDEZ et al. 2018), shape differences in correlation with reproductive traits could be detected between species and the sexes in chapter III and IV. Interestingly in chapter III, morphological differences could be found separating the two subgenera within the genus *Tylototriton* substantiate the genetic data. One reproductive trait correlated with phylogeny and morphology, making both mechanisms hard to disentangle, but mating mode turned out to be a valid explanatory variable explaining morphological variation irrespective of phylogenetic relationship in crocodile newts (chapter III). It described divergent shape differences in cranial morphology between species and divergent SD patterns between the sexes (chapter III & IV). Beside their conservative morphology, analyses in chapter IV revealed divergent allometric trajectories between crocodile newt species, whereas allometries were constant between males and females. This suggests that species endure different selective pressure, likely due to the broad variation of habitats those newts inhabit (HERNANDEZ 2016), whereas in the sexes those stayed constant during species differentiation. The interaction of different allometric trajectories may leads then to differences in shape changes of males and females in different species. Nevertheless, in the humerus, shape differences were present between the different mating modes, but unexpectedly this trait did not explain variation in SD trajectories between species. Thus, further mechanisms must add additional variation and promote different SD trajectories in urodeles. Finally, knowledge about ecological traits forming morphology of hard



tissues are of main interest for the reconstruction of ancient species. To fully understand how certain patterns evolved during large evolutionary timescales, extinct taxa must be included into analyses. In chapter III, I could show that specimens of extinct pleurodeline newts of the genus *Chelotriton* exhibit large morphological variation and thus, likely represent different taxonomic units. Future studies are needed to provide deeper insight into the taxonomic relationships of fossil specimens from different deposits and geological strata. Further, I could reveal morphological similarity of *Chelotriton* specimens from one deposit with extant members exhibiting a more terrestrial orientated reproduction i.e., *Echinotriton* and *Yaotriton*, which is in concordance with the strata in which the remains of this extinct genus were excavated (WESTPHAL 1980).

This thesis provides essential new insights into morphological variation in urodeles between species and the sexes and its evolutionary origin and gives further hints on future research directions. It could be shown that multivariate approaches in a phylogenetic context are needed to further reveal potential sources of morphological variation especially in context of sexual dimorphism. The different reproductive systems are at least partly valid in explaining morphological differences between the different species of crocodile newts and SD trajectories.



## Future directions

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In future research, experimental studies are required that explicitly test evolutionary theories and selection pressures leading to SD. As single morphological structures likely experience different selective forces, these experiments must set a well-defined goal to unravel the different selection pressures and its magnitudes. Although food niche partitioning or agonistic male competition is often addressed in context with head size and shape dimorphism, clear evidence for such kind of selection pressures are missing in urodeles so far. A good knowledge on the ecology and life history of the studied species is mandatory to be able to define potential ecological traits that may be exposed to selection pressures that result in SSD and SShD. Crocodile newts represent a potentially good model system for the investigation of how reproductive biology shapes patterns of sexual dimorphism. In many species of *Tylototriton* still less is known on their biology and diversity. Further, sometimes it is unclear whether species exhibit inter-population variability or plasticity in their reproductive biology. More clarity is needed here to provide proper analyses. Inclusion of extinct members can provide further knowledge on the evolutionary history of morphological variation. A revision of *Chelotriton* is strikingly needed to clarify phylogenetic relationships within this genus and within pleurodeline newts. Then further studies could investigate the presence of SDs in these ancient species as already proved in other extinct tetrapods, providing a deeper insight into their reproductive biology and their evolutionary history. Broad scale studies covering members of an entire monophyletic taxon are needed to fully reveal evolutionary mechanisms forming the shape of males and females in different directions. Therefore, the family Salamandridae is and will represent an excellent model system.





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# Appendix

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**RESEARCH ARTICLE**

# Flesh and bone: An integrative approach towards sexual size dimorphism of a terrestrial salamander (genus *Salamandrina*)

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**Abstract**

Males and females face different selection pressures due to a sexually biased investment into reproduction. This often results in different morphologies. Sexual size dimorphisms (SSD) can give us important hints on the evolution and biology of a species. Salamanders are a perfectly suited system for investigating SSD, including a diversity of reproductive modes and behaviors, and patterns of SSD combined with life history traits in a phylogenetic context help us to understand the evolution of these processes. Because spectacled salamanders (genus *Salamandrina*) are the phylogenetically most basal taxon of the Salamandridae, they play a key role in reconstructing the evolutionary pattern of SSD. Combining extensive external and skeletal measurements of the cranium, limbs, and the pelvic girdle using high-resolution micro Computer Tomography ( $\mu$ CT) yielded an integrative analysis of expressed SSD of morphology and osteology of *Salamandrina perspicillata*. Multivariate analysis of external characters showed that males generally had larger cloacae, heads, and limbs relative to body size, while females had larger trunks. Analysis of osteology confirmed this pattern but also revealed new dimorphic characters in the cranium and the pelvic girdle. Dimorphic characters in external morphology and osteology are likely linked to the different reproductive roles of the sexes and support sexual rather than ecological selection as the primary force acting on the phenotype of the phylogenetically basal salamandrids.

**KEYWORDS**

micro CT, morphometry, osteology, sexual selection, sexual shape dimorphism

## 1 | INTRODUCTION

Sexual dimorphism (SD), differences in morphology between males and females, is a common feature in the animal kingdom (e.g., Fairbairn, 2007), whereby differences in body size and its proportions are generally referred to as sexual size dimorphism (SSD; e.g., Kupfer, 2007; Malmgren & Thollessen, 1999; Monnet & Cherry, 2002). Various selective forces are thought to drive the divergent morphology of males and females. (a) The fecundity theory hypothesizes that larger females have a selective advantage by producing more or larger offspring (Darwin, 1871; Kupfer, Nabhitabhata, & Himstedt, 2004); (b) the sexual selection theory states that in species with intrasexual male competition larger and stronger males gain more access to receptive females and hence have a higher fitness (Andersson, 1994; Shine, 1979); while the (c) ecological niche partitioning theory assumes that males and females gain an advantage

through using different resources, minimizing intersexual competition and resulting in sex-specific adaptations (Hedrick & Temeles, 1989; Shetty & Shine, 2002). In anurans and urodeles, 90% and 61%, respectively, exhibit female-biased body size dimorphism (Kupfer, 2007; Shine, 1979). Caecilian amphibians are insufficiently investigated, with female-biased body size dimorphism known for only a few species (Kupfer, 2009; Maerker, Reinhard, Pogoda, & Kupfer, 2016).

The sexes can also be dissimilar in ways other than overall body size: divergence in body shape is also possible, and quite common. However, research on sexual shape dimorphism (SShD) is much scarcer than on SSD (e.g., Alarcón-Ríos, Velo-Antón, & Kaliontzopoulou, 2017; Gidaszewski, Baylac, & Klingenberg, 2009; Ivanović, Sotiroopoulos, Furtula, Džukić, & Kalezić, 2008; Schwarzkopf, 2005). As cryptic intersexual differences in shape or body proportions are not captured if only body length is analyzed (Reinhard, Renner, & Kupfer, 2015; Schwarzkopf, 2005) SShD is only detectable by multivariate

morphometrics (e.g., Malmgren & Thollesson, 1999; Reinhard & Kupfer, 2015).

While mammalian osteology is well investigated in terms of SD (e.g., Plavcan, 2001), comparative osteology was neglected in most other vertebrates. However, it is important to determine whether externally visible dimorphisms are based on differences in soft tissue, such as muscles, or are linked to differentially shaped bones, as this would lead to different conclusions regarding function (Howard & Kluge, 1985; Lynch, 1971; Navas & James, 2007; Oka, Ohtani, Satou, & Ueda, 1984). Cryptic not externally visible dimorphic traits, may additionally be found in the skeleton. A major advantage of skeletal features is the solidity of the bones, which makes them less prone to artefactual alterations. Explicitly, amphibians are known to shrink after preservation (Lee, 1982; Verrell, 1985a). As bones have lower water content, they are expected to be less affected by the shrinkage process or the physical constitution of the individual than soft tissue. Whereas many studies in anurans showed dimorphic humeri (e.g., Duellman, 1970; Duellman & Savitzky, 1976; Howard & Kluge, 1985; Lynch, 1971; Padhye, Jadhav, Sulakhe, & Dahanukar, 2015) or ilia (Blain, Lózano-Fernández, & Böhme, 2015), the morphometric osteology of salamanders was rarely investigated (Herre, 1952; Ivanović et al., 2008; Ivanović & Kalezić, 2012).

True salamanders of the family Salamandridae are a well-suited system for investigating SSD and SShD because they exhibit variable reproductive systems and modes and have highly diverse life history strategies (Kupfer, 2007; Sparreboom, 2014). Although, the majority of species show female-biased SSD in body size, there are about 19% of salamander species exhibiting male-biased SSD (Kupfer, 2007; Shine, 1979). As a variety of sexually dimorphic traits were found among salamandrids, including larger trunks in females or larger cloacae, limbs and heads in males (e.g., Bovero, Sotgiu, Castellano, & Giacomina, 2003; Malmgren & Thollesson, 1999; Marvin, 2009; Reinhard et al., 2015) we assumed that certain patterns of SSD and SShD manifested very early in the ancestral line of Salamandridae.

Spectacled salamanders (genus *Salamandrina*) represent the phylogenetically most basal taxon of the Salamandridae (Zhang, Papenfuss, Wake, Qu, & Wake, 2008), thus they likely hold a key role in the reconstruction and understanding of the evolutionary patterns of SSD in this family. Spectacled salamanders rarely reach an overall length greater than 10 centimeters (Romano et al., 2009; Zuffi, 1999). After metamorphosis, *Salamandrina* spends its entire life in terrestrial habitats. Only gravid females enter small streams for a short time period for oviposition. Mating takes place on land, males courting the females (Zuffi, 1999). As these differences in life history traits between male and female spectacled salamanders are similar to other salamandrids, a variety of SSD and SShD is expected.

Our aim was to investigate SSD and SShD of *Salamandrina* by combining two approaches (a) studying the overall bauplan externally and (b) to apply micro Computer Tomography ( $\mu$ CT) to obtain data on SSD of various osteological characters for the first time in a salamander. We hypothesize that *Salamandrina* exhibits patterns of SD already known in derived groups within the family. Male-biased characters are expected in cloacal size, proportions of the limbs and head dimensions, while the trunk measurements are expected to be female-biased. Further, we hypothesize that the osteological investigations

will correlate with the external measurements and also reveal new skeletal dimorphic characters. We discuss the external and osteological patterns of SD in relation to the biology of *Salamandrina*. Finally, we provide a phylogenetic comparison of SSD within the family Salamandridae.

## 2 | MATERIALS AND METHODS

### 2.1 | Study species

The genus *Salamandrina* Fitzinger, 1826 comprises two species and is endemic to the Italian peninsula. We studied specimens of the Northern spectacled salamander, *Salamandrina perspicillata* (Savi, 1821), which were obtained from various natural history museum collections housed in Germany and Switzerland (see Supporting Information Table S1). We assumed that all examined individuals had been preserved for more than a decade and had finished the initial shrinkage process (see also Malmgren & Thollesson, 1999). Severely damaged, obviously starved, or dehydrated individuals were excluded from analysis. In total, 92 individuals, 45 males, and 47 females, from various locations in Northwestern Italy (provinces of Liguria and Tuscany, see Supporting Information Table S1) were measured. They were subsequently sexed to avoid a measurement bias while processing specimens. The salamanders were sexed by internal inspection of gonads (Francis, 1934) and cloacal wall morphology (Romano, Bruni, & Paoletti, 2009; Vignoli, Silici, Brizzi, & Bologna, 2010). Well-developed gonads, cloacal papillae and ridges, and a SVL >29 mm in all individuals indicated maturity (Francis, 1934; Zuffi, 1999).

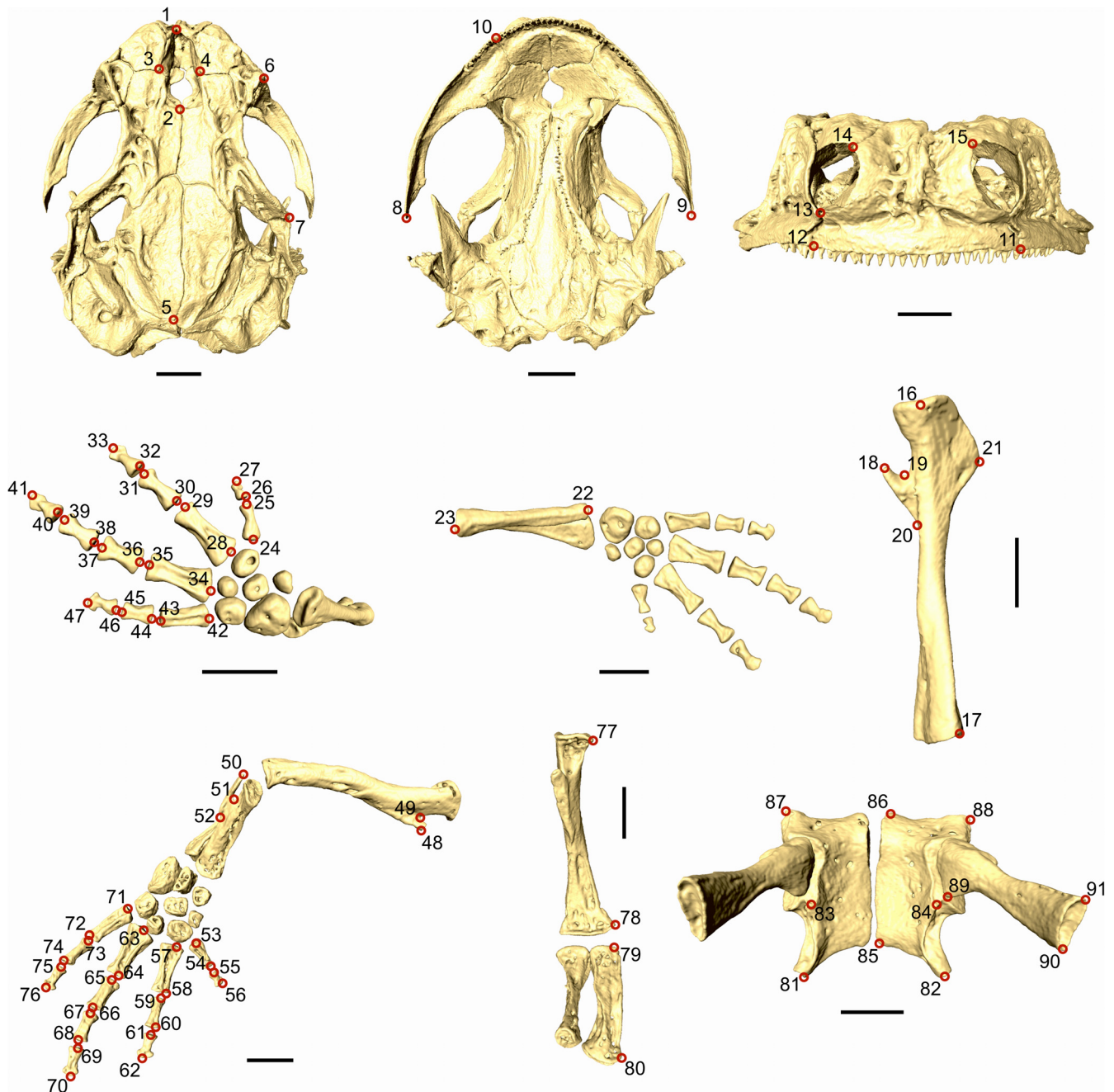
### 2.2 | External morphometry

To investigate SSD in *S. perspicillata*, 45 external characters were selected (Table 1). Bilateral characters were measured on the right side. Measurements were taken to the nearest 0.01 mm using a digital caliper (Rok International Industry, Ltd.). For minute characters, a stereomicroscope (Wild Herrbrug) was used. Furthermore, digit length was assessed from photographs using the method of Kaczmarek et al. (2015).

Hands and feet, respectively, were placed dorsal side up on an ocular micrometer and enclosed by a second glass slide to obtain straightened digits without compression (Balogová et al., 2015). Digital photographs were taken with a Canon EOS 550D DSLR with an attached EF-S 60 mm Macro lens from a fixed distance. A tps file was built separately for hands and feet using the software tpsUtil (Rohlf, 2016a), and the file was processed in the software tpsDig2 (Rohlf, 2016b), setting a scale and landmarks on each photograph. Seven landmarks were defined for hands and feet and were digitized (see Supporting Information Figure S1). The newly generated tps files contained landmark data. The program CoordGen8 (including the package TMorphGen) (Sheets, 2014) was used to calculate length measurements from landmark data subsequently processed in Excel (Microsoft Corp.). For digits measured twice (right and left side), a mean was calculated.

**TABLE 1** Morphometric characters of the head, trunk, and limbs for the external analysis of SSD in northern spectacled salamanders *S. perspicillata*. For each measurements referring to limbs, these were kept perpendicular to the body axis to assure standardized measurements. Note that the toe measurements start with the second toe as the first toe is reduced and nonexistent in *Salamandrina*

Abbreviation	Character	Definition
Head		
HL	Head length	Tip of snout to margin of neck
HNL	Head-neck length	Tip of snout to the anterior margins of forelimbs
HW	Head width behind eyes	Width at the posterior margin of eyes
HH	Head height behind eyes	Height at the posterior margin of eyes
ML	Mouth length	Tip of mandible to the corner of the mouth
ND	Internarial distance	Measured from their medial margins
EL	Eye diameter	Horizontal diameter from medial to lateral canthus
IOD	Interorbital distance	Distance between medial canthus
END	Eye-naris distance	Medial canthus of eye to lateral margin of nostril
SEL	Snout-eye length	Tip of snout to medial canthus of eye
Trunk		
SVL	Snout-vent length	Tip of snout to posterior margin of cloacal lips
TLL	Tail length	Posterior margin of cloacal lips to tail tip
TTL	Total length	Tip of snout to tail tip
AGD	Axilla-groin distance	Space between axilla to groin
TRL	Trunk length	Anterior margin of forelimb to anterior margin of cloaca
SHW	Shoulders width	Anterior margins of forelimb at junction to body
CHW	Chest width	Distance between the axilla
GG	Width of trunk at groin level	Distance between groins
CLL	Cloaca length	Anterior to posterior margin of cloacal lips
CLW	Cloaca width	Left to right margin in the middle of CLL
FSL	Fissure length	Anterior to posterior margin of fissure
TLHB	Tail height at base	Behind posterior margin of cloaca
TLHM	Tail height at mid-tail	Measured at 50% of tail length
TLWB	Tail width at base	Behind posterior margin of cloaca
TLWM	Tail width at mid-tail	Measured at 50% of tail length
Forelimb		
ARM	Total arm length	Axilla to longest finger
UAL	Upper arm length	Axilla to tip of elbow
LAL	Lower arm length	Tip of elbow to wrist
HAL	Hand length	Wrist to longest finger
UAD	Upper arm diameter	At mid-arm in dorsoventral plane
LAD	Lower arm diameter	At mid-arm in dorsoventral plane
1FL	First finger length	After Kaczmarek, Kubicka, Tryjanowski, & Hromada, 2015, see methods
2FL	Second finger length	After Kaczmarek et al., 2015, see methods
3FL	Third finger length	After Kaczmarek et al., 2015, see methods
4FL	Fourth finger length	After Kaczmarek et al., 2015, see methods
Hind limb		
LEG	Total leg length	Groin to longest toe
ULL	Upper leg length	Groin to tip of knee
LLL	Lower leg length	Tip of knee to ankle
FOL	Foot length	Ankle to longest toe
ULD	Upper leg diameter	At mid-leg in dorsoventral plane
LLD	Lower leg diameter	At mid-leg in dorsoventral plane
2TO	Second toe length	After Kaczmarek et al., 2015, see methods
3TO	Third toe length	After Kaczmarek et al., 2015, see methods
4TO	Fourth toe length	After Kaczmarek et al., 2015, see methods
5TO	Fifth toe length	After Kaczmarek et al., 2015, see methods



**FIGURE 1** Definition of landmarks (red circles) on the skeleton of the cranium (upper row), forelimb (middle row), hind limb (bottom, left and middle) and pelvic girdle (bottom, right) of northern spectacled salamander *S. perspicillata* to obtain length measurements for analysis of SSD. In Table 2 landmarks for each measurement are given. Scale bar = 1 mm

### 2.3 | Osteological morphometry

To analyze the osteology of the salamanders, we carried out high resolution  $\mu$ CT scans using a Bruker SkyScan1272 device at the Natural History Museum of Stuttgart. Twenty individuals per sex were randomly chosen for scans (see Supporting Information Table S1). Scans were performed without a filter, at 50 kV and 200 mA at a resolution of 15  $\mu$ m. Rotation steps were set at 0.4°, with an exposure time of 309 ms per frame. Depending on size and specimen position, the reconstructions resulted in approximately 2,000 to 4,000 slices at an image resolution of 1344  $\times$  1344 pixels. For volume rendering, segmentation and 3D surface modeling, the data were imported into

Amira® 6.2 (Visualization Sciences Group). For subsequent data acquisition and analysis, we segmented the cranium, right fore- and hindlimb, and the pelvic girdle and created surface-files. For analysis, we decided on traditional morphometry via length measurements instead of a geometric morphometrics (GM) approach, because moveable body parts such as limbs are challenging to consistently arrange in the same posture, and would introduce artificial variation. In addition, the definition of an adequate number of clear landmarks to capture shape is problematic for long or very small bones (phalanges), making differences in shape difficult to assess. Length measurements of skeletal elements were obtained using landmarks and calculating the Euclidean distance between two landmarks. Landmarks were specified from the



**TABLE 2** Morphometric characters of the head, limbs, and pelvic girdle for the osteological analysis of SSD in northern spectacled salamanders *S. perspicillata*. For landmark definition see Figure 1

Abbreviation	Character	Landmarks used
Cranium		
SL	Cranium length	1,5
SW	Cranium width	8,9
MXL	Maxillary length	8,10
PMW	Premaxillary width	11,12
UND	Naris distance at upper edge	14,15
ND	Naris diameter	13,14
NCL	Nasal cavity length	1,2
NCW	Nasal cavity width	3,4
OL	Orbit length	6,7
Forelimb		
HUL	Humerus length	16,17
HUW	Humerus width	18,21
UL	Ulna length	22,23
CdhH	Crista dorsalis humeri height	18,19
CdhL	Crista dorsalis humeri length	18,20
CAR1	Carpal I length	24,25
FPh1.1	Finger phalange 2.1 length	26,27
CAR2	Carpal II length	28,29
FPh2.1	Finger phalange 3.1 length	30,31
FPh2.2	Finger phalange 3.2 length	32,33
CAR3	Carpal III length	34,35
FPh3.1	Finger phalange 4.1 length	36,37
FPh3.2	Finger phalange 4.2 length	38,39
FPh3.3	Finger phalange 4.3 length	40,41
CAR4	Carpal IV length	42,43
FPh4.1	Finger phalange 5.1 length	44,45
FPh4.2	Finger phalange 5.2 length	46,47
Hind limb		
FML	Femur length	77,78
FIBL	Fibula length	79,80
THL	Trochanter height	48,49
TSI	Tibial spine length from invagination	50,51
TSB	Tibial spine length from basis	50,52
TAR2	Tarsal II length	53,54
TPh2.2	Toe phalange 2.1 length	55,56
TAR3	Tarsal III length	57,58
TPh3.2	Toe phalange 3.1 length	59,60
TPh3.3	Toe phalange 3.2 length	61,62
TAR4	Tarsal IV length	63,64
TPh4.2	Toe phalange 4.1 length	65,66
TPh4.3	Toe phalange 4.2 length	67,68
TPh4.4	Toe phalange 4.3 length	69,70
TAR5	Tarsal V length	71,72
TPh5.2	Toe phalange 5.1 length	73,74
TPh5.3	Toe phalange 5.2 length	75,76
Pelvis		
PVL	Pelvis length	85,86
aPVW	Anterior pelvis width	87,88

(Continues)

**TABLE 2** (Continued)

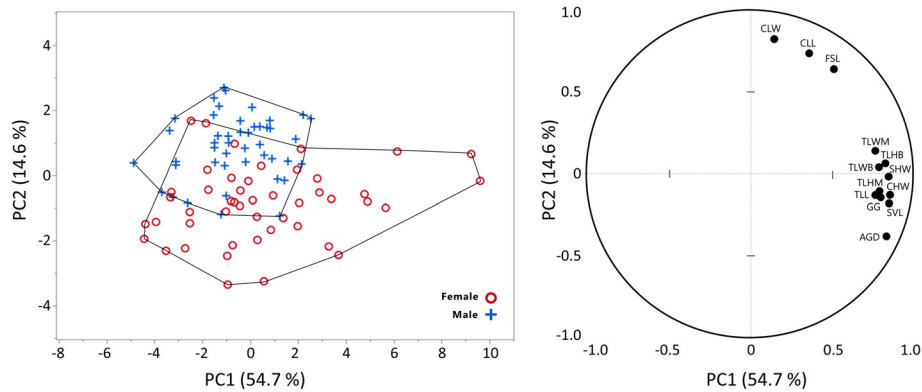
Abbreviation	Character	Landmarks used
pPVW	Posterior pelvis width	81,82
mPVW	Medial pelvis width	83,84
ISCHW	Ischium width	82,85
PBW	Pubis width	86,88
ILL	Ilium length	89,90
ILW	Ilium width on dorsal condyle	90,91

surface models, using IDAV Landmark Editor (Wiley et al., 2005). A total of 14 landmarks were defined on the cranium, 31 on the fore- and 32 on the hind-limb and another 12 landmarks on the pelvic girdle (Figure 1). From these landmarks, nine measurements of the cranium, 17 of the fore- and hind-limb, and another eight length measurements of the pelvic girdle (Table 2) were calculated for the analysis of SSD.

## 2.4 | Statistical analysis

Some specimens contained missing values for certain characters due to damage or an unfavorable fixation posture, making accurate measurements impossible. We filled in gaps using the missMDA package (Josse & Husson, 2016), implemented in R version 3.3 (© The R Core Team 2016). According to Lovich and Gibbons (1992), we calculated the sexual dimorphism index (SDI) for body size (SVL). We checked each variable for normality by eye using histogram plots. Non-normal variables were transformed using a LOG10 or exponential transformation. To test for general patterns of SSD, *t* tests (equal variances, two-tailed) were performed on each character. Further, a principle component analysis (PCA) was performed, to test for SSD and SShD separately, via *t* tests (two-tailed) on PC scores. For each body part (head/cranium, forelimb, hind limb, trunk, pelvic girdle) a separate PCA was conducted (see Bernardes et al., 2017; Malmgren & Thollesson, 1999). As in some body parts some measurements were highly collinear, the following variables were excluded to enhance the PCA scores: HNL, TRL, and TTL. For the osteological limb measurements, the sums of phalanges plus metacarpals and metatarsals, respectively, were calculated to represent entire digit length, further reducing the number of variables. The first two principal components were considered for subsequent analysis. As PC1 always had high positive loadings for all characters, it was interpreted as a general measure of size of the specific body part, while PC2 represented a shape variable, exhibiting positive and negative loadings for various characters (see, e.g., Bookstein et al., 1985; Malmgren & Thollesson, 1999).

To avoid the bias of individual differences in size, SSD in each character was assessed using an analysis of covariance (ANCOVA) with PC1 scores of the respective PCA as a covariate, correcting for size of the respective body part (see Romano, Bruni, & Paoletti, 2009). However, this analysis does not correctly scale character dimensions in relation to the entire body architecture (BA). The traditional trait characterizing body size in salamanders, and also in lizards, is SVL (Kupfer, 2007; Reinhard & Kupfer, 2015; Seglie, Roy, & Giacomini, 2010). Kratochvíl, Fokt, Rehák, and Frynta (2003) determined that scaling for size by one, often sexually dimorphic character, is not adequate. Therefore, we conducted another PCA with characters



**FIGURE 2** Scatterplot of principal component scores (left) and factor loading plot (right) for the first two principal components of a principle component analysis to describe trunk morphology of *S. perspicillata*. Although, morphospace of the sexes is largely overlapping both, PC1 and PC2 differ significantly (see text). Characters best separating male and female are cloaca-related measures and axilla-groin distance. Abbreviations see Table 1

representing the entire BA, including representative characters such as HL, HW, HH, SVL, TLL, AGD, GG, CHW, CLL, CLW, ARM, and LEG. The resulting PC1 (henceforth referred to as PC1\_BA) was then used as a covariate, first in a multivariate analysis of covariance (MANCOVA) to assess overall differences in morphology and osteology in each body part, and then in an ANCOVA on each character to assess which characters were sexually dimorphic relative to the entire BA. Statistical analyses were carried out in JMP version 12 (© SAS Institute Inc.) and SPSS version 24 (© SPSS Inc.).

### 3 | RESULTS

#### 3.1 | External morphometry

The SDI indicated that females were on average 6% larger than males in body size (SVL). A direct intersexual comparison revealed no SSD in head and limb characters, except in foot length, which was male-biased. Male cloacal dimensions were larger, while for trunk length and width the opposite was the case (see Supporting Information Table S2). Comparisons of PC axes revealed neither SSD nor SShD in the head and limbs, but for trunk measurements, both PC axes were significant. PC1 (explaining 54.7% of the variance) represented a general measure of size ( $t = 2.09$ ,  $p = .04$ ) and PC2 (explaining 14.6% of the variance) showed the highest positive loadings for cloacal measurements and lower negative loadings for body length measures, representing an optimal ratio between cloacal size and body length (Figure 2;  $t = 7.99$ ,  $p < .0001$ ). Adjusting for size of single body parts using the respective PC1 revealed another set of dimorphic characters: head width and lower leg length were larger in females relative to head and hind limb size, respectively (HW,  $F = 4.83$ ,  $p = .03$ ; LLL,  $F = 8.32$ ,  $p = .005$ ). Cloacal size was larger in males, while females had larger trunks but no SD in forelimb measurements was found (Supporting Information Table S3).

Multivariate analysis (MANCOVA) correcting for the entire BA, revealed significant intersexual differences in trunk and limb morphology but not in head morphology (Table 3).

In addition to a size dimorphic cloaca, males exhibited a greater head length and limb dimensions, including upper and lower limb

diameters, relative to their BA. Again trunk characters such as groin distances were larger in females than in males (Table 4, Supporting Information Figure S2a–c).

#### 3.2 | Osteological morphometry

Direct comparison of osteological measurements revealed a diversity of dimorphic characters. Males exhibited larger values for SL, NCL, OL, HUL, UL, FIBL, many of the digit lengths and pubis width, whereas females exhibited larger ilium width (Supporting Information Table S4). Comparisons of PC axes revealed SSD and SShD in both limbs, but not in the cranium or pelvic girdle. The forelimb was dimorphic in both PC1 (explaining 56.3%,  $t = 2.38$ ,  $p = .023$ ) and PC2 (explaining 16.4%,  $t = 2.33$ ,  $p = .025$ ), while the hind limb was dimorphic in PC1 (explaining 55.8%,  $t = 2.94$ ,  $p = .006$ ) but not PC2 (explaining 13.1%,  $t = 1.99$ ,  $p = \text{n.s.}$ ), indicating only differences in size but not the shape of the latter. Body part size-adjusted analyses via ANCOVA changed the overall observed pattern, that is, the cranium was the most dimorphic body part relative to its size. Males exhibited a longer orbit, skull, and nasal cavity, while females had a wider skull and premaxilla and a longer maxilla (Figure 3, Supporting Information Table S5).

In terms of forelimb osteology females had a weakly larger humeral crista dorsalis (CdhL,  $F = 4.13$ ,  $p = .049$ ) but also some other osteological traits of the forelimb (HUW, CdhH, UL) showed slight tendencies towards a female biased size. When comparing the hindlimb osteology between the sexes females showed longer femora

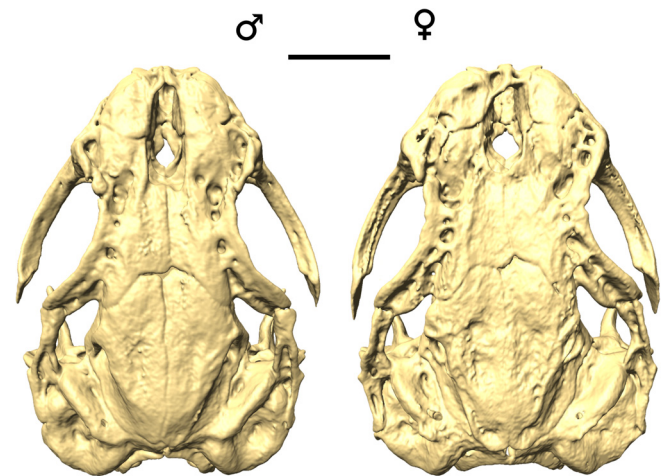
**TABLE 3** Analysis of SShD in overall external morphology (head, fore- and hind-limb and trunk) in northern spectacled salamanders *S. perspicillata* ( $n = 45$  males;  $n = 47$  females). Results of multivariate analysis of covariance (MANCOVA). Analyses were performed with PC1\_BA as covariate correcting for overall BA

Body part	Wilks' Lambda	F	p
Head	0.86	1.47	n.s.
Trunk	0.399	8.93	<.0001
Forelimb	0.68	3.76	<.001
Hind limb	0.597	5.4	<.0001

**TABLE 4** Analysis of SSD of 45 external characters in northern spectacled salamanders *S. perspicillata* ( $n = 45$  males;  $n = 47$  females). Results of analysis of covariance (ANCOVA). Analyses were performed with PC1\_BA as covariate correcting for overall BA

Character	F	p	Sex bias
Head			
HL	6.61	<.05	M
HW	0.08	n.s.	-
HH	0.05	n.s.	-
ML	1.73	n.s.	-
IND	1.31	n.s.	-
EL	1.36	n.s.	-
IOD	0.24	n.s.	-
END	0.08	n.s.	-
SEL	2.29	n.s.	-
Trunk			
SVL	14.68	<.001	F
TLL	0.58	n.s.	-
AGD	68.94	<.0001	F
SHW	0.99	n.s.	-
CHW	3.11	n.s.	-
GG	7.22	<.01	F
CLL	33.04	<.0001	M
CLW	24.93	<.0001	M
FSL	31.64	<.0001	M
TLHB	0.42	n.s.	-
TLHM	0.14	n.s.	-
TLWB	0.75	n.s.	-
TLWM	0.23	n.s.	-
Forelimb			
ARM	9.34	<.01	M
UAL	1.87	n.s.	-
LAL	4.89	<.05	M
HAL	7.49	<.01	M
UAD	5.16	<.05	M
LAD	11.14	<.01	M
1FI	3.58	n.s.	-
2FI	17.43	<.0001	M
3FI	10.7	<.01	M
4FI	2.18	n.s.	-
Hind limb			
LEG	11.07	<.01	M
ULL	3.13	n.s.	-
LLL	1.86	n.s.	-
FOL	19.18	<.0001	M
ULD	7.3	<.001	M
LLD	6.97	<.05	M
1TO	8.86	<.01	M
2TO	18.64	<.0001	M
3TO	14.2	<.001	M
4TO	3.05	n.s.	-

(FML,  $F = 4.66$ ,  $p = .038$ ) and a larger trochanter (THL,  $F = 4.5$ ,  $p = .049$ ), while males had longer overall fourth digits (4TPh,  $F = 7.86$ ,  $p = .008$ ). A SD in the pelvic girdle remained, that is, males had a



**FIGURE 3** Comparison of a male (ZMB 72302) and female (ZMB 72363) cranium of *S. perspicillata* of the same length (6.3 mm) to illustrate shape dimorphism. Scale bar = 2 mm

wider pubis but females had a wider ilium (PBW,  $F = 5.67$ ,  $p = .023$ ; ILW,  $F = 36.61$ ,  $p < .0001$ ).

The multivariate analyses (MANCOVA) showed an overall intersexual difference in osteology in all four skeletal subunits (Table 5). The ANCOVA including PC1\_BA as a covariate supported the SSD pattern of external osteology, that is, males had longer cranial characters such as the size of the nasal cavity and were larger in various limb characters, including pubis width, whereas females had a wider ilium (Table 6; Supporting information Figure S2D-F).

## 4 | DISCUSSION

### 4.1 | Integrative analyses of SSD

We studied the patterns of SSD and SShD of a phylogenetically most basal salamandrid, *S. perspicillata* by integrating external morphology and osteology. As the analysis of two similar morphological and osteological trait sets revealed an overall quite identical pattern of dimorphism we concluded that soft tissue does not significantly mask or produce SSD. However, a dimorphic external morphology was not always linked to osteology. Externally wider limbs in males did not correspond to a dimorphic shape of long bones as the cristae for muscle attachment appeared similar and were of equal size between the

**TABLE 5** Analysis of SShD in overall osteology in cranium, forelimb, hind limb, and pelvic girdle measurements in northern spectacled salamanders *S. perspicillata* ( $n = 20$  males and 20 females). Results of multivariate analysis of covariance (MANCOVA). Analyses were performed with PC1\_BA as covariate correcting for overall BA

Body part	Wilks' lambda	F	p
Cranium	0.411	4.61	<.001
Forelimb	0.557	2.56	<.05
Hind limb	0.4	4.83	<.001
Pelvis	0.394	5.77	<.001

**TABLE 6** Analysis of SSD in 36 osteological characters of northern spectacled salamanders *S. perspicillata* ( $n = 20$  males and  $n = 20$  females). Results of analysis of covariance (ANCOVA). Analyses were performed with PC1\_BA as covariate correcting for overall BA

Character	F	p	Sex bias
Cranium			
SL	16.78	<.001	M
SW	0.07	n.s.	-
MXL	1.54	n.s.	-
PMW	1.63	n.s.	-
UND	0.67	n.s.	-
ND	0.22	n.s.	-
NCL	8.58	<.01	M
NCW	0.05	n.s.	-
OL	6.18	<.05	M
Forelimb			
HUL	11.54	<.01	M
HUW	0.11	n.s.	-
UL	14.81	<.001	M
CdhH	3.91	n.s.	-
CdhL	2.63	n.s.	-
1FPh	2.21	n.s.	-
2FPh	4.37	<.05	M
3FPh	6.43	<.05	M
4FPh	10.4	<.01	M
Hind limb			
FML	1.79	n.s.	-
THL	0.12	n.s.	-
FIBL	14.3	<.001	M
TSI	0.39	n.s.	-
TSB	0.16	n.s.	-
2TPh	4.74	<.05	M
3TPh	14.85	<.001	M
4TPh	20.48	<.0001	M
5TPh	9.13	<.01	M
Pelvis			
PVL	0.12	n.s.	-
aPVW	1.24	n.s.	-
pPVW	0.92	n.s.	-
mPVW	0.49	n.s.	-
ISCHW	1.85	n.s.	-
PBW	7.46	<.05	M
ILL	1.05	n.s.	-
ILW	32.04	<.0001	F

sexes. Taking osteology into account enabled us to identify dimorphic characters previously unknown in salamanders. We detected a higher quantity of dimorphic characters by incorporating osteological traits into our analyses than found when analyzing external morphology. Quantitative osteology via three-dimensional  $\mu$ CT models also is more precise than manual measurements because digitized object can easily be magnified and rotated. The models are not affected by mechanical pressure and specific bones can be segmented for measurement.

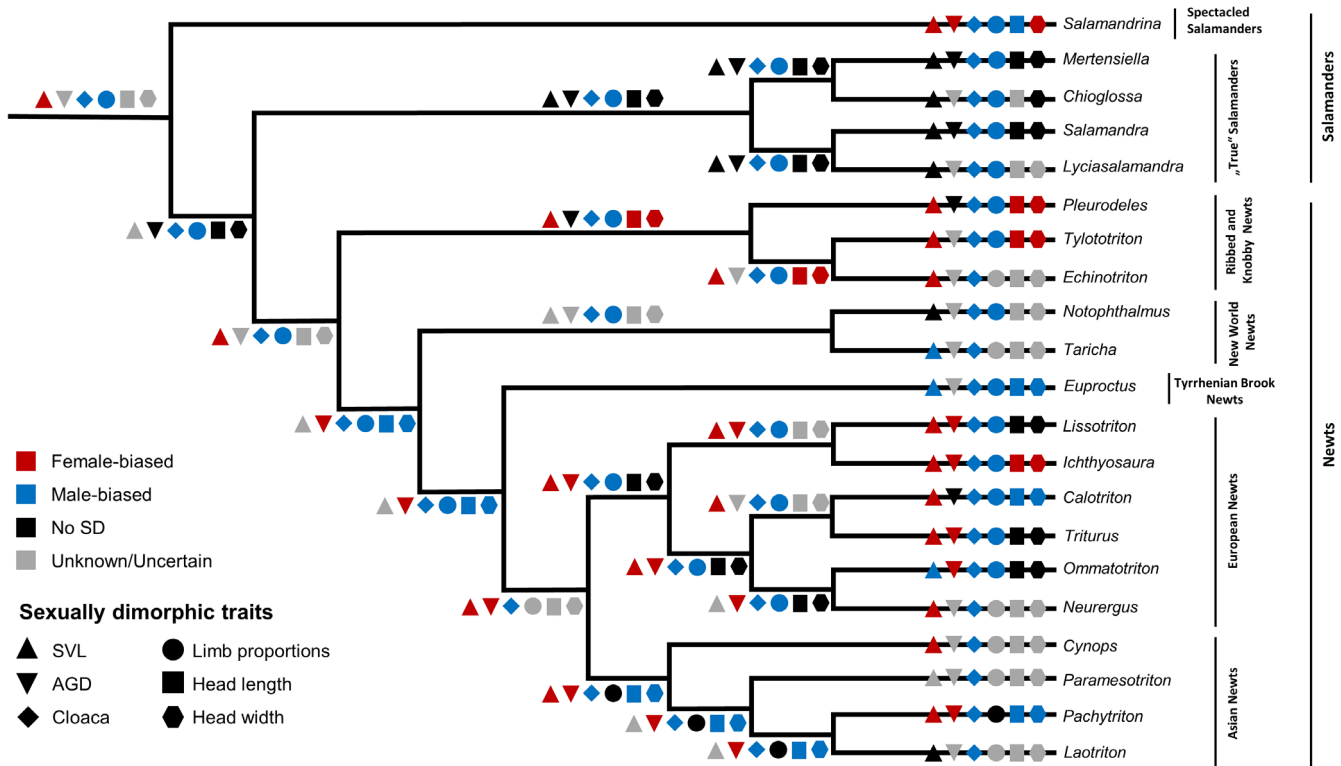
## 4.2 | Dimorphism, selection, and evolution

Longer and wider trunks in female salamanders meet the assumptions of the fecundity advantage hypothesis (Kupfer, 2007; Shine, 1988), and are commonly found among salamanders of various families (e.g., Bakkegard & Guyer, 2004; Malmgren & Tholleson, 1999; Marvin, 2009; Reinhard & Kupfer, 2015; Xiong, Liu, Li, Zhang, & Min, 2017). As trunk size is directly related to pleuroperitoneal cavity size limiting number and size of eggs this trait is directly linked to female reproductive success (Halliday & Verrell, 1986; Kalezic, Crnobrnja, Dorovic, & Dzukic, 1992; Salthé, 1969; Verrell & Francillon, 1986). Furthermore, male salamanders prefer larger females likely due to their higher reproductive output (Verrell, 1985b, 1986).

Internally fertilizing salamanders deposit spermatophores on the substrate, which then are picked up by the female cloaca. In newts, a male deposits several spermatophores during a single mating event, but only a small proportion enters the female cloaca, therefore, representing a high male investment (Halliday, 1990). Males produce high amounts of pheromones to direct females to collect spermatophores (Sever, 2003). Although other glands, such as mental and caudal glands in plethodontid salamanders, also contribute to successful copulation, the cloacal glands are assumed to be a primary source of courtship pheromones (e.g., Houck & Reagan, 1990; Rollmann, Houck, & Feldhoff, 1999; Sever, 1989). Thus, male salamanders are expected to be under positive selection for productive cloacal glands. To meet the requirements of spermatophore and pheromone production, male salamanders have a much higher glandular masses around the cloacal fissure than females (Sever, Verrell, Halliday, Griffiths, & Waights, 1990). As the cloaca of terrestrial *Salamandrina* appears rather discrete compared to other European newts and salamanders the cloaca was thought to be of equal size in both sexes (Brizzi, Del-fino, & Calloni, 1988, 1989 in Zuffi, 1999). Our study revealed that this is not the case in *Salamandrina*, albeit the cloacal size differences are not as obvious as in other urodeles. Also we found a male-biased pubis width and wider pubes might accommodate more space for larger cloacae.

The complex courtship display of male newts and salamanders is a primary source of selection acting on limb size. Consequently a male-biased dimorphic limb length and diameter is present in many salamander and newts (see Figure 4).

Generally salamander feet are subject to rapid phenotypic evolution (Salvidio, Crovetto, & Adams, 2015 for a plethodontid salamander). Relatively longer limbs in male salamanders could stabilize courtship performance by providing a firm foothold (Franzen, 1999; Malmgren & Tholleson, 1999; Reinhard et al., 2015; Reinhard & Kupfer, 2015). Wider limbs usually accommodate a higher muscle mass. Longer limbs might be advantageous for covering larger distances (Wittlinger, Wehner, & Wolf, 2006) as terrestrial males tend to walk longer distances (for *Salamandrina*: Barbieri, 1991; Vanni, 1980 in Zuffi, 1999) while seeking receptive females. *Salamandrina* females had differentially shaped hind limbs, that is, when adjusted for limb size, females exhibited relatively longer femora, more developed trochanters and had wider ilia. Female spectacled salamanders oviposit in small creeks and ponds and attach eggs to submerged structures (Barbieri, 1991; Vanni, 1980 in Zuffi, 1999). Differentially shaped limb



**FIGURE 4** Phylogenetic relationships of Salamandridae and the distribution of SD of six, widely investigated characters. Characters are displayed for each genus and at internal nodes of the phylogeny. Grey symbols indicate noninvestigated characters or that a species of a given genus express both directions of SSD preventing conclusions on the ancestral state. Internal node characters were plotted after a  $> 50\%$  majority rule, while known character states were given priority over “unkown/uncertain” states. Equivocal characters received an “unkown/uncertain”—grey marking. The phylogeny was modified from Zhang et al. (2008). References are listed in the supporting information

bones and enlarged muscles (e.g., *Musculus extensor iliotalialis*, *M. ilio-femoralis* or *M. ilio-fibularis sensu Francis, 1934*) are likely supporting aquatic oviposition.

Sexual selection predicts that in species in which males face intra-sexual competition, males develop traits to be more competitive, for example, larger heads (Bakkegard & Guyer, 2004; Bovero et al., 2003). In contrast, the ecological niche theory predicts that females will evolve larger head sizes to maximize food intake, because females spend proportionally more resources on reproduction (Shetty & Shine, 2002; Shine, 1989). Male *S. perspicillata* exhibited longer heads than females, but relative to head size, females had wider heads in our sample. As our results differed from a previous study (Romano, Bruni, & Paoletti, 2009), which reported more slender heads in females the contradictory patterns of SSD could be explained with the variable ecological parameters at the sample sites (Angelini et al., 2015; Kalezic et al., 1992; Schäuble, 2004; Serra-Cobo, Uiblein, & Martínez-Rica, 2000). Specimens examined herein originated from sites close to the coast of the Tyrrhenian Sea, while Romano, Bruni, and Paoletti (2009) studied a population further inland in the Apennine mountains. As Romano and Ficetola (2010) already demonstrated differences in body size along the distributional range of *S. perspicillata* we cannot rule out the possibility that differences in shape also occur. Most studies on salamanders revealed wider heads in males (Bakkegard & Guyer, 2004; Fontenet & Seigel, 2008; Marvin, 2009) rather than in females (Seglie et al., 2010). From a few observations, it is known that male

spectacled salamanders sometimes face agonistic behavior (Utzeri, Antonelli, & Angelini, 2005; Zuffi, 1999). Spectacled salamanders mainly feed on collembolans (Costa et al., 2015), rather small prey obviously not triggering evolution of larger and wider heads to increase food intake rather than increasing feeding frequency or efficiency. Apparently there is no difference in prey types between the sexes (Costa et al., 2015), supporting sexual selection rather than ecological niche divergence to explain a head shape dimorphism.

We confirmed a longer nasal cavity of male *S. perspicillata*. Already Herre (1935) illustrated crania of *Salamandrina* and pointed out intersexual differences in nasal cavity size but prior to our study this was not investigated further. Salamanders and newts largely depend on olfactory rather than visual cues to find food, receptive conspecifics (e.g., Dawley, 1984; Luthardt & Roth, 1983; Schubert, Houck, Feldhoff, Feldhoff, & Woodley, 2008; Treer et al., 2013), and for homing (e.g., Madison, 1969; Twitty, Grant, & Anderson, 1964). Sever (2003) emphasized the significance of the nasal cavity for the biology of salamanders. An enlarged male nasal cavity of *S. perspicillata* likely is linked to an increased volume of the olfactory epithelium. As male *S. perspicillata* highly depend on olfactory cues for mating (Cedrine & Fasolo, 1971; Dawley, 1984; Schubert et al., 2008) a larger nasal cavity increases olfactory performance (e.g., Dawley, 1992; Dawley & Crowder, 1995). However, some histological or further non-invasive  $\mu$ CT analyses with stained epithelia are needed to confirm our osteological results.

### 4.3 | CONCLUSIONS

SSD in external morphology such as a male-biased cloaca and limb size and larger trunks in females reported herein confirmed earlier findings on other salamanders. Although, the analysis of external and osteological characters revealed a similar pattern, we detected some novel dimorphic characters in osteology previously unknown for salamanders. Especially the functional morphology of a longer nasal cavity and pubis in males and a wider ilium in females should be further investigated.

Patterns of SShD are complex as proportions of different body parts diverged relative to each other (e.g., limbs to trunk), and divergence also occurred within body parts, for instance in bone shape or proportion of the cranium or the limbs. Patterns of SD of both sexes are likely caused by different selective forces to enhance reproductive success. Fecundity selection drives female body size such as trunk length, whereas male traits are likely selected for multiple mates and to increase successful spermatophore transfer. Also, environmental and geographical parameters shape the direction and strength of selection and more recently the heritability of, for example, head shape was demonstrated in plethodontid salamanders (Adams, 2011). Such a variety of factors prevent to, clearly disentangle the selective forces acting on a specific sexually size dimorphic trait.

Taking phylogeny into account, we showed that SSD in selected traits such as cloaca, limb, and body size dimorphism likely evolved in the ancestor of the family Salamandridae (Figure 4). There were some reversals from ancestral female-biased to male-biased body size among newts (e.g., the genera *Ommatotriton* and *Euproctus*). However, for many other traits, the current data is insufficient to draw conclusions. SD in head morphology is much more variable and might respond more sensitively or variably to environmental parameters.

To achieve a comprehensive view of the evolutionary history and patterns of SD among Salamandridae, further taxa should be investigated and more traits must be set into a phylogenetic context. Whereas European salamandrid genera and species received much attention still additional data on the morphologies, reproductive biology and life histories of Asian salamanders are needed to test robust evolutionary hypotheses. Including osteological characters in the analysis of SD gives the opportunity to consider fossil specimens to trace the evolutionary history of dimorphic traits.

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### AUTHOR CONTRIBUTIONS

AK and PP designed the study. PP carried out the  $\mu$ CT scanning, processed the specimens and took all measurements and carried out the statistical analysis. Both authors wrote and approved all versions of the manuscript.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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## Supporting Information

Table S1: Table of specimens examined of Northern spectacled salamanders *Salamandrina perspicillata* from collections of various Natural History Museums in Germany and Switzerland taken for the analysis of SSD and SShD.

Specimens marked with \* were chosen for mCT scanning by random.

Abbreviations: MTKD – Museum für Tierkunde, Dresden; NMBS –

Naturhistorisches Museum Basel; SMF – Senckenberg Naturmuseum,

Frankfurt; SMNS – Staatliches Museum für Naturkunde Stuttgart; ZMB –

Museum für Naturkunde Berlin; ZSM – Zoologische Staatssammlung München:

<b>Institutioncode</b>	<b>Catalognumber</b>	<b>Locality</b>	<b>Sex</b>
MTDK	2815*	N-Italy	f
MTDK	3215*	Genoa	m
MTDK	6467*	Genoa	f
MTDK	6468	Genoa	f
MTDK	6469*	Genoa	f
MTDK	6470*	Genoa	f
MTDK	13760	N-Italy	f
MTDK	13761	N-Italy	m
MTDK	22138*	Cardoso	m
MTDK	22139	Cardoso	m
MTDK	23900*	Cardoso	m
MTDK	33345	Cardoso	m
MTDK	34321	Cardoso	f
MTDK	35355	Grosseto	m
MTDK	35356	Grosseto	f
MTDK	35357	Grosseto	m
MTDK	37078	Cardoso	m
MTDK	43225*	N-Italy	f
MTDK	43226*	N-Italy	m
MTDK	43227	N-Italy	f
MTDK	44565*	Cardoso	m
MTDK	44566*	Cardoso	m
NMBS	361	Genoa	f
NMBS	362	Genoa	f
NMBS	363*	Genoa	f
NMBS	365*	Genoa	m
NMBS	369*	Genoa	m
NMBS	3076*	Genoa	m
NMBS	7232	Genoa	f
NMBS	7560	Genoa	f
SMF	235	Genoa	f
SMF	236*	Genoa	f
SMF	237	Genoa	f
SMF	238*	Genoa	m
SMF	239	Genoa	m
SMF	250	Portofino	m

SMF	251	Portofino	m
SMF	338	Genoa	m
SMF	339	Genoa	f
SMNS	1545.1	Genoa	m
SMNS	1545.2*	Genoa	m
SMNS	1545.3	Genoa	f
SMNS	1545.4	Genoa	f
SMNS	1545.5*	Genoa	f
SMNS	1545.6	Genoa	m
SMNS	1545.7	Genoa	f
ZMB	38238	Genoa	f
ZMB	72342	Genoa	f
ZMB	72343	Genoa	f
ZMB	72344	Genoa	f
ZMB	72345*	Genoa	m
ZMB	72346	Genoa	f
ZMB	72347	Genoa	f
ZMB	72348	Genoa	m
ZMB	72349*	Genoa	m
ZMB	72350	Genoa	m
ZMB	72351*	Genoa	f
ZMB	72352*	Genoa	f
ZMB	72353	Genoa	m
ZMB	72354*	Genoa	f
ZMB	72355*	Genoa	f
ZMB	72356*	Genoa	f
ZMB	72357*	Genoa	m
ZMB	72358*	Genoa	m
ZMB	72359	Genoa	f
ZMB	72360*	Genoa	m
ZMB	72361	Genoa	m
ZMB	72362*	Genoa	m
ZMB	72363*	Genoa	f
ZMB	72364*	Genoa	f
ZMB	72365	Genoa	f
ZMB	72366	Genoa	m
ZMB	72367*	Genoa	f
ZMB	72368	Genoa	f
ZMB	72369	Genoa	m
ZMB	72370*	Genoa	m
ZMB	72371	Genoa	m
ZMB	72372	Genoa	m
ZMB	72373	Genoa	m
ZMB	72374*	Genoa	f
ZMB	72375	Genoa	m
ZMB	72376*	Genoa	f
ZMB	72377*	Genoa	f
ZMB	72378	Genoa	m
ZSM	1251/0/1	Genoa	f
ZSM	1251/0/2	Genoa	m
ZSM	1254/0/1	N-Italy	f
ZSM	1254/0/2	N-Italy	f
ZSM	198/2010*	Cardoso	m
ZSM	199/2010	Cardoso	m
ZSM	864/1920/1*	Genoa	m
ZSM	864/1920/4*	Genoa	f

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Table S2: Analysis of sexual size dimorphism of external morphology of Northern spectacled salamander *Salamandrina perspicillata* (n = 45 males, n = 47 females). Given are the mean (M), standard deviation (SD) and range of 45 characters. P-values from t-tests (two-tailed;  $\alpha = 0.05$ ) are shown in last column. All measurements in mm.

Character	Males (n = 45)		Females (n = 47)		P
	M $\pm$ SD	Range	M $\pm$ SD	Range	
Head					
HL	7.80 $\pm$ 0.46	6.56 - 8.74	7.81 $\pm$ 0.56	6.81 - 9.22	n.s.
HNL	10.88 $\pm$ 0.72	8.45 - 12.26	10.97 $\pm$ 0.90	9.44 - 13.19	n.s.
HW	6.01 $\pm$ 0.30	5.43 - 6.76	6.16 $\pm$ 0.45	5.37 - 7.35	n.s.
HH	2.80 $\pm$ 0.23	2.31 - 3.23	2.86 $\pm$ 0.27	2.35 - 3.77	n.s.
ML	5.37 $\pm$ 0.36	4.38 - 6.23	5.41 $\pm$ 0.45	4.38 - 6.44	n.s.
IND	1.84 $\pm$ 0.24	1.37 - 2.41	1.85 $\pm$ 0.25	1.35 - 2.38	n.s.
EL	2.14 $\pm$ 0.16	1.72 - 2.61	2.14 $\pm$ 0.16	1.74 - 2.54	n.s.
IOD	3.73 $\pm$ 0.25	3.12 - 4.22	3.83 $\pm$ 0.27	3.37 - 4.72	n.s.
END	1.74 $\pm$ 0.15	1.46 - 2.00	1.78 $\pm$ 0.22	1.38 - 2.77	n.s.
SEL	2.13 $\pm$ 0.26	1.44 - 2.67	2.08 $\pm$ 0.23	1.73 - 2.75	n.s.
Trunk					
SVL	34.34 $\pm$ 2.31	29.06 - 39.90	36.34 $\pm$ 3.54	29.00 - 46.10	<.01
TLL	42.87 $\pm$ 4.33	34.00 - 50.31	44.44 $\pm$ 6.66	34.83 - 61.76	n.s.
TTL	77.22 $\pm$ 6.23	64.70 - 88.78	80.87 $\pm$ 9.93	65.37 - 107.60	<.05
AGD	15.83 $\pm$ 1.38	12.35 - 19.87	17.95 $\pm$ 2.33	13.41 - 24.60	<.0001
TRL	20.13 $\pm$ 1.99	16.70 - 26.09	22.42 $\pm$ 3.30	16.97 - 33.40	<.0001
SHW	4.70 $\pm$ 0.57	3.32 - 5.94	4.83 $\pm$ 0.79	3.27 - 7.14	n.s.
CHW	4.23 $\pm$ 0.49	3.16 - 5.23	4.59 $\pm$ 0.80	3.16 - 6.74	<.05
GG	3.65 $\pm$ 0.39	3.05 - 4.75	3.98 $\pm$ 0.55	3.09 - 5.26	<.01
CLL	3.76 $\pm$ 0.39	2.99 - 4.67	3.39 $\pm$ 0.45	2.59 - 4.48	<.0001
CLW	2.20 $\pm$ 0.37	1.36 - 3.11	1.84 $\pm$ 0.34	0.71 - 2.53	<.0001
FSL	2.77 $\pm$ 0.30	2.00 - 3.23	2.50 $\pm$ 0.43	1.77 - 3.54	<.001
TLHB	2.66 $\pm$ 0.23	2.00 - 3.16	2.78 $\pm$ 0.38	1.93 - 3.93	n.s.
TLHM	2.43 $\pm$ 0.24	1.85 - 3.00	2.56 $\pm$ 0.37	1.93 - 3.39	n.s.
TLWB	1.86 $\pm$ 0.14	1.46 - 2.16	1.93 $\pm$ 0.25	1.39 - 2.70	n.s.
TLWM	1.37 $\pm$ 0.12	1.08 - 1.62	1.42 $\pm$ 0.23	1.00 - 2.16	n.s.
Forelimb					
ARM	11.64 $\pm$ 0.95	9.66 - 13.70	11.58 $\pm$ 0.95	10.19 - 14.78	n.s.
UAL	4.16 $\pm$ 0.33	3.47 - 4.93	4.17 $\pm$ 0.34	3.52 - 5.12	n.s.
LAL	4.26 $\pm$ 0.35	3.41 - 5.03	4.23 $\pm$ 0.39	3.48 - 5.50	n.s.
HAL	3.23 $\pm$ 0.40	2.15 - 3.98	3.18 $\pm$ 0.40	2.31 - 4.29	n.s.
UAD	1.25 $\pm$ 0.23	0.73 - 1.64	1.22 $\pm$ 0.24	0.88 - 1.74	n.s.
LAD	1.20 $\pm$ 0.21	0.84 - 1.62	1.14 $\pm$ 0.25	0.62 - 1.92	n.s.
1FL	0.42 $\pm$ 0.08	0.27 - 0.61	0.41 $\pm$ 0.09	0.22 - 0.63	n.s.
2FL	1.66 $\pm$ 0.18	1.29 - 1.98	1.60 $\pm$ 0.16	1.33 - 2.08	n.s.
3FL	2.07 $\pm$ 0.21	1.65 - 2.46	2.02 $\pm$ 0.21	1.64 - 2.57	n.s.
4FL	1.02 $\pm$ 0.17	0.56 - 1.34	1.01 $\pm$ 0.16	0.65 - 1.34	n.s.
Hind limb					
LEG	12.51 $\pm$ 0.90	10.55 - 14.25	12.35 $\pm$ 0.95	10.69 - 15.01	n.s.
ULL	4.10 $\pm$ 0.33	3.33 - 4.74	4.05 $\pm$ 0.38	3.28 - 4.95	n.s.
LLL	4.57 $\pm$ 0.37	3.80 - 5.53	4.60 $\pm$ 0.36	3.79 - 5.42	n.s.
FOL	3.84 $\pm$ 0.38	2.98 - 4.79	3.69 $\pm$ 0.36	3.04 - 4.70	<.05
ULD	1.42 $\pm$ 0.26	0.72 - 2.10	1.36 $\pm$ 0.32	0.78 - 2.21	n.s.
LLD	1.32 $\pm$ 0.20	0.90 - 1.76	1.28 $\pm$ 0.26	0.87 - 1.98	n.s.

2TO	0.53 ± 0.11	0.29 - 0.80	0.51 ± 0.10	0.32 - 0.77	n.s.
3TO	1.73 ± 0.19	1.27 - 2.10	1.66 ± 0.18	1.35 - 2.18	n.s.
4TO	2.43 ± 0.23	1.96 - 2.94	2.35 ± 0.24	1.87 - 2.97	n.s.
5TO	1.30 ± 0.17	0.93 - 1.66	1.27 ± 0.26	0.34 - 1.83	n.s.

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Table S3: Analysis of sexual size dimorphism of external morphology of Northern spectacled salamander *Salamandrina perspicillata* (n = 45 males, n = 47 females) with ANCOVA accounting for general size of the different body parts via first principle component (PC1) from separate PCA's per body part (head, trunk, forelimb, hind limb).

<b>Character</b>	<b>F</b>	<b>P</b>	<b>Sex bias</b>
Head			
HL	1.38	n.s.	–
HW	4.83	<.05	F
HH	0.26	n.s.	–
ML	0.35	n.s.	–
IND	0.37	n.s.	–
EL	0.57	n.s.	–
IOD	2.97	n.s.	–
END	0.05	n.s.	–
SEL	1.89	n.s.	–
Trunk			
SVL	7.01	<.01	F
TLL	0.16	n.s.	–
AGD	45.06	<.0001	F
SHW	2.63	n.s.	–
CHW	2.54	n.s.	–
GG	7.01	<.01	F
CLL	34.2	<.0001	M
CLW	30.5	<.0001	M
FSL	38.4	<.0001	M
TLHB	0.04	n.s.	–
TLHM	0.005	n.s.	–
TLWB	0.2	n.s.	–
TLWM	0.2	n.s.	–
Forelimb			
ARM	2.44	n.s.	–
UAL	1.74	n.s.	–
LAL	0.42	n.s.	–
HAL	0.04	n.s.	–
UAD	0.05	n.s.	–
LAD	1.22	n.s.	–
1FI	0.1	n.s.	–
2FI	3	n.s.	–
3FI	1.13	n.s.	–
4FI	0.11	n.s.	–
Hind limb			
LEG	0.92	n.s.	–
ULL	0.1	n.s.	–
LLL	8.32	<.01	F
FOL	2.27	n.s.	–

ULD	0.07	n.s.	-
LLD	0.19	n.s.	-
1TO	0	n.s.	-
2TO	1.5	n.s.	-
3TO	0.7	n.s.	-
4TO	0.08	n.s.	-

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Table S4: Analysis of sexual size dimorphism of osteology of Northern spectacled salamander *Salamandrina perspicillata* (n = 20 males and females). Given are the means (M), standard deviation (SD) and range of 36 characters. P-values from t-tests (two-tailed;  $\alpha = 0.05$ ) are shown in last column. All measurements in mm.

Character	Males (n = 20)		Females (n = 20)		P
	M $\pm$ SD	Range	M $\pm$ SD	Range	
Cranium					
SL	6.34 $\pm$ 0.36	5.64 - 6.91	6.10 $\pm$ 0.24	5.73 - 6.66	<.05
SW	5.99 $\pm$ 0.33	5.42 - 6.58	5.96 $\pm$ 0.29	5.47 - 6.52	n.s.
MXL	4.30 $\pm$ 0.47	3.79 - 5.85	4.37 $\pm$ 0.22	4.03 - 4.87	n.s.
PMW	2.33 $\pm$ 0.22	2.05 - 2.77	2.38 $\pm$ 0.23	2.00 - 2.84	n.s.
UND	1.33 $\pm$ 0.11	1.15 - 1.55	1.28 $\pm$ 0.20	0.89 - 1.59	n.s.
ND	0.76 $\pm$ 0.07	0.68 - 0.93	0.75 $\pm$ 0.06	0.57 - 0.86	n.s.
NCL	1.86 $\pm$ 0.14	1.63 - 2.16	1.73 $\pm$ 0.13	1.48 - 1.91	<.01
NCW	0.85 $\pm$ 0.12	0.67 - 1.14	0.86 $\pm$ 0.11	0.61 - 1.01	n.s.
OL	2.84 $\pm$ 0.21	2.53 - 3.33	2.71 $\pm$ 0.11	2.57 - 2.93	<.05
Forelimb					
HUL	4.18 $\pm$ 0.19	3.77 - 4.55	3.97 $\pm$ 0.30	3.45 - 4.60	<.05
HUW	1.26 $\pm$ 0.08	1.11 - 1.39	1.26 $\pm$ 0.08	1.10 - 1.50	n.s.
UL	3.03 $\pm$ 0.29	2.52 - 3.40	2.66 $\pm$ 0.36	2.22 - 3.49	<.01
CdhH	0.22 $\pm$ 0.04	0.13 - 0.30	0.25 $\pm$ 0.06	0.16 - 0.35	n.s.
CdhL	0.88 $\pm$ 0.11	0.56 - 1.05	0.92 $\pm$ 0.10	0.72 - 1.10	n.s.
1FPh	0.88 $\pm$ 0.10	0.62 - 1.08	0.83 $\pm$ 0.10	0.70 - 1.04	n.s.
2FPh	2.15 $\pm$ 0.19	1.67 - 2.42	2.05 $\pm$ 0.17	1.65 - 2.34	n.s.
3FPh	2.68 $\pm$ 0.26	2.01 - 3.05	2.49 $\pm$ 0.31	1.54 - 2.95	<.05
4FPh	1.71 $\pm$ 0.14	1.42 - 2.00	1.59 $\pm$ 0.15	1.29 - 1.83	<.01
CAR1	0.59 $\pm$ 0.06	0.43 - 0.72	0.57 $\pm$ 0.06	0.49 - 0.70	n.s.
FPh1.1	0.29 $\pm$ 0.04	0.20 - 0.38	0.27 $\pm$ 0.05	0.18 - 0.35	n.s.
CAR2	1.00 $\pm$ 0.09	0.73 - 1.11	0.92 $\pm$ 0.08	0.74 - 1.08	<.01
FPh2.1	0.62 $\pm$ 0.08	0.47 - 0.70	0.60 $\pm$ 0.06	0.48 - 0.71	<.05
FPh2.2	0.53 $\pm$ 0.07	0.40 - 0.63	0.52 $\pm$ 0.04	0.42 - 0.59	n.s.
CAR3	1.03 $\pm$ 0.09	0.78 - 1.18	0.96 $\pm$ 0.09	0.76 - 1.16	<.05
FPh3.1	0.62 $\pm$ 0.07	0.41 - 0.72	0.56 $\pm$ 0.06	0.38 - 0.66	<.05
FPh3.2	0.53 $\pm$ 0.08	0.37 - 0.66	0.48 $\pm$ 0.09	0.15 - 0.59	n.s.
FPh3.3	0.50 $\pm$ 0.09	0.30 - 0.62	0.49 $\pm$ 0.08	0.25 - 0.58	n.s.
CAR4	0.78 $\pm$ 0.06	0.65 - 0.92	0.73 $\pm$ 0.06	0.65 - 0.86	<.01
FPh4.1	0.49 $\pm$ 0.07	0.29 - 0.60	0.45 $\pm$ 0.06	0.31 - 0.54	n.s.
FPh4.2	0.44 $\pm$ 0.04	0.36 - 0.50	0.40 $\pm$ 0.05	0.29 - 0.48	<.05
Hind limb					
FML	3.84 $\pm$ 0.23	3.32 - 4.26	3.75 $\pm$ 0.19	3.45 - 4.18	n.s.
THL	0.29 $\pm$ 0.04	0.25 - 0.42	0.30 $\pm$ 0.04	0.23 - 0.40	n.s.
FIBL	2.71 $\pm$ 0.16	2.37 - 2.95	2.51 $\pm$ 0.24	2.18 - 3.12	<.01
TSI	0.73 $\pm$ 0.12	0.55 - 1.08	0.71 $\pm$ 0.11	0.43 - 0.84	n.s.
TSB	1.23 $\pm$ 0.17	0.95 - 1.50	1.21 $\pm$ 0.14	1.01 - 1.45	n.s.
2TPh	1.09 $\pm$ 0.09	0.91 - 1.26	1.03 $\pm$ 0.10	0.91 - 1.27	<.05
3TPh	2.41 $\pm$ 0.15	2.05 - 2.69	2.24 $\pm$ 0.17	1.92 - 2.56	<.01
4TPh	3.23 $\pm$ 0.23	2.75 - 3.64	2.93 $\pm$ 0.22	2.50 - 3.31	<.001
5TPh	2.22 $\pm$ 0.17	1.84 - 2.57	2.04 $\pm$ 0.21	1.58 - 2.38	<.01
TAR2	0.74 $\pm$ 0.06	0.64 - 0.85	0.68 $\pm$ 0.06	0.61 - 0.84	<.01
TPh2.2	0.35 $\pm$ 0.05	0.19 - 0.42	0.35 $\pm$ 0.04	0.27 - 0.43	n.s.
TAR3	1.12 $\pm$ 0.08	0.95 - 1.25	1.03 $\pm$ 0.07	0.92 - 1.19	<.001

TPh3.2	0.71 ± 0.05	0.61 - 0.79	0.66 ± 0.06	0.56 - 0.78	<.01
TPh3.3	0.58 ± 0.07	0.41 - 0.66	0.55 ± 0.04	0.45 - 0.62	n.s.
TAR4	1.20 ± 0.08	1.03 - 1.32	1.11 ± 0.08	1.00 - 1.34	<.001
TPh4.2	0.77 ± 0.06	0.64 - 0.89	0.70 ± 0.05	0.58 - 0.78	<.002
TPh4.3	0.66 ± 0.07	0.53 - 0.76	0.58 ± 0.09	0.27 - 0.70	<.01
TPh4.4	0.60 ± 0.08	0.31 - 0.69	0.55 ± 0.10	0.18 - 0.67	n.s.
TAR5	1.02 ± 0.07	0.87 - 1.19	0.95 ± 0.09	0.74 - 1.14	<.01
TPh5.2	0.67 ± 0.06	0.55 - 0.79	0.60 ± 0.09	0.29 - 0.76	<.01
TPh5.3	0.53 ± 0.06	0.36 - 0.60	0.50 ± 0.09	0.20 - 0.63	n.s.
Pelvis					
PVL	2.08 ± 0.12	1.91 - 2.38	2.07 ± 0.09	1.95 - 2.28	n.s.
aPVW	2.87 ± 0.22	2.61 - 3.55	2.78 ± 0.37	1.83 - 3.47	n.s.
pPVW	2.14 ± 0.24	1.68 - 2.54	2.06 ± 0.27	1.68 - 2.58	n.s.
mPVW	1.79 ± 0.16	1.52 - 2.23	1.74 ± 0.20	1.42 - 2.06	n.s.
ISCHW	1.20 ± 0.12	0.93 - 1.43	1.14 ± 0.14	0.80 - 1.42	n.s.
PBW	1.33 ± 0.23	1.09 - 1.80	1.18 ± 0.20	0.64 - 1.53	<.05
ILL	1.75 ± 0.16	1.50 - 2.06	1.70 ± 0.15	1.54 - 2.08	n.s.
ILW	0.62 ± 0.06	0.51 - 0.76	0.68 ± 0.07	0.56 - 0.85	<.01

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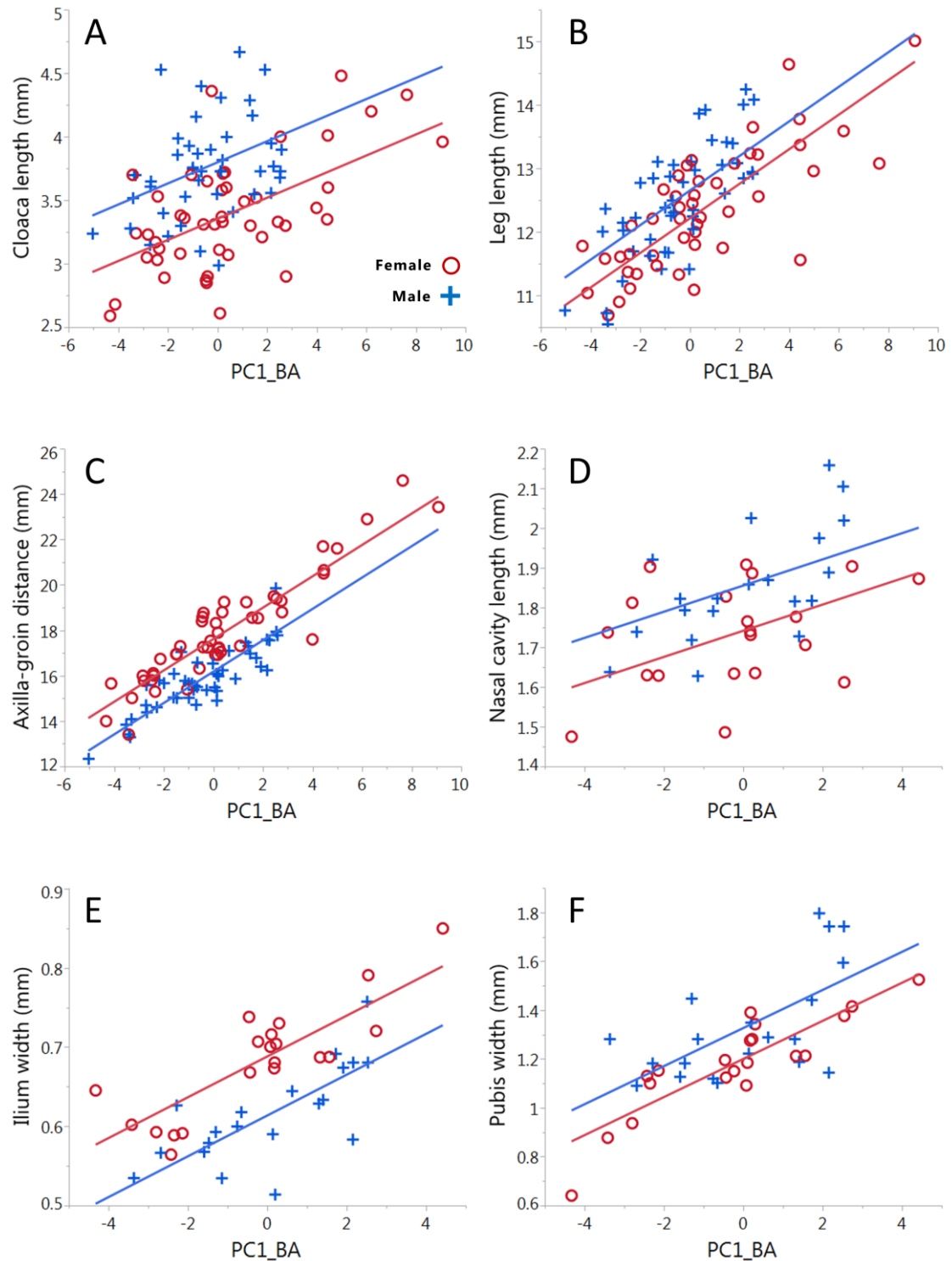
Table S5: Analysis of sexual size dimorphism of osteology of Northern spectacled salamander *Salamandrina perspicillata* (n = 20 males and females) with ANCOVA accounting for general size of the different body parts via first principle component (PC1) from separate PCA's per body part (cranium, forelimb, hind limb, pelvic girdle).

<b>Character</b>	<b>F</b>	<b>P</b>	<b>Sex bias</b>
Cranium			
SL	10.06	<.01	M
SW	4.67	<.05	F
MXL	7.31	<.05	F
PMW	4.37	<.05	F
UND	0.03	n.s.	–
ND	0.03	n.s.	–
NCL	5.72	<.05	M
NCW	0.02	n.s.	–
OL	5.09	<.05	M
Forelimb			
HUL	1.24	n.s.	–
HUW	3.8	n.s.	–
UL	3.71	n.s.	–
CdhH	4.02	n.s.	–
CdhL	4.13	<.05	F
1FPh	0.06	n.s.	–
2FPh	0.24	n.s.	–
3FPh	0	n.s.	–
4FPh	1.63	n.s.	–
Hind limb			
FML	4.66	<.05	F
THL	4.5	<.05	F
FIBL	1.39	n.s.	–
TSI	0.48	n.s.	–
TSB	2.17	n.s.	–
2TPh	0	n.s.	–
3TPh	2.38	n.s.	–
4TPh	7.86	<.01	M
5TPh	1.14	n.s.	–
Pelvis			
PVL	0	n.s.	–
aPVW	0.17	n.s.	–
pPVW	0.21	n.s.	–
mPVW	0	n.s.	–
ISCHW	0.8	n.s.	–
PBW	5.67	<.05	M
ILL	0.2	n.s.	–
ILW	36.61	<.0001	F

Fig. S1: External images of hand (left) and feet (right) of Northern spectacled salamander *Salamandrina perspicillata* with drawn landmarks (dots) and digit length measurements (lines) measured using tpsDig2 and CoordGen8 software to estimate digit length. One grid cell = 0.5 mm



Fig. S2: Scatterplots of some representative sexually dimorphic characters of the external morphology (A – C) and osteology (D – F) in the Northern spectacled salamander *Salamandrina perspicillata*. X-axis represents the first PC of a PCA including representative characters of the entire body to represent a general measure of size of the whole body architecture.



**Additional references supporting figure 4: Phylogeny of Salamandridae with mapped characters of sexual size dimorphism.**

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# Sexual shape dimorphism in the cranium and pelvic girdle of Northern spectacled salamanders, *Salamandrina perspicillata*, investigated via 3D geometric morphometrics

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**Abstract.** Sexual shape dimorphism (SShD) is still a neglected research topic, likely because SShD is often very subtle. Since shape differences between the sexes are difficult to detect by applying traditional morphometrics, only recently-emerged morphometric methodologies such as geometric morphometrics (GM) have highlighted their significance in evolutionary and morphology research. Here we provide an analysis of a three-dimensional morphometric data set of the cranial and, for the first time in a salamander, pelvic osteology of the small terrestrial spectacled salamander *Salamandrina perspicillata*. We also compare our GM results with prior results achieved via traditional linear morphometrics on the same species. Male and female salamanders differed both in cranial and pelvic girdle shape but not in absolute size. The shape but not size differences revealed by GM were congruent with the traditional morphometrics. We discuss intersexual shape differences in an evolutionary context and compare the features of both methods.

**Key words.** Amphibia, Caudata, Salamandridae, sexual dimorphism, sexual selection, osteology, allometry, morphology, traditional morphometrics.

## Introduction

Sexual size dimorphism (SSD), i.e., phenotypic size differences between the sexes, has been investigated at many different taxonomic levels (see FAIRBAIRN et al. 2007). In contrast, research on sexual shape dimorphism (SShD), i.e., differences in morphology between males and females irrespective of size, has only just begun to rise as a new field of research in evolutionary biology (e.g., IVANOVIĆ et al. 2008, GIDASZEWSKI et al. 2009, ALARCÓN-RÍOS et al. 2017). While other phenotypic differences between males and females (e.g., ornamentation, coloration, body size, etc.) are easily detectable, SShD is more subtle (MALMGREN & THOLLESSON 1999, SHETTY & SHINE 2002), requiring denser data collection, larger sample sizes, and more advanced analytical approaches (POGODA & KUPFER 2018). Sexual shape dimorphism is often associated with ecological niche partitioning and life history strategies that differ between the sexes (e.g., HEDRICK & TEMELES 1989, HERREL et al. 1999, SHETTY & SHINE 2002, KUPFER 2007).

Geometric morphometrics (GM) is a highly valuable methodology to fulfil most of the aforementioned requirements and has received much attention by researchers in

the last decades (e.g., ADAMS et al. 2004, KALIONTZOPOULOU 2011). Shape data facilitate new insights into diverse aspects of morphological evolution and ecological adaptations that have led to the variety of morphology noted today (e.g., FAIRBAIRN 1997, ADAMS 2010, IVANOVIĆ et al. 2011, BERTRAND et al. 2019, GRAY et al. 2019). Also, in herpetology, GM is increasingly outcompeting traditional morphometrics via linear measurements (KALIONTZOPOULOU 2011). Geometric morphometrics has been shown to be more capable of assessing subtle shape variation in particular (e.g., BLANCO & GODFREY 2006, ABDEL-RAHMAN et al. 2009, ARENDT 2010, BRENO et al. 2011, SCHMIEDER et al. 2015, ILIĆ et al. 2019), making this method a valuable approach to accurately quantify SShD (ABDEL-RAHMAN et al. 2009, GÓMEZ-VALDÉS et al. 2012, BERNS & ADAMS 2013). The higher sensitivity of GM to shape variations enables researchers to differentiate even between populations of a single species (ADAMS & ROHLF 2000, IVANOVIĆ & KALEZIĆ 2012). The different approaches of the methods may lead to confusion about the meaning of SSD and SShD. In traditional morphometrics, differences within a linear measurement were regularly interpreted as size dimorphism. When a measurement is seen

in relation to the entire body size, or when several length measurements are taken into account, it is often addressed as shape dimorphism. In GM, deviations in landmark configuration, which represent shape data, are interpreted as SShD, whereas SSD is deduced from differences in the centroid size (CS).

Although tailed lissamphibians are a less speciose group than anurans they have developed a remarkable variation in morphology (PETRANKA 1998, SPARREBOOM 2014). In anurans, 90% of species exhibit a female-biased SSD, while in urodeles, this is only the case in about 61% of the species, and nineteen percent of salamanders exhibit a male-biased SSD (SHINE 1979, KUPFER 2007, AMAT 2019). Because salamanders and newts also have diverse reproductive modes and mating systems related to a variety of life history traits, they constitute a highly suited system for the investigation of various forms of sexual dimorphism. As yet, studies on SShD in salamanders are scarce and mainly based on external morphology (e.g., MALMGREN & THOLLESSON 1999, ALCORN et al. 2013, ZHANG et al. 2014, ÜZÜM et al. 2015, ALTUNIŞIK 2017). Typical SSD in urodeles include longer limbs, crania and larger cloacae in males, whereas females exhibit a longer trunk relative to overall body size (e.g., MALMGREN & THOLLESSON 1999, BOVERO et al. 2003, ALCORN et al. 2013, ZHANG et al. 2014, REINHARD & KUPFER 2015, REINHARD et al. 2015, POGODA & KUPFER 2018, XIONG et al. 2019). However, contrary patterns (e.g., SEG-LIE et al. 2010, AMAT et al. 2015, ALARCÓN-RÍOS et al. 2017, CVIJANOVIC et al. 2017) and further, more subtle morphological differences between male and female salamanders have also been reported (e.g., KACZMARSKI et al. 2015). Micro-computed tomography ( $\mu$ CT) enables non-destructive access also to the osteology and combined with GM, closes a gap in morphology research on salamanders and newts with regard to dimorphism (IVANOVIĆ & KALEZIĆ 2012, POGODA & KUPFER 2018). Although this research field is still at the beginning (BROECKHOVEN & DU PLESSIS 2018), the osteology of urodeles still harbours novel patterns of SShD otherwise not quantifiable in external morphology (IVANOVIĆ & KALEZIĆ 2012, POGODA & KUPFER 2018).

In order to reconstruct and understand the evolutionary patterns of sexual dimorphism, phylogenetically basal taxa such as spectacled salamanders (genus *Salamandrina*) in the Salamandridae, the true salamanders, play a key role (ZHANG et al. 2008). Spectacled salamanders comprise two species endemic to the Italian Peninsula. The entire life cycle of metamorphosed individuals is terrestrial, and only females will enter water bodies for oviposition during a short period of the year (ZUFFI 1999). Males take over the active part during the mating season, actively looking for and courting females and are involved in antagonistic interactions (ZUFFI 1999, UTZERI et al. 2005). These differences in life history traits likely lead to differences not only in SSD but also in SShD so far undiscovered. Investigations employing traditional morphometrics already provided valuable hints as to the existence of SShD in *Salamandrina* (ROMANO et al. 2009, POGODA & KUPFER 2018). Especially the pelvic region plays an important role during

reproduction, being essential for egg and spermatophore deposition over and above locomotion. It is therefore quite inexplicable that this region has received so little attention in research on sexual dimorphism.

In order to provide new insights into the intersexual morphological variation of salamanders, we applied a 3D GM approach on the osteology of the cranium and pelvic girdle of the phylogenetically basal *Salamandrina perspicillata* (SAVI, 1821). Second, we wanted to compare our outcome with former results on SShD obtained from linear measurements on the same set of specimens (see POGODA & KUPFER 2018), providing the first comparison of these methods in adult amphibians.

## Material and methods

We studied SShD in the Northern spectacled salamander, *Salamandrina perspicillata*, distributed in the northern part of the Italian Peninsula. For the analysis of SShD in the osteology of *S. perspicillata*, we carried out high-resolution  $\mu$ CT scans with a Bruker SkyScan1272 scanner. Forty specimens of *S. perspicillata*, 20 males and females each, were randomly sampled from various natural history collections (Supplementary Table S1, see also POGODA & KUPFER 2018). Scans of the entire salamander body were performed without a filter at 50 kV and 200 mA at a resolution of 15  $\mu$ m and rotation steps were set at 0.4° with an exposure time of 309 ms per frame. Surface modelling was carried out using the software Amira® 6.2 (Visualisation Science Group). For the 3D GM analyses of SShD, we decided to limit these to the cranium and pelvic girdle as these two body regions exhibit limited kinetic movement and provide valuable structures for landmark settings. To capture the entire shape variation, 43 and 20 three-dimensionally fixed landmarks were digitized by one author on the cranium and pelvic girdle, respectively (Fig. 1) using IDAV Landmark Editor (WILEY et al. 2005a, WILEY et al. 2005b).

As all details of traditional morphometrics can be found in POGODA & KUPFER (2018), we provide only the main details herein. Linear measurements of the osteology were obtained by calculating the Euclidean distance between two landmarks. We calculated nine and eight distances, respectively, for the cranium and the pelvic girdle of *Salamandrina perspicillata* (Table 1). Statistical analyses were carried out using t-test and analysis of co-variance (ANCOVA), correcting either for the body size or the respective body region via the first principal component of a principal component analysis (PCA). The results are summarized in Table 1.

Geometric morphometrics was performed in R version 3.5.3 (R Development Core Team 2019) using the packages geomorph v.3.1.3, RRPP v. 0.4.3 and Morpho v.2.7 (SCHLAGER 2017, COLLYER & ADAMS 2018, ADAMS et al. 2019). A generalized Procrustes alignment (GPA) was carried out by the function 'gpagen' to remove variation due to location, rotation and scale of the individual specimens.

The resultant output is a matrix of shape coordinates – so-called Procrustes coordinates for each landmark and the centroid size (CS) for each specimen (ZELDITCH et al. 2012). Centroid size was calculated as the square root of the summed squared distances of each landmark from the centroid (BOOKSTEIN 1997, ZELDITCH et al. 2012) and is a measure of scale in geometric morphometrics independent of shape. First, we investigated allometric shape changes of shape data on logCS and second, we tested for unique allometry patterns in males and females by multivariate regressions, using the generic function ‘procD.lm’. Significance testing of regressions was performed by permutation procedures with 10.000 iterations as incorporated in the RRPP package (COLLYER & ADAMS 2018, ADAMS et al. 2019). Shape changes were visualized by warping the mean shape to the shapes at the minimum and maximum logCS by a thin-plate spline approach with the function ‘plotRefToTarget’. As both sexes did not show different allometric trajectories, allometry was removed from the shape data for subsequent analysis by transforming the residuals from multivariate regression of shape to logCS, using the generic function ‘procD.lm’ and applying these to the mean shape values. With the allometry-free shape data, a PCA was performed with the function ‘gm.prcomp’

to visualize the occupied morphospaces of the sexes. With the function ‘procD.lm’, we applied a Procrustes ANOVA to test whether males and females differed in their shapes and logCS’. For visualizing shape changes, we warped the overall mean shape to the mean shapes of males and females. Shape changes for the cranium were always magnified by the factor of three and for the pelvic girdle by the factor of two to facilitate visualization.

## Results

Both cranium and pelvis exhibited allometric shape changes (Table 2), but we did not detect different allometric trajectories in males and females (Table 2). Allometric shape changes in the cranium were diverse (Fig. 2), i.e., smaller crania were more roundly shaped, and exhibited a wider neurocranium and more elaborate maxillary bones. Furthermore, the maxillary and premaxillary were more upwardly curved, and the occipital region was directed straight backwards and was much bulkier than in larger crania. Smaller pelvic girdles exhibited a relatively longer and narrower ischiopubis, and smaller ilia appeared in a more vertical position than in larger pelvises (Fig. 3).

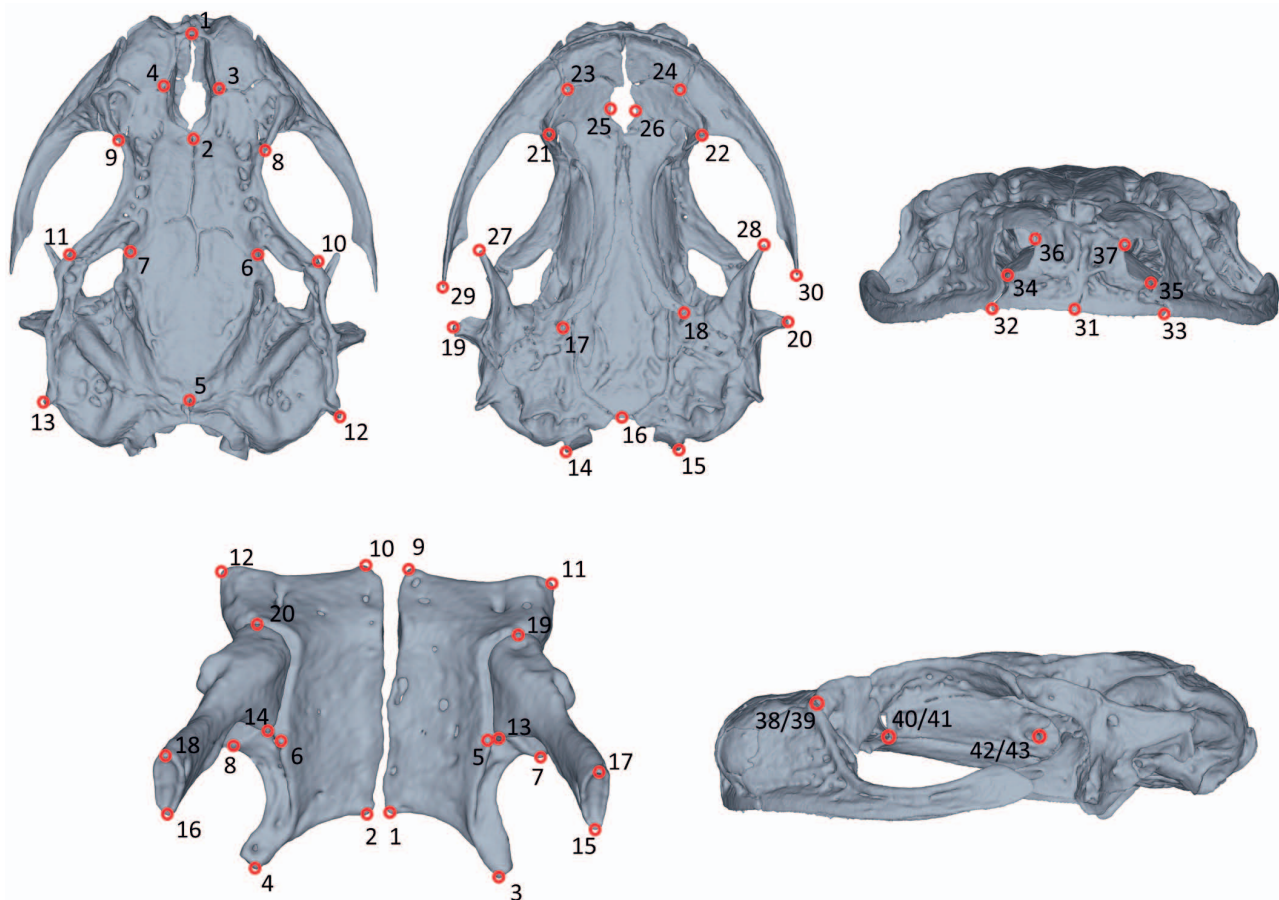


Figure 1. Definition of three-dimensional landmarks (circles) on the cranial and pelvic (lower left) skeleton of Northern spectacled salamanders, *Salamandrina perspicillata*, for the analysis of SShD.

Table 1. Linear morphometric characters of the cranium and pelvic girdle for the osteological analysis of SSD in Northern spectacled salamanders, *Salamandrina perspicillata*, and statistical results as in our prior study (see POGODA & KUPFER 2018). For landmark definition see Figure 1. The respective ANCOVA analyses used as covariate either <sup>1</sup>) PC1 scores of a PCA including only characters of the respective body part to correct for individual size variation in those, or of a <sup>2</sup>) PCA including characters from the entire body to account for individual body size. For more details see POGODA & KUPFER (2018). + marks female-biased characters.

Linear character	Landmarks used	t-test	ANCOVA <sup>1</sup> (PC1 of body part)	ANCOVA <sup>2</sup> (PC1 of entire body)
Cranium				
Cranium length	1; 5	< 0.05	< 0.01	< 0.001
Cranium width	29; 30	n.s.	< 0.05 <sup>+</sup>	n.s.
Maxillary length	29; 32	n.s.	< 0.05 <sup>+</sup>	n.s.
Premaxillary width	32; 33	n.s.	< 0.05 <sup>+</sup>	n.s.
Naris distance at upper edge	36; 37	n.s.	n.s.	n.s.
Naris diameter	34; 36	n.s.	n.s.	n.s.
Nasal cavity length	1; 2	< 0.01	< 0.05	< 0.01
Nasal cavity width	3; 4	n.s.	n.s.	n.s.
Orbit length	10; 39	< 0.05	< 0.05	< 0.05
Pelvic girdle				
Pelvis length	1; 9	n.s.	n.s.	n.s.
Anterior pelvis width	11; 12	n.s.	n.s.	n.s.
Posterior pelvis width	3; 4	n.s.	n.s.	n.s.
Medial pelvis width	5; 6	n.s.	n.s.	n.s.
Ischium width	1; 3	n.s.	n.s.	n.s.
Pubis width	9; 11	< 0.05	< 0.05	< 0.05
Ilium length	13; 15	n.s.	n.s.	n.s.
Ilium width on dorsal condyle	15; 17	< 0.01 <sup>+</sup>	< 0.0001 <sup>+</sup>	< 0.0001 <sup>+</sup>

Table 2. Analysis of static and unique allometry in shape data of the cranium and pelvic girdle of *Salamandrina perspicillata*. Results of multivariate regressions of shape data on logCS and sex. Significant p-values are given in bold.

	Cranium			Pelvic girdle		
	DF	F	P	DF	F	P
Static allometry: shape ~ logCS	1	2.52	<b>0.0003</b>	1	5	<b>&lt; 0.0001</b>
Unique allometry: shape ~ sex*logCS	1	0.87	0.64	1	1.86	0.054

In the PCA morphospace of the cranium, the first two principal components (PC) explained 15.9 and 11.3%, respectively, of the variance (Fig. 4). The sexes occupied separate ranges within the morphospace, although one male specimen deviated particularly strongly from it. Procrustes ANOVA revealed a strong effect of sex on cranial shape but not on logCS (Table 3). Males exhibited a longer snout and occipital region with a narrower neurocranium and less elaborate maxillary bones. Thus, female crania appeared more circularly shaped and the maxillary bones were shorter in males than in females. In the pelvic girdle analysis, the first two PCs explained 26 and 16.2% of the variance, respectively (Fig. 5). Although morphospace ranges of each sex of PC1 and PC2 overlapped widely, the

Procrustes ANOVA revealed a significant shape difference between the sexes (Table 3). The logCS did not differ between males and females, however. The ischiopubis was narrower in females than in males, especially in the anterior part (Fig. 5). Furthermore, the left and right halves of the ischiopubis were arranged in a flat V-shape in males. In the dorsal part of the ilia, males exhibited torsion towards the sagittal plane and were in a more vertical position, whereas in females the ilia were wider at their dorsal condyle.

## Discussion

### Evolution of sexual shape dimorphism

We investigated SShD in the cranium and, for the first time in a salamander, in the pelvic girdle via three-dimensional shape data. Salamandrid salamanders and newts have been shown to exhibit a high evolvability of allometric relations even within a genus (IVANOVIĆ et al. 2007, CVIJANOVIĆ et al. 2014, IVANOVIĆ & ARNTZEN 2017) and species (IVANOVIĆ & KALEZIĆ 2012). When cranial size increases the occipital region will decrease and the size of the facial region increase in vertebrates (HANKEN & HALL 1993) and this is also reflected by our data (Fig. 2). As in newts (IVANOVIĆ & KALEZIĆ 2012), the pattern of cranial SShD of *Salamandrina perspicillata* follows the direction of size-related shape changes. Thus, selection in favour of size in one sex could

cause dimorphism in shape. In *S. perspicillata*, cranial size is relatively longer in males while it is wider in females, suggesting that shape differences are at least partly due to allometric shape changes. In the pelvic girdle, we detected no size dimorphism and hence, other mechanisms must explain the observed shape differences.

Male *S. perspicillata* exhibited a slimmer and longer cranium and shorter maxillaries than females. Concurrent with snout elongation, the nasal cavity was anteriorly found to be enlarged in males. These patterns were confirmed by linear measurements (Table 1, see also POGODA & KUPFER 2018). When compared to the extent of SSD and SShD in other animals, the detected sexual dimorphism in salamanders appears relatively small (see FAIRBAIRN et al. 2007). The driving forces in the evolution of subtle shape differences between males and females are hard to pinpoint in an evolutionary context, as benefits for the one or the other sex are difficult to identify. Often seemingly conflicting findings can complicate interpretations of new discoveries. While ROMANO et al. (2009) found females with more slender heads than males in Northern spectacled salamanders, different ecological parameters at their sample site might have caused slightly different ecological

selection pressures on the investigated population in contrast to the one investigated herein (ROMANO & FICETOLA 2010). This may have led to different directions of evolution of single traits between the sexes (KALEZIĆ et al. 1992, SCHÄUBLE 2004, ANGELINI et al. 2015). When sexual selection is linked to male intrasexual competition, the development of male traits is selected for increasing competitiveness. Competitiveness between amphibians is often associated with larger heads to increase biting performance (BAKKEGARD & GUYER 2004, MARVIN 2009). In spectacled salamanders, the head is longer in males than in females relative to body size. On the other hand, females exhibit relatively wider crania (see POGODA & KUPFER 2018), but the overall cranial size, as defined by CS, does not differ. Nevertheless, males tend to have a slightly larger CS, and allometric shape changes of larger crania are congruent with shape changes in males (see above, Figs 2, 4), indicating selection for competitiveness. Antagonistic behaviour has occasionally been observed in male spectacled salamanders (ZUFFI 1999, UTZERI et al. 2005), but it is unknown whether this behaviour is of importance in terrestrial salamanders and whether elongated snouts are of advantage in antagonistic male-male competition still await experimen-

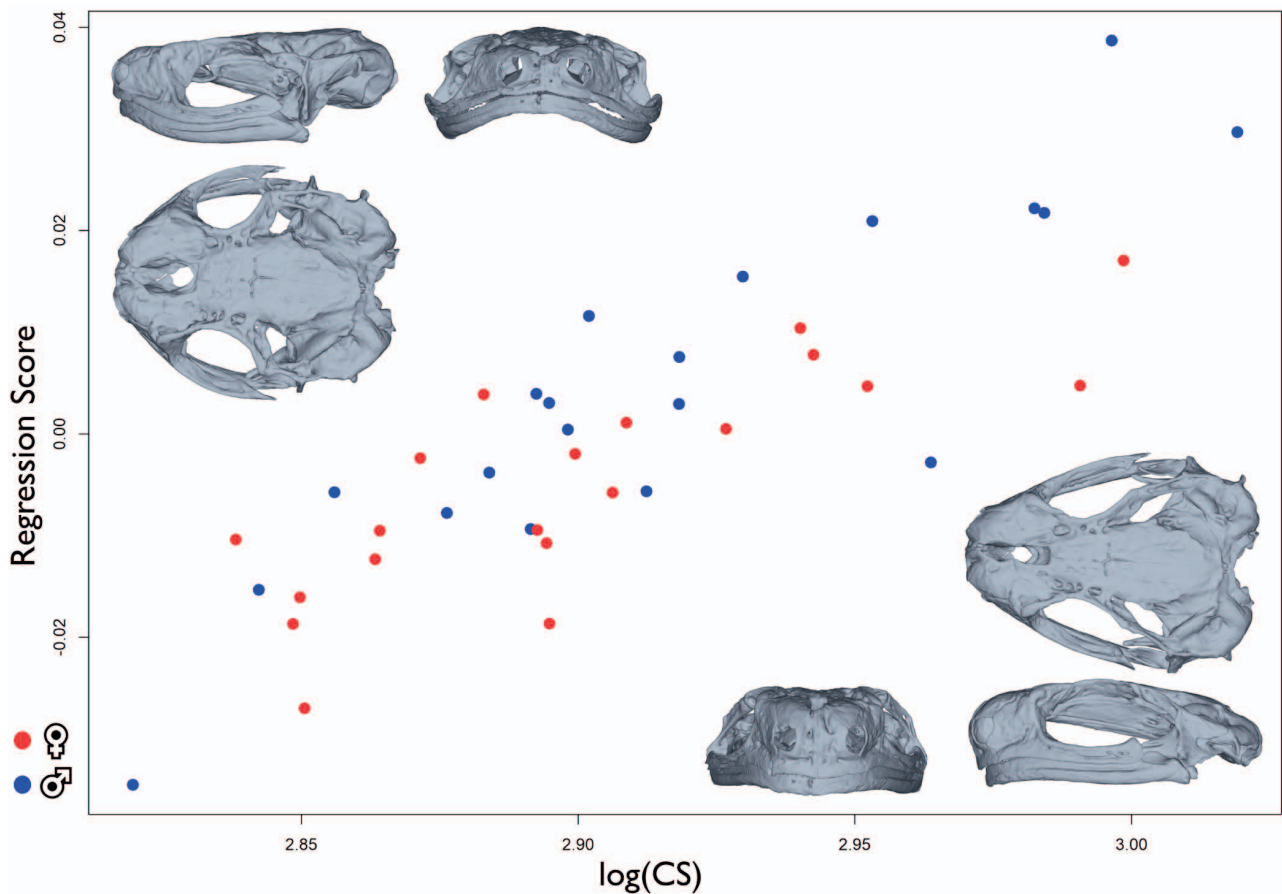


Figure 2. Common allometry estimated by multivariate regression of cranial shape on cranial size (as  $\log(\text{CS})$ ) of Northern spectacled salamanders, *Salamandrina perspicillata*. The shape for the smallest (upper left) and largest (lower right) values of cranial size ( $\log(\text{CS})$ ) were visualized from the mean shape as warped 3D-meshes. The shape changes are magnified by the factor of three.

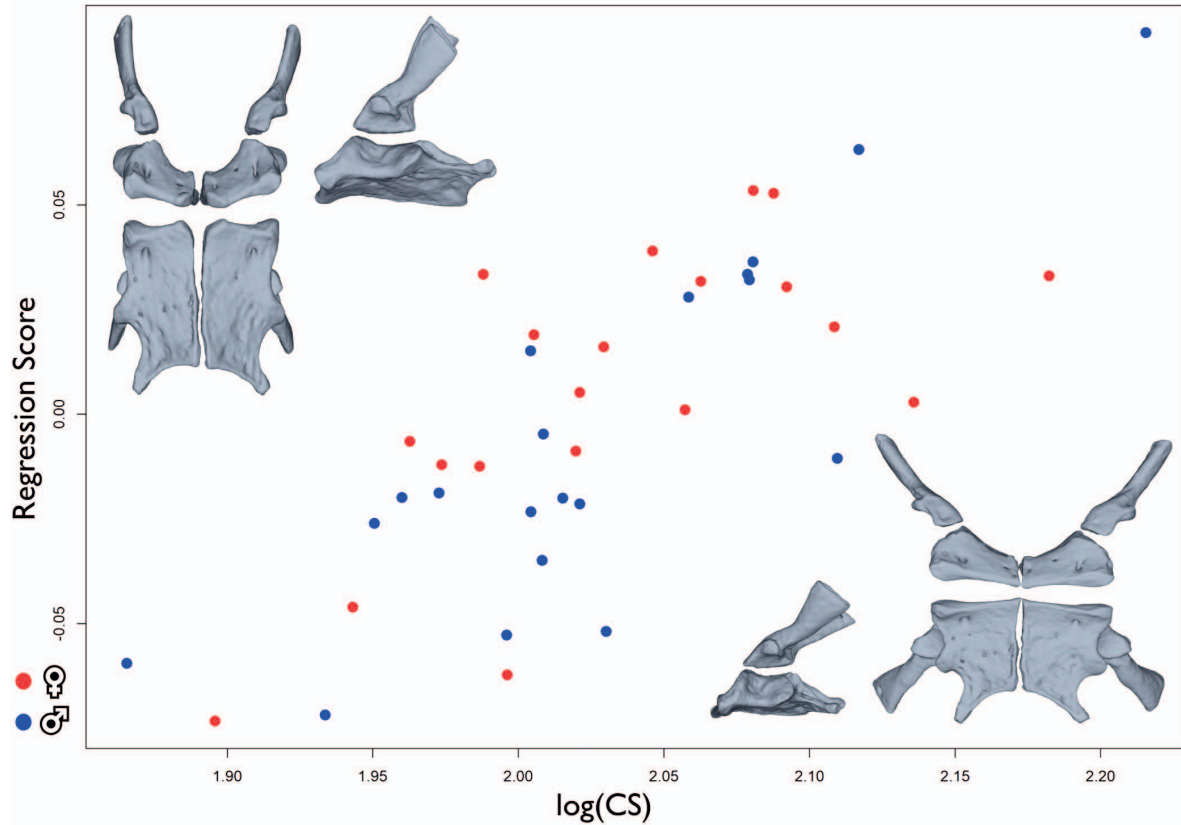


Figure 3. Common allometry estimated by multivariate regression of pelvis shape on pelvis size (as  $\log(\text{CS})$ ) of Northern spectacled salamanders, *Salamandrina perspicillata*. The shape for the smallest (upper left) and largest (lower right) values of pelvis size ( $\log(\text{CS})$ ) were visualized from the mean shape as warped 3D-meshes. The shape changes are magnified by the factor of two.

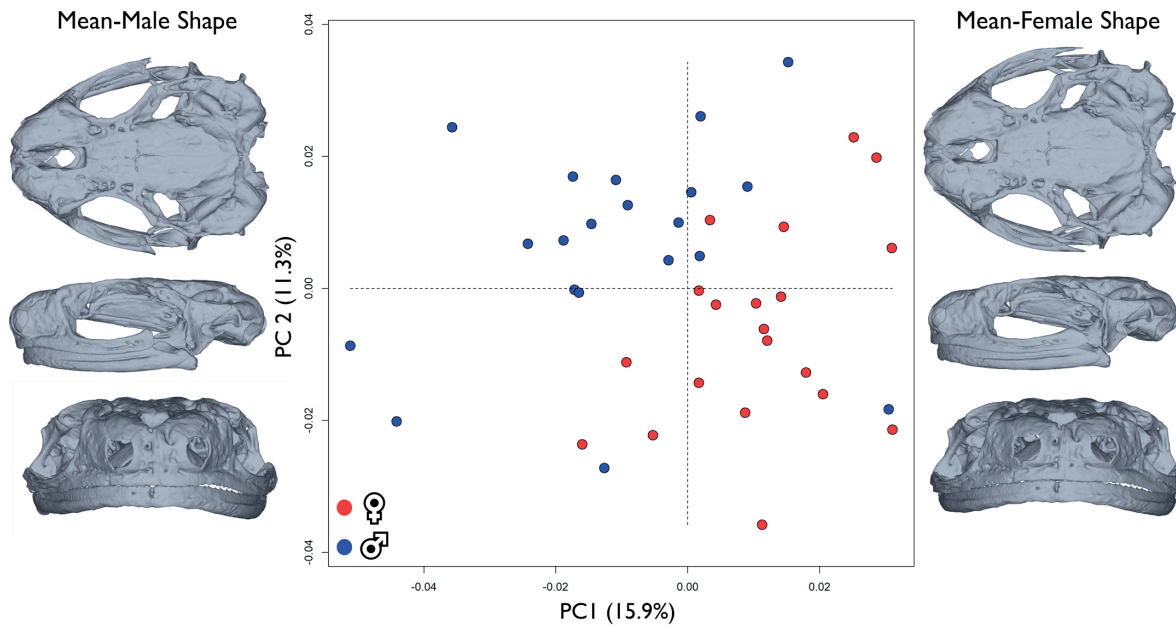


Figure 4. Morphospace for cranial shape data in the Northern spectacled salamander, *Salamandrina perspicillata*, built by the first and second axes of the principal component analysis of 43 three-dimensional landmarks. The mean shape for males (left) and females (right) were visualized from the mean shape as warped 3D-meshes. The shape changes are magnified by the factor of three.

Table 3. Analysis of SShD in shape data of the cranium and pelvic girdle of *Salamandrina perspicillata*. Results of ANOVA of sex on shape and logCS, respectively. Significant p-values are given in bold.

	Cranium			Pelvic girdle		
	DF	F	P	DF	F	P
shape ~ sex	1	3.35	<b>&lt; 0.0001</b>	1	3.45	<b>0.0012</b>
logCS ~ sex	1	1.04	0.3	1	0.13	0.7

tal evidence. On the other hand, the development of cranial elongation could be associated with an elongated nasal cavity partly accommodating the vomeronasal organ (e.g., DAWLEY 1992, ALCORN et al. 2013). A larger nasal cavity might increase olfactory performance of males and thus, increase their ability to find receptive females (DAWLEY 1984, SCHUBERT et al. 2008, MARVIN 2009). Also, cranial SShD in *S. perspicillata* could be related to ecological niche divergence, i.e., when males and females use different food sources, minimizing intersexual competition (e.g., SHINE 1989). Currently, ecological niche divergence is still awaiting evidence in *Salamandrina* (COSTA et al. 2015).

Intersexual shape differences in the pelvic girdle are in accordance with our previous findings from using traditional morphometrics (Table 1, POGODA & KUPFER 2018). Females exhibited a wider dorsal condyle of the ilium,

while males exhibited a wider pubis. Additional shape differences were disclosed by GM (Fig. 5) – steeper ilia and a more V-shaped arrangement of the ischiopubis in males. The tilted female ilia might contribute to the wider groin width found in external morphology (POGODA & KUPFER 2018). Pelvis shape likely is adapted to different reproductive requirements in males and females, i.e., males deposit spermatophores on land, while females deposit eggs on stones and plant material in lentic and lotic water bodies. The male cloacal glands producing the spermatophores may require an enlarged vent, while the tilted ilia in females may provide a larger parturient canal to facilitate egg passage. A wider ilia condyle in association with adapted femur shape (see POGODA & KUPFER 2018) may accommodate different mechanical requirements for aquatic egg deposition and more space for limb muscle attachment, as aquatic movement requires more resources than moving in terrestrial habitats.

### Tradition and modernism

We carried out the first comparative analysis of a salamander osteology dataset gathered via traditional and 3D geometric morphometrics. Both methods yielded similar results regarding SShD, but the outcome concerning SSD was different. Traditional morphometrics revealed a longer cranium in males, whereas GM did not reveal differences in CS. This is likely attributable to the different calcula-

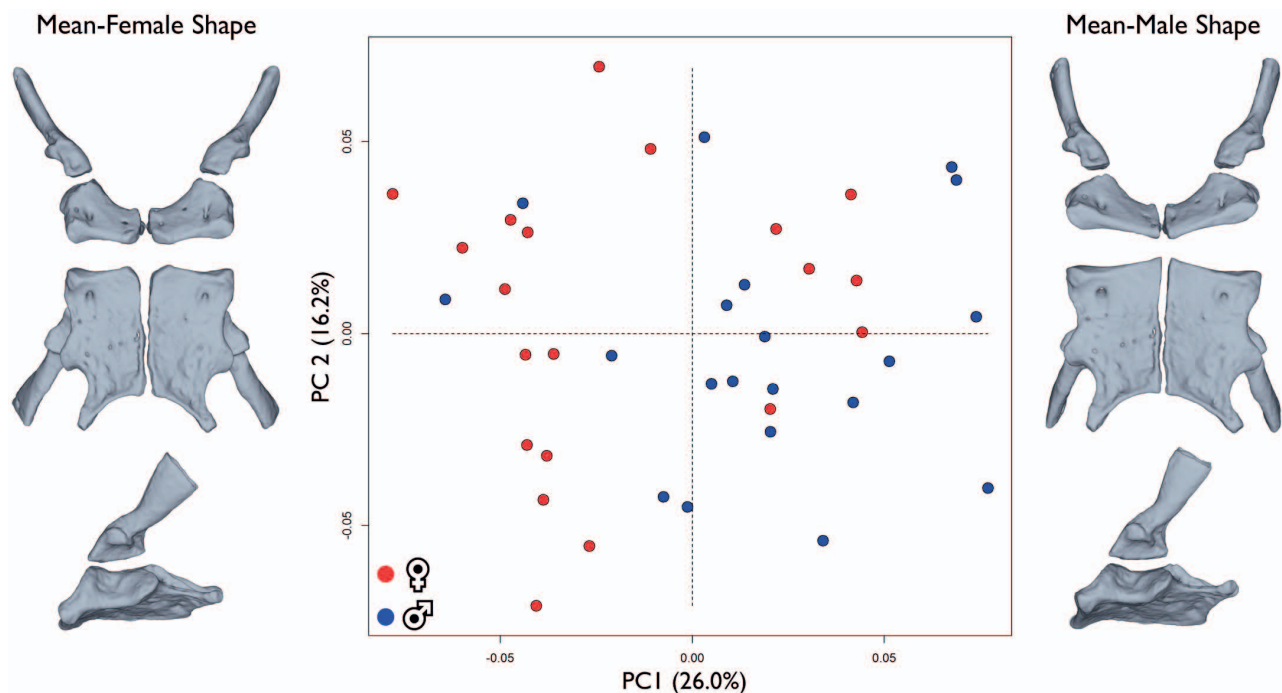


Figure 5. Morphospace for pelvis shape data in the Northern spectacled salamander, *Salamandrina perspicillata*, built by the first and second axes of the principal component analysis of 20 three-dimensional landmarks. The mean shape for males (right) and females (left) were visualized from the mean shape as warped 3D-meshes. The shape changes are magnified by the factor of two.

tions and perspectives of the methods. In the traditional approach, the statement about a larger cranium is based on one or two linear measurements, while GM takes into account the entire landmark configuration and thus, the entire morphology of the single body region to estimate CS. The question is here whether, e.g., a longer cranium is also a larger one. The statistical outcome from linear morphometrics depends also on the statistical approach. In our prior study, using linear measurements, we also corrected for overall body part size and body size. Therefore, we used the first PC of a PCA including all measurements of the respective body part and body (see POGODA & KUPFER 2018). If all characters are correlated positively with the first PC it will generally be interpreted as a size component. Cranial width turned out female-biased relative to cranial size but not to body size (Table 1), demonstrating that size and shape differences are very complex and obviously difficult to differentiate between. Both methods substantially depended on the selection of measured distances and landmarks, respectively (e.g., ARENDT 2010). Including more linear measurements into a dataset to capture the entire shape variation would likely represent a higher workload than the digitizing of additional landmarks for GM analyses. Furthermore, the measurement error is probably higher when using linear morphometrics, as the specimen has to be handled differently for every measurement, while in GM, once the specimen is digitized, either by, e.g., a standardized photograph or CT scan, one has not to worry about altering the morphology of the specimen. This underlines the superiority of GM versus linear morphometrics in gathering even subtle shape differences (e.g., ADAMS & ROHLF 2000, ARENDT 2010, GABELAIA et al. 2018, this study). While 2D GM can be easily achieved by photographs, 3D GM depends to a large extent on CT-scanning, which is still relatively expensive and time-consuming, even though 3D images of external morphology can be deduced from photographs (GABELAIA et al. 2018). Nevertheless, this leads to the focus of 3D GM on osteology (e.g., CLAUDE et al. 2004, GRAY et al. 2017, IVANOVIĆ & ARNTZEN 2017, BERTRAND et al. 2019) while external morphology is mostly covered by 2D GM (e.g., ARENDT 2010, SCHMIEDER et al. 2015, ILIĆ et al. 2019, POGODA et al. 2020).

Geometric morphometrics is nowadays the method of choice in morphology research, because it is more accurate and facilitates the handling of more specimens. Linear measurements can be extracted easily from landmark data as well. If one considers both traditional and geometric morphometrics with adequate data, conclusions on shape differences are expected to be consistent. Concerning size differences of single body parts in relation to body size, GM exhibits some weaknesses. A combination of both methods could yield further advances in the research of sexual dimorphism. If morphometric measurements such as snout-vent length will be included into the analysis of shape data, further information on the relations of the shapes of single body parts up to the entire body are possible, leading to new and broader insights into morphological variation of the species investigated.

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### Supplementary data

The following data are available online:

Supplementary Table S1. Table of specimens examined of northern spectacled salamanders *Salamandrina perspicillata* (SAVI, 1821) for the analysis of SSHD.

**Supplementary Table S1.** Table of specimens examined of northern spectacled salamanders *Salamandrina perspicillata* (SAVI, 1821) for the analysis of SShD. Abbreviations: MTKD – Museum für Tierkunde, Dresden; NMBS – Naturhistorisches Museum Basel; SMF – Senckenberg Naturmuseum, Frankfurt am Main; SMNS – Staatliches Museum für Naturkunde Stuttgart; ZMB – Museum für Naturkunde Berlin; ZSM – Zoologische Staatssammlung München.

Institutioncode	Catalognumber	Locality	Sex
MTDK	2815	N-Italy	f
MTDK	3215	Genoa	m
MTDK	6467	Genoa	f
MTDK	6469	Genoa	f
MTDK	6470	Genoa	f
MTDK	22138	Cardoso	m
MTDK	23900	Cardoso	m
MTDK	43225	N-Italy	f
MTDK	43226	N-Italy	m
MTDK	44565	Cardoso	m
MTDK	44566	Cardoso	m
NMBS	363	Genoa	f
NMBS	365	Genoa	m
NMBS	369	Genoa	m
NMBS	3076	Genoa	m
SMF	236	Genoa	f
SMF	238	Genoa	m
SMNS	1545.2	Genoa	m
SMNS	1545.5	Genoa	f
ZMB	72345	Genoa	m
ZMB	72349	Genoa	m
ZMB	72351	Genoa	f
ZMB	72352	Genoa	f
ZMB	72354	Genoa	f
ZMB	72355	Genoa	f
ZMB	72356	Genoa	f
ZMB	72357	Genoa	m
ZMB	72360	Genoa	m
ZMB	72362	Genoa	m
ZMB	72363	Genoa	f
ZMB	72364	Genoa	f
ZMB	72367	Genoa	f
ZMB	72370	Genoa	m
ZMB	72373	Genoa	f
ZMB	72374	Genoa	f
ZMB	72376	Genoa	f
ZMB	72377	Genoa	f
ZSM	198/2010	Cardoso	m
ZSM	864/1920/1	Genoa	m
ZSM	864/1920/4	Genoa	f

# Cranial shape evolution of extant and fossil crocodile newts and its relation to reproduction and ecology

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## Abstract

The diversity of the vertebrate cranial shape of phylogenetically related taxa allows conclusions on ecology and life history. As pleurodeline newts (the genera *Echinotriton*, *Pleurodeles* and *Tylototriton*) have polymorphic reproductive modes, they are highly suitable for following cranial shape evolution in relation to reproduction and environment. We investigated interspecific differences externally and differences in the cranial shape of pleurodeline newts via two-dimensional geometric morphometrics. Our analyses also included the closely related but extinct genus *Chelotriton* to better follow the evolutionary history of cranial shape. *Pleurodeles* was morphologically distinct in relation to other phylogenetically basal salamanders. The subgenera within *Tylototriton* (*Tylototriton* and *Yaotriton*) were well separated in morphospace, whereas *Echinotriton* resembled the subgenus *Yaotriton* more than *Tylototriton*. Oviposition site choice correlated with phylogeny and morphology. Only the mating mode, with a random distribution along the phylogenetic tree, separated crocodile newts into two morphologically distinct groups. Extinct *Chelotriton* likely represented several species and were morphologically and ecologically more similar to *Echinotriton* and *Yaotriton* than to *Tylototriton* subgenera. Our data also provide the first comprehensive morphological support for the molecular phylogeny of pleurodeline newts.

## KEYWORDS

*Chelotriton*, cranium, *Echinotriton*, fossil salamander, geometric morphometrics, paleoecology, reproductive biology, shape evolution, *Tylototriton*

## 1 | INTRODUCTION

One of the most complex structures in tetrapods is the cranium, an almost static component. It is composed of various different bones that fuse during ontogeny to build a robust capsule for most of the sensory organs and functional units for various tasks such as food intake and the perception of the environment. This variety

of tasks and the structural design forced and allowed vertebrates to evolve very distinct cranial morphologies. Accordingly, the analogous evolution of similar shapes in phylogenetically non-related taxa can be used to draw conclusions on their ecology, especially the niches they occupy (e.g. Herrel et al., 2004; Dean et al., 2007; Herrel et al., 2008). Despite its enormous morphological variation, the cranial skeleton still allows conclusions on a species'

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ecology, e.g. the dentition is usually closely related to the diet (Hotton, 1955; Strait, 1993), and this relation is of special importance for the reconstruction of ancient lineages. As the skeleton usually represents the only remains in the fossil record, it is most promising to carry out comparative osteology that includes extant taxa to draw conclusions about the ecology of extinct vertebrates. Furthermore, by linking morphological traits of extant species to their environment, ecology and life history in a comparative way, it is possible to obtain insights into the evolutionary history of extinct taxa. For example, patterns of countershading in extant species allow conclusions to be made concerning predator-prey interactions or the habitat of dinosaurs (Brown et al., 2017; Smithwick et al., 2017).

True salamanders of the family Salamandridae evolved a variety of cranial shapes (Ivanović and Arntzen, 2017). The taxon of Pleurodelini, often referred to as 'primitive newts', represent a basal group of Salamandridae comprising three extant genera (Zhang et al., 2008; Veith et al., 2018). Whereas the three species of ribbed newts, genus *Pleurodeles* Michahelles, 1830, inhabit a restricted range in southwest Europe and North Africa along the Mediterranean, crocodile newts are much more diverse and are currently assigned to two genera. *Echinotriton* Nussbaum and Brodie, 1982 comprises three species, inhabiting the Ryu-Kyu archipelago, Japan and east China (Chang, 1932; Hou et al., 2014). *Tylosotriton* Anderson, 1871 includes 25 species divided into two subgenera: *Tylosotriton* Anderson, 1871 and *Yaotriton* Dubois and Raffaelli, 2009 (Dubois and Raffaelli, 2009). *Tylosotriton* is widely distributed from east Nepal to east and central China, southwards to Myanmar, central Vietnam, Laos and Thailand (Wang et al., 2018; Zaw et al., 2019). Apparently, crocodile newts have a quite conservative morphological evolution (Hernandez et al., 2018), leading to a high number of species mainly recognized by genetic studies in recent years. On the other hand, distinct diagnostic morphological characters are only sparsely available (e.g. Nishikawa et al., 2013; Phimmachak et al., 2015a; Qian et al., 2017; Grismer et al., 2018; Zaw et al., 2019).

Pleurodeline newts are polymorphic in their reproductive mode and mating strategy (Kieren et al., 2018), including terrestrial and aquatic mating, as well as the choice of oviposition sites (Kuzmin et al., 1994; Ziegler et al., 2008; Igawa et al., 2013; Bernardes et al., 2017). Whereas some species use a ventral amplexus similar to terrestrial salamandridae, others perform a circular mating dance comparable to European newts (Dasgupta, 1994; Roy and Mushahidunnabi, 2001; Fleck, 2010a; 2010b; Wang et al., 2017; Gong et al., 2018). Ribbed and crocodile newts occupy various habitats along the latitudinal and altitudinal gradient from tropical lowland rainforests to montane forests and grassy landscapes (Bernardes et al., 2013; Hernandez et al., 2017; Hernandez et al., 2019). Their diverse ecology may result in indistinct morphological adaptations hard to access with traditional morphological approaches. Additionally, pleurodeline newts are represented by several fossil taxa. Three extinct species of *Tylosotriton* were described from Germany (Noble, 1928; Herre, 1935; 1949), today being recognized as members of other fossil newt genera (Estes,

1981; Nussbaum and Brodie, 1982; Böhme and Ilg, 2003). The most prominent one is the genus *Chelotriton* Pomel, 1853, currently consisting of four nominally described species (Goldfuss, 1831; Pomel, 1853; Westphal, 1980; Bailon, 1989) of which *Chelotriton paradoxus* is the best known. *Chelotriton* is known from Spain to east Europe from the Eocene to Miocene (about 50–11 mya). Based on unique morphological characters, *Chelotriton* was assigned to the tribe Pleurodelini by various authors and is regarded as more closely related to crocodile newts, i.e. the genera *Echinotriton* and *Tylosotriton*, than to *Pleurodeles* (Marjanović and Witzmann, 2015; Schoch et al., 2015). In recent years, several exceptionally well-preserved specimens of *Chelotriton* have been excavated from localities in southwest Germany (Figure 1; Roček and Wuttke, 2010; Schoch et al., 2015).

Via two-dimensional (2D) geometric morphometrics of external head and skull morphology, accessed via micro-computed tomography ( $\mu$ CT) scans, we investigated how cranial shape of ribbed and crocodile newts differs interspecifically. We tested how cranial morphology relates to selected ecological and reproductive traits. Fossil *Chelotriton* specimens from deposits of Randeck Maar and Enspel Crater Lake were included in the analyses of cranial morphology to obtain further hints on the relationship between extant and extinct taxa and to draw conclusions on the ecology of *Chelotriton* based on morphology–ecology correlations of extant taxa. The overall aim was to obtain novel insights into the evolution of cranial shape in relation to ecology of selected, phylogenetically basal salamandrids.

## 2 | MATERIALS AND METHODS

We have investigated the crania of 157 newt specimens covering 21 of 31 extant species (68%) which are currently ascribed to the Pleurodelini (Frost, 2018). With additional data from the literature, we covered information on up to 26 species (see below and Table 1). As populations of *Echinotriton andersoni* originating from the island of Okinawa and the Amami archipelago showed deep divergence and are under debate as to whether they should be recognized as distinct taxonomic units (Hayashi et al., 1992; Honda et al., 2012; Kurabayashi et al., 2012), we treated those separately in our analyses. To exclude additional variation due to sexual dimorphism in extant species, only male specimens were analysed, except for a photograph of the *Echinotriton maxiquadratus* holotype, which is a female. Additionally, eight well-preserved fossil specimens of the genus *Chelotriton* from deposits of the Randeck Maar, Baden-Wuerttemberg (17–15 Ma, mammal zone MN5, see Böhme, 2003; Rasser et al., 2013) and Enspel Crater lake, Rhineland-Palatine (24.8–24.6 Ma, mammal zone MP28, see Roček and Wuttke, 2010; Schindler and Wuttke, 2010), both in Germany, were included in the analysis (Figure 1; Schoch et al., 2015). Currently, specimens of *Chelotriton* from these deposits are tentatively associated with the type species *C. paradoxus* (Schoch et al., 2015).

**FIGURE 1** Well-preserved cranial skeletons of fossil *Chelotriton* specimens from the Enspel crater lake, Rhineland-Palatine (a,b) and the deposits of the Randeck Maar, Baden-Wuerttemberg (c), Germany



## 2.1 | Landmark data acquisition

We investigated the cranial shape of pleurodeline salamanders by 2D geometric morphometric (GM) approaches. Two-dimensional analysis was preferred over three-dimensional analysis as the crania of fossils newts were too flat to apply 3D GM for comparison between extant and extinct samples (see below). First, we took standardized pictures of the extant representatives in dorsal and right lateral view of the cranium (Figure 2). As we could not access all representative species, we further searched in the literature for suitable images of crocodile newts. Figures from published work were extracted for *E. maxiquadratus* from Hou et al. (2014), *Tylotriton anguliceps* from Le et al. (2015), *Tylotriton broadoridgus* and *Tylotriton liuyangensis* from Yang et al. (2014), *Tylotriton ngarsuensis* from Grismer et al. (2018), *Tylotriton notialis* from Stuart et al. (2010), a topotypic *Tylotriton verrucosus*, holotype of *Tylotriton shanorum* and *Tylotriton uyenoii* from Nishikawa et al. (2014) and *Tylotriton kachinorum* from Zaw et al. (2019).

Second, extant and fossil specimens were scanned via  $\mu$ CT to allow investigation of the cranial skeleton. Scans were carried out either with a Bruker SkyScan1272 or within the X-ray imaging laboratory at the Institute for Photon Science and Synchrotron Radiation, Karlsruhe Institute of Technology (KIT), employing a microfocus X-ray tube (XWT-225, X-RAY WorX) and a flat panel detector (XRD 1621 CN14 ES, Perkin Elmer) in combination with a custom-designed mechanical sample manipulator. For the datasets measured at KIT, OCTOPUS 8.6 (Inside Matters) was used to perform the tomographic reconstruction. Due to the time-consuming procedure of  $\mu$ CT scanning, only a subsample per species

was scanned (Table 1, Supporting Information Table S1). In some species with larger distribution areas or presumably different morphologies among localities, additional specimens were analyzed via  $\mu$ CT. In total, 121 specimens including fossils were  $\mu$ CT-scanned (Table S1). The scan resolution for extant newts was either 20.1 (SkyScan) or 21.3  $\mu$ m (KIT-scanner). *Chelotriton* specimens were scanned at 35.2  $\mu$ m resolution. Three-dimensional reconstructions were processed in AMIRA® 6.5 (Visualisation Science Group). Flattened and distorted during fossilization, *Chelotriton* specimens did not allow sufficient reconstruction in a three-dimensional space. Nevertheless, to reconstruct a morphology which is most likely to represent its original dorsal shape, retrodeformation by algorithmic symmetrization using the software IDAV LANDMARK EDITOR v.3.7 (<http://graphics.idav.ucdavis.edu/research/EvoMorph>) was performed to reduce asymmetrical distortion in the fossil crania of the Enspel specimens (Tallman et al., 2014). Landmark configurations for retrodeformation were specifically adapted to each single fossil specimen, as deformation was different in each specimen. We also employed several retrodeformations with different sets of landmarks to receive results appearing as symmetrical as possible but simultaneously not diverging too much from the original shape (Supporting Information Figure S1). The only sample from Randeck Maar appears to be symmetrical and was therefore not retrodeformed. Two-dimensional images of skulls were taken in dorsal view and, additionally, skull images of extant taxa were taken in the right lateral view (Figure 2) to allow comparison with external morphology. In the following, 'head shape/morphology' will refer to external cranial morphology including soft tissue, 'skull shape/morphology' to the osteology, and

**TABLE 1** Sample sizes per species of pleurodeline salamandrids for 2D geometric morphometrics analyses of cranial morphology

Species	n – external	n – osteology
<i>Echinotriton andersoni</i> – Okinawa	8	5
<i>Echinotriton andersoni</i> – Amami	4	3
<i>Echinotriton maxiquadratus</i>	1*	–
<b><i>Tylototriton (Tylototriton) anguliceps</i></b>	5*	2
<b><i>Tylototriton (Yaotriton) asperrimus</i></b>	6	7
<b><i>Tylototriton (Yaotriton) broadoridgus</i></b>	1*	–
<i>Tylototriton (Yaotriton) hainanensis</i>	1	1
<i>Tylototriton (Tylototriton) himalayanus</i>	12	9
<b><i>Tylototriton (Tylototriton) kachinorum</i></b>	7*	–
<i>Tylototriton (Tylototriton) kweichowensis</i>	10	5
<b><i>Tylototriton (Yaotriton) liuyangensis</i></b>	1*	–
<i>Tylototriton (Yaotriton) lizhenchangi</i>	3	2
<b><i>Tylototriton (Tylototriton) ngarsuensis</i></b>	2*	–
<b><i>Tylototriton (Yaotriton) notialis</i></b>	2*	1
<i>Tylototriton (Yaotriton) panhai</i>	3	3
<b><i>Tylototriton (Tylototriton) panwaensis</i></b>	3	3
<i>Tylototriton (Tylototriton) podichthys</i>	3	3
<i>Tylototriton (Tylototriton) shanjing</i>	13	9
<b><i>Tylototriton (Tylototriton) shanorum</i></b>	6*	4
<i>Tylototriton (Tylototriton) taliangensis</i>	10	10
<b><i>Tylototriton (Tylototriton) uyenoi</i></b>	13*	9
<i>Tylototriton (Tylototriton) verrucosus</i>	17*	14
<i>Tylototriton (Yaotriton) vietnamensis</i>	11	8
<i>Tylototriton (Yaotriton) wenxianensis</i>	7	5
<i>Tylototriton (Tylototriton) yangi</i>	2 <sup>+</sup>	–
<i>Tylototriton (Yaotriton) zieglerei</i>	10	5
<i>Pleurodeles waltl</i>	5	5
<i>Chelotriton Enspel</i>	–	7
<i>Chelotriton Randeck</i>	–	1

\*Species where pictures were additionally taken from literature.

<sup>+</sup>Species where only life specimens were available. Species where holotype and/or paratype material is also included are given in bold.

'cranial shape/morphology' more generally to cranial morphology irrespective of the dataset analyzed herein.

For the analyses of head morphology, we digitized 16 landmarks and 20 semilandmarks in dorsal view and seven landmarks and 20 semilandmarks in lateral view. On skull images, 22 landmarks and 60 semilandmarks in dorsal view and 10 landmarks and 20 semilandmarks in lateral view were digitized (Figure 2, Supporting Information Table S2). Landmark digitization was carried out by one author using tpsUtil and tpsDig (Rohlf, 2016a; 2016b). Specimens were randomly shuffled. To test for accuracy of landmark placement, each landmark configuration was tested by digitizing one specimen five times and five other specimens of the same species. Procrustes distance to the

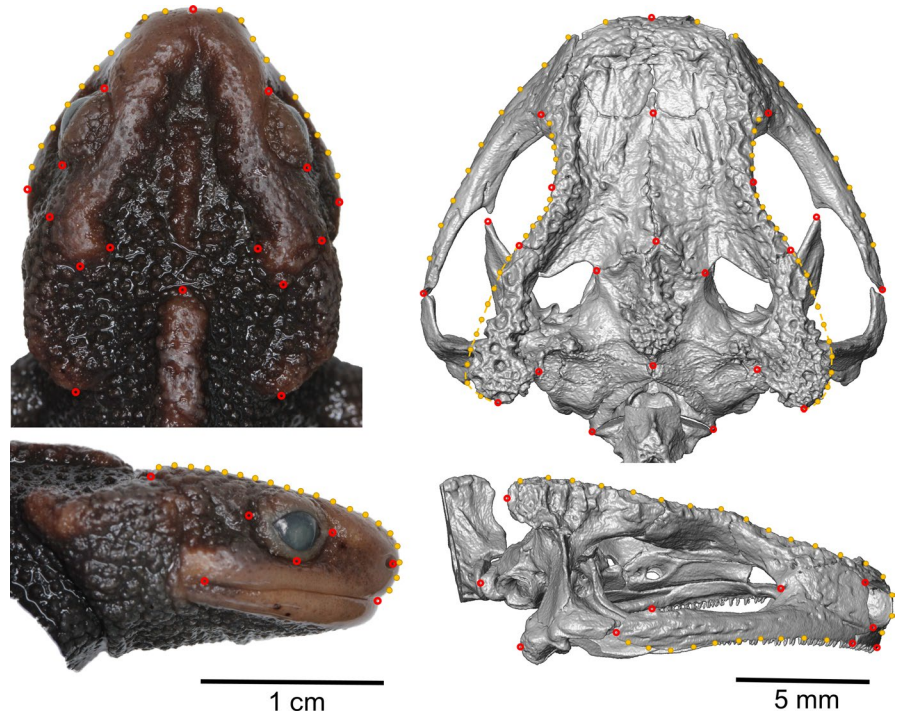
mean shape of replicates and interindividuals were tested against each other to test whether intraindividual landmark placement was consistent in comparison with landmark placement between different individuals.

## 2.2 | Geometric morphometrics

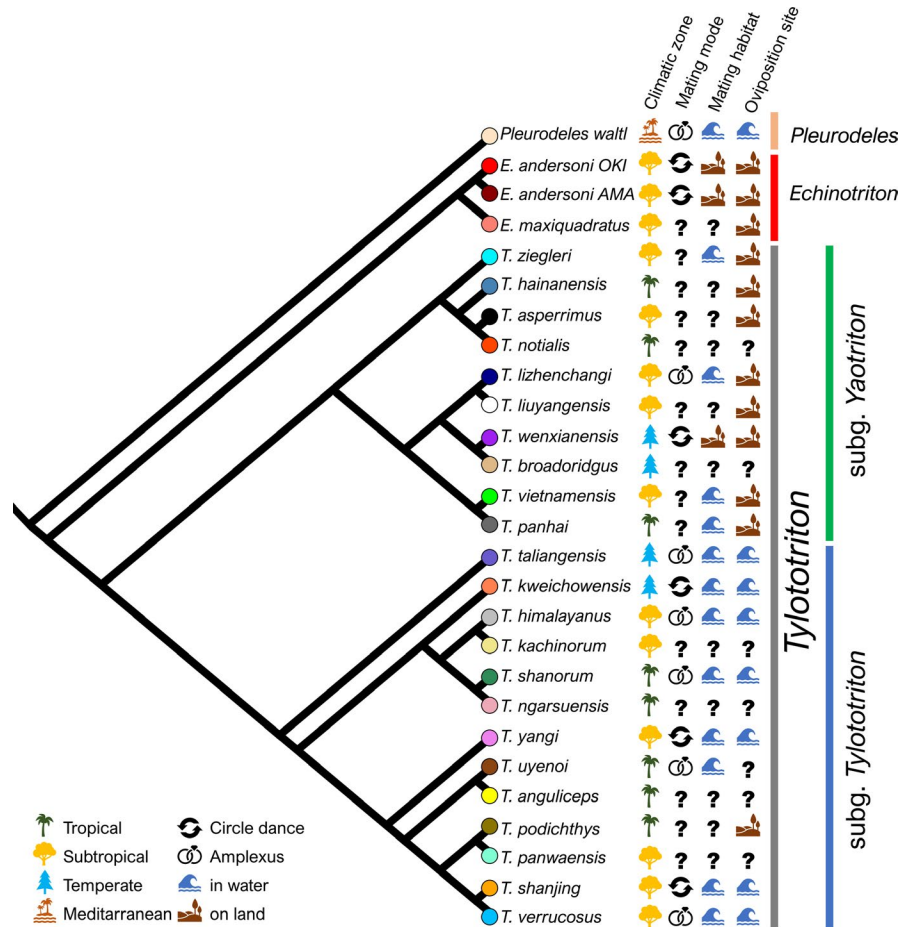
Two-dimensional geometric morphometrics analysis was performed in R version 3.5.3 (R Development Core Team, 2019) using the packages geomorph v.3.1.1 and RRPP v. 0.4.1 (Collyer and Adams, 2018; Adams et al., 2019). The procedure of analysis was equal for each dataset. Missing landmarks were estimated by applying thin plate spline approach using the function 'estimate.missing', as complete landmark configurations are needed for subsequent procedures. The estimation of missing landmarks was done separately for extant and extinct members in order not to mix up shape variation. In extant specimens in total, three landmarks in two specimens were missing only, whereas in fossils, 105 of 672 landmarks were missing due to locally unsuitable preservation. Three fossils had preserved the entire landmark configuration. Two fossil specimens accumulated most of the missing landmarks, one having 50 and 44, respectively, missing landmarks comprised mainly of semilandmarks. A generalized Procrustes alignment (GPA) was employed with the function 'gpagen' to remove variation due to location, rotation and scale of the samples (Rohlf and Slice, 1990). Simultaneously, semilandmarks were slid by minimizing bending energy (Bookstein, 1997a; Perez et al., 2006). This resulted in a new dataset of so-called Procrustes coordinates of each landmark and centroid size (CS) for each sample. Centroid size is a measure of scale in geometric morphometrics being independent of shape and is calculated as the square root of the summed squared distances of each landmark from the centroid (Bookstein, 1997b; Zelditch et al., 2012). Ivanović and Arntzen (2017) showed that the allometric shape component explains a relatively low amount of shape variation within Salamandridae and even less within pleurodeline newts. Thus, we removed allometry, which is beyond the scope in this study, from the datasets to emphasize other potential sources of variation. Allometry-free shapes were generated by transforming the residuals from multivariate regression of shape to log(CS) using the generic function 'procD.lm' and applying these to the mean shape values. Allometry-free shapes were used to explore cranial shape. First, a principal component analysis (PCA) was performed on the covariance matrix of the Procrustes shape coordinates with the function 'plotTangentSpace'. To test the effect of species and genus on cranial shape and log(CS), we performed a Procrustes analysis of variance (ANOVA) using the function 'procD.lm'. A pairwise comparison of species and genera was carried out *post hoc* to clarify which species and genera were different from each other. Species with only one sample were excluded from *post hoc* testing. Alpha level for multiple testing was adjusted via Bonferroni correction.

For further analysis, species means were calculated from Procrustes coordinates and for log(CS). Again, a PCA was conducted on the species' mean shapes. Visualization including phylogeny

**FIGURE 2** Landmark configurations in dorsal (top) and lateral (bottom) view of the external (left) and osteological (right) cranial morphology used in 2D geometric morphometric analyses of cranial shape of pleurodeline newts. Red circles denote landmarks, yellow dots semilandmarks



**FIGURE 3** Phylogeny of pleurodeline newts including the genera *Pleurodeles*, *Echinotriton* and *Tylototriton* following Zaw et al., 2019. Data on distribution in climatic zones and life history traits (mating mode, mating habitat, oviposition site) are illustrated. Color codes of tree tips correspond to settings in subsequent figures



was performed using the function 'plotGMPhyloMorphoSpace', creating a plot of principal components for a set of Procrustes coordinates. Internal nodes were calculated by the squared-changed

parsimony method (Rohlf, 2002; Klingenberg and Gidaszewski, 2010). We tested whether cranial shape was affected by phylogeny using the function 'physignal' on different taxonomic levels



within pleurodeline newts. This function estimates the phylogenetic signal using the *Kmult* statistics, assuming the Brownian motion model of evolution (Adams, 2014a). We followed the most recent phylogeny of Zaw et al. (2019), including all species used in this study (Figure 3). Contrary to Fei et al. (2012), we treated *Tylototriton kweichowensis* and *T. taliangensis* as members of the subgenus *Tylototriton* and did not follow the concept of recognizing additional subgenera accommodating the species. Evolutionary divergence for the two populations of *E. andersoni* from Okinawa and the Amami archipelago was taken from Kurabayashi et al. (2012). Skull shape in dorsal view was analyzed once without and once with the *Chelotriton* datasets for two reasons: to allow a better comparability of datasets for external morphology and osteology in extant newts and to help examine whether fossil specimens potentially add variation to the morphospace in a particular direction, and hence affect the morphospace of the extant members (Pérez-Ben et al., 2019).

Finally, we tested whether cranial morphology correlates with ecological and reproductive biology via phylogenetic ANOVA (Procrustes ANOVA and regression models in a phylogenetic context assuming the Brownian motion model of evolution) using the function 'procD.pgls' (Adams, 2014b). We collected available data in the literature on the following traits: mating mode (amplexus, mating dance), mating habitat (terrestrial, aquatic) and oviposition site (terrestrial, aquatic) (Figure 3). Further, species distribution area was assigned to one of the following main biomes: tropical, subtropical, temperate, Mediterranean according to Kottke et al. (2006) and Woodward et al. (2004) (Figure 3). Significance testing

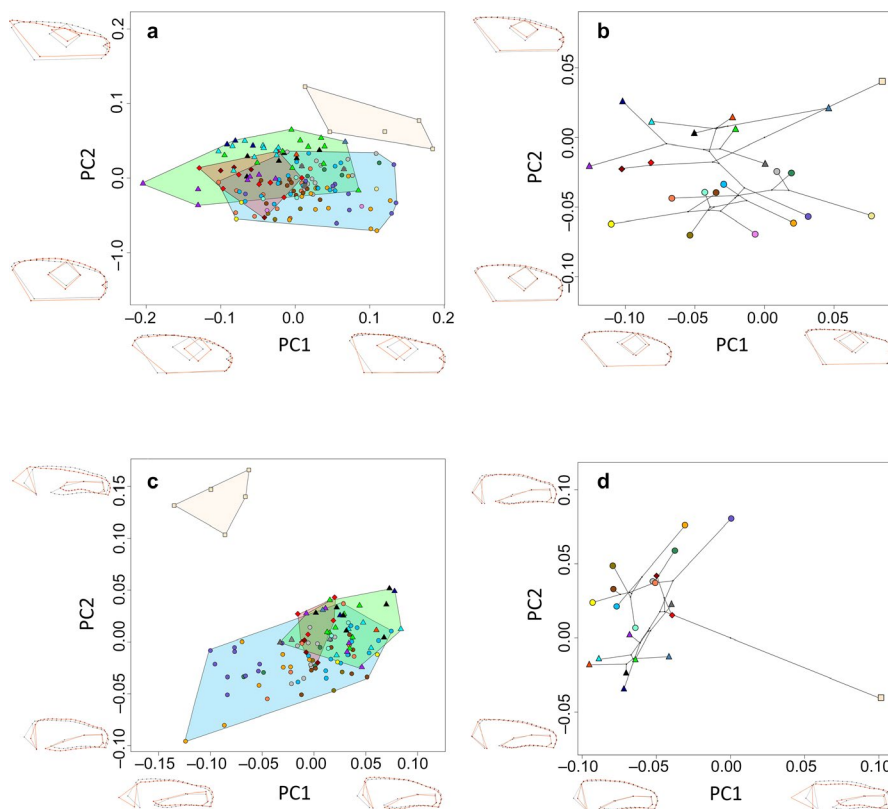
was performed by permutation procedures with 10,000 iterations implemented in the RRPP package (Collyer and Adams, 2018; Adams et al., 2019).

### 3 | RESULTS

#### 3.1 | Lateral head morphology

In terms of head morphology, principal component (PC) 1 explained 55.3% and PC2 14.2% of the observed variance. Ribbed newts were well separated from the crocodile newts, whereas the latter largely overlapped in their morphospace (Figure 4a). *Echinotriton andersoni* from Okinawa and Amami were not separated. Positive PC1 scores were associated with a posterior eye position and labial angle. Further, the naris was positioned more dorsally, and the dorsolateral ridge ended at a more anterior and dorsal position, leading to a straight occiput line connecting the most posterior end of the dorsolateral ridge and labial angle. Negative PC1 scores comprised an anterior position of the eye and labial angle with a more ventrally positioned naris and posterior end of the dorsolateral ridge. The occiput was more diagonal. High loadings of PC2 were associated with a flattened head with a straight cranial roof and an anterior shift of the occiput, whereas negative PC 2 values were associated with a dorsoventrally raised head with a convex cranial roof above the orbit and a posteriorly shifted occiput.

In the analysis on species means, PC1 explained 60.3% and PC2 18.6% of the variance. The morphological changes along PC axes

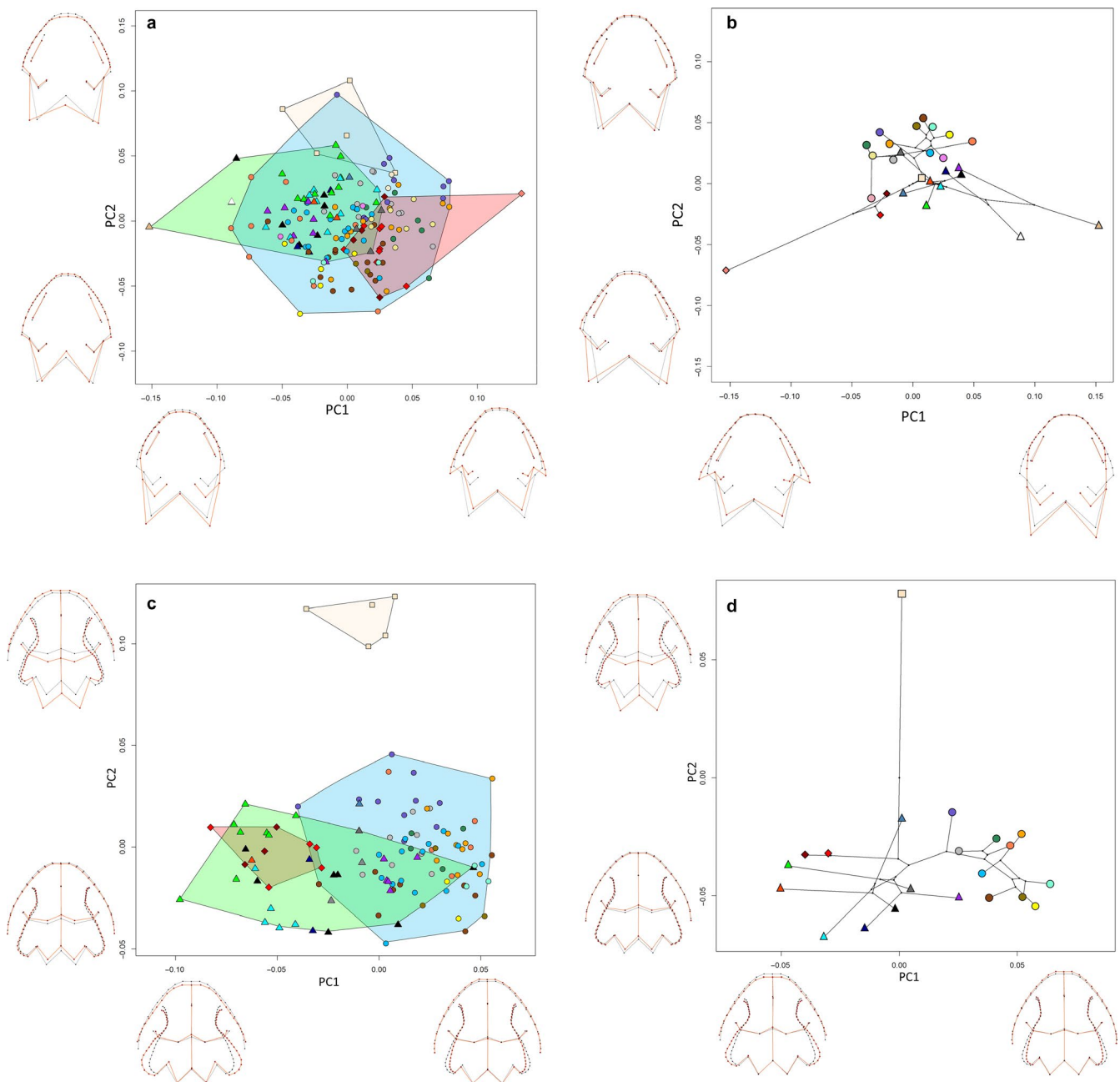


**FIGURE 4** PCA plots of GPA-aligned, allometry-free shapes of cranial morphology in lateral view of pleurodeline newts. Black wireframe corresponds to the mean shape, red wireframe represents the shape at the extreme value of the respective PC axes. (a) External morphology of all specimens. (b) External morphology of species mean shapes. (c) Osteology of all specimens. (d) Osteology of species mean shapes. Triangles correspond to the members of the subgenus *Yaotriton*, ellipses to subgenus *Tylototriton*, diamonds to *Echinotriton*, and square to *Pleurodeles*. For color code, see Figure 2

were similar, as described above, but less pronounced (Figure 4b). The subgenera *Yaotriton* and *Tylototriton* were morphologically well separated in the morphospace along the second PC axis. *Echinotriton* and *Pleurodeles* morphologically resembled *Yaotriton* more than *Tylototriton*.

In skull morphology, PC1 explained 31.0% and PC2 22.6% of the observed variance. *Pleurodeles* occupied a separate area in the morphospace along the PC2 axis, whereas *Echinotriton* and *Tylototriton* overlapped (Figure 4c). Positive PC1 scores were associated with a

slender snout tip, a ventrally moved maxillary tip, a lower dorsolateral ridge and a shorter but higher occiput, which is tilted forward. Negative PC1 scores were associated with a bulkier snout tip, dorsally moved maxillary tip and dorsolateral ridge, and a posterior elongated occiput which is orientated almost perpendicularly. Positive values on the PC2 axis were associated with an uplift and shortening of the maxillary, an elongated snout tip, a flatter skull roof slightly elongated posteriorly and a backward tilted occiput with posteriorly moved occipital condyles.



**FIGURE 5** PCA plots of GPA-aligned, allometry-free shapes of cranial morphology in dorsal view of pleurodeline newts excluding *Chelotriton*. Black wireframe corresponds to the mean shape, red wireframe represents the shape at the extreme value of the respective PC axes. (a) External morphology of all specimens. (b) External morphology of species mean shapes. (c) Osteology of all specimens. (d) Osteology of species mean shapes. Triangles correspond to the members of the subgenus *Yaotriton*, ellipses to subgenus *Tylototriton*, diamonds to *Echinotriton* and square to *Pleurodeles*. For color code see Figure 2

Morphospace on species means was rotated along both axes, PC1 (42.1%) and PC2 (28.7%). *Pleurodeles* occupied the extreme value along PC1 and both subgenera *Yaotriton* and *Tylotriton* again were distinct. Whereas *E. andersoni* from Okinawa was closer to *Yaotriton*, specimens from the Amami archipelago fell within the morphospace of the subgenus *Tylotriton* (Figure 4d). Positive PC1 scores were associated with a slightly bulkier snout tip, a flatter skull, a shorter and uplifted maxilla, a perpendicular occiput and posteriorly shifted occipital condyle. Negative PC1 scores were associated with an only slight elongation of the maxillary and an anteriorly moved occipital condyle. Positive scores on PC2 were associated with an elongation of the maxilla, a perpendicular orientation of the occiput, an elevation of the anterior skull roof and a drop of the posterior end of the dorsolateral ridge. Negative scores on PC2 were associated with a shortening of the maxilla, a slight backward tilt of the occiput, an uplift of the posterior end of the dorsolateral ridge and a slight drop of the anterior skull roof.

### 3.2 | Dorsal head morphology

In dorsal view, PCA on head morphology explained 25.6% on PC1 and 15.3% on PC2 of the observed variance. Although *Pleurodeles* occupied the morphospace at the end of PC2, it still largely overlapped with the morphospace of *Tylotriton* subgenera (Figure 5a). *Pleurodeles* was best separated from crocodile newts along a gradient of PC2 and PC4 (10.1%, Supporting Information Figure S2). *Tylotriton* subgenera overlapped with both *Yaotriton* and *Echinotriton*, covering almost the entire morphospace of pleurodeline newts (Figure 5a). *Echinotriton andersoni* overlapped with both *Tylotriton* subgenera, whereas *E. maxiquadratus* was well separated. This was also the case for *Tylotriton (Yaotriton) broadoridgus* at the other side of the morphospace. There was no clear separation of Okinawa and Amami populations. With positive scores of PC1, head morphology was associated with anterior parotoid tips, slender and anteriorly moved dorsolateral ridges, slightly prolonged snout, a wider cranium and a more posterior widest head width. Negative PC1 scores were associated with posterior shift of parotoid tips and dorsolateral ridges, the latter one also being wider, having a shorter snout and a slender cranium with a more anterior position of the largest head width. Positive scores on PC2 were associated with a truncated snout, posterior position of eyes and of the vertebral ridge, and distally shifted parotoid tips. Negative scores were associated with a pointier snout tip, anteriorly shifted eyes and end of vertebral ridge and proximal parotoid tips.

In the morphospace of species means, PC1 explained 48.8% and PC2 16.9% of the variance. Subgenera *Tylotriton* and *Yaotriton* were well separated except for *T. (Y.) panhai* falling into the morphospace of subgenus *Tylotriton* (Figure 5b). *Echinotriton* morphospace was well separated from *Tylotriton*, with only a slight overlap. *Echinotriton maxiquadratus* fell far apart from *Tylotriton* compared with *E. andersoni*. *Tylotriton (Y.) liuyangensis* and *T. (Y.) broadoridgus* also fell far apart from their other congeners. *Pleurodeles* fell right

into the middle of the occupied morphospace. Ribbed newts were separated well from crocodile newts along PC3, explaining 12.2% of variance (Supporting Information Figure S3). Positive scores on PC1 were associated with posterior shift of parotoid tips and dorsolateral ridges and wider dorsolateral ridges. Negative PC1 scores were associated with anterior shifts of the parotoid tips and dorsolateral ridges, narrower ridges and a wider head. Positive scores of PC2 were associated with a slight elongation and narrowing of the cranium and a proximal shift of parotoid tips, whereas negative PC2 scores were linked to a truncation of the snout, widening of the cranium and a distal shift of the parotoid tips. Additionally, positive PC3 scores were associated with a posterior and proximal movement of eyes, an anterior shift of the widest head position and a posterior shift of the terminal point of the vertebral ridge. Negative PC3 scores were related to an anterior shift of the terminal point of the vertebral ridge. Otherwise, morphological changes were only marginal.

PC1 explained 23.6% and PC2 explained 22.1% of the observed variance in osteology. Again, *Pleurodeles* were well separated from all other groups (Figure 5c). The subgenera of *Tylotriton* overlapped within about half of their respective morphospace. *Echinotriton* settled within the space of *Yaotriton*, only slightly overlapping with subgenus *Tylotriton*. Positive scores on PC1 were associated with an elongation of the snout tip and maxilla, the anterior shift of orbit edges and slender dorsolateral ridges. Negative PC1 scores were related to the shortening of the snout tip and maxilla, a posterior shift of the orbit edges and widening of dorsolateral ridges. Positive scores of PC2 were associated with a protruding and blunter snout, a shorter maxilla, a posterior shift of the fronto-parietal suture, a posterior shift of the occipital condyles and parietals, slender dorsolateral ridges and a slender cranial roof between orbits. Negative PC2 scores were associated with a slightly truncated snout and an anterior shift of the fronto-parietal suture, occipital condyles and parietals.

PC1 of species mean shapes explained 27.9% and PC2 25.9% of morphospace variance. Notably, *Pleurodeles* appeared far apart from all crocodile newts but also *Yaotriton* and *Tylotriton* were well separated without overlapping (Figure 5d) and *Echinotriton* resembled *Yaotriton* more than species of subgenus *Tylotriton*. The morphological changes along PC axes were similar to those described before.

### 3.3 | Morphology of fossil *Chelotriton*

When including fossil crocodile newts, PC1 explained 23.6% and PC2 22.1% of the variance. *Chelotriton* of the two deposits occupied different positions in the morphospace. *Chelotriton* from Enspel were located at the upper left quadrant, whereas *Chelotriton* from Randeck fell into the lower right quadrant (Figure 6a). Neither lay within the morphospace of extant taxa, whereas *Pleurodeles* was morphologically distinct and occupied a different part of the morphospace. Morphological changes along PC1 axis were similar to the skull dataset excluding *Chelotriton* described above. The second

PC axis changed orientation, positive scores comprised an anterior shift of the fronto-parietal suture and the occiput, prolonged maxilla, a wider skull roof between orbits and more laterally orientated pterygoids. Negative scores along PC2 comprised a shortening of the maxilla, posterior shift of the fronto-parietal suture, an elongation of the occiput including the dorsolateral ridges and a narrower skull roof between orbits.

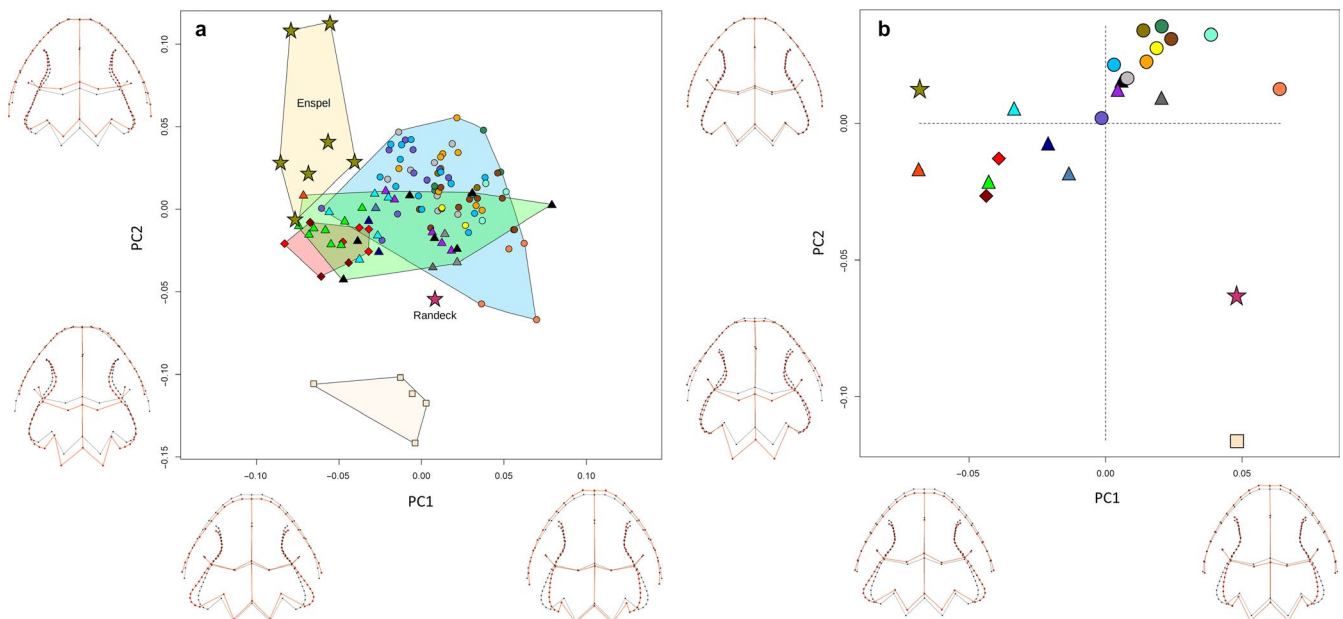
On species means, PC1 explained 27.9% and PC2 25.9% of the observed variance. The subgenera *Yaotriton* and *Tylototriton* were also well separated, although *Tylototriton* (*Tylototriton taliangensis* fell within the morphospace of *Yaotriton* (Figure 6b). Again, neither of the fossil *Chelotriton* fell into the morphospace of *Tylototriton*, *Echinotriton* or *Pleurodeles*. Specimens from Enspel were located closer to *Yaotriton*, whereas the Randeck specimen was placed in-between *Tylototriton* subgenera and *Pleurodeles*. Morphological changes along PC axes were similar to those described before, with the exception that positive PC1 scores additionally comprised a slight anterior shift of the fronto-parietal suture and occiput, and the opposite was true for negative scores.

### 3.4 | Phylogeny, ecology and shape

In all datasets, a phylogenetic signal was present (Table 2). The influence of phylogeny was still strong within the genus *Tylototriton*, whereas within its subgenera it was only detectable among subgenus *Tylototriton*, and only in dorsal morphology. Shape differed interspecifically and generically in all datasets (see Table 3). Post-hoc testing revealed that all pleurodeline newt genera were morphologically

distinct in cranial shape (Supporting Information Tables S2, S6, S14 and S18). Only in lateral skull view was no difference among *Tylototriton* and *Echinotriton* detected, whereas *Pleurodeles* showed a distinct cranial morphology from all other pleurodeline newts (Supporting Information Table S10). The two populations of *E. andersoni* were different neither in cranial shape or size. In lateral view, both *Echinotriton* populations were less distinct in their morphology from *Tylototriton* (Supporting Information Tables S1 and S9). In dorsal view, especially *E. andersoni* from Okinawa appeared morphologically distinct from many *Tylototriton* species (Supporting Information Tables S5 and S13). *Yaotriton* subgenera showed only little interspecific differences in various datasets, whereas divergence within *Tylototriton* subgenera was more marked (Tables S1, S5, S9 and S13). In particular, *T. (T.) kweichowensis* and *T. (T.) taliangensis* were distinct from several consubgenera. Within *Yaotriton*, *T. (Y.) vietnamensis* and *T. (Y.) zieglerei* were highly divergent from *Tylototriton* subgenera, but *T. (T.) shanjing* and *T. (T.) taliangensis* appeared morphologically distinct from most *Yaotriton* species.

Fossil *Chelotriton* from Enspel (the single *Chelotriton* from Randeck was excluded due to sample size) exhibited a cranial morphology distinct from nearly all other pleurodeline newts (Supporting Information Table S17). Centroid size differed interspecifically in all datasets but on the genus level, only head and skull morphology including *Chelotriton* was different (Table 3). Post hoc tests revealed that the dorsal head morphology of *Tylototriton* was smaller than in *Echinotriton* (Supporting Information Table S4). Further, *Chelotriton* was larger than all other genera and species of pleurodeline newts except *T. (T.) kweichowensis* (Supporting Information Tables S19 and S20). In dorsal view, populations from Okinawa of *E. andersoni*



**FIGURE 6** PCA plots of GPA-aligned, allometry-free shapes of osteological, cranial morphology in dorsal view of pleurodeline newts including *Chelotriton*. Black wireframe corresponds to the mean shape, red wireframe represents the shape at the extreme value of the respective PC axes. (a) PCA plot for all specimens. (b) PCA on species mean shapes. Triangles correspond to the members of the subgenus *Yaotriton*, ellipses to subgenus *Tylototriton*, diamonds to *Echinotriton*, the square to *Pleurodeles*, stars to *Chelotriton*. For color code, see Figure 2

tended to be larger than many *Tylostotriton*. Only *T. (T.) kweichowensis* developed a similar cranial size, being even larger than *E. andersoni* from the Amami archipelago and most other congeners (Supporting Information Tables S3, S7, S11 and S15). Within *Yaotriton*, *T. (Y.) asperrimus* had the largest cranial size, but only *T. (Y.) vietnamensis* and *T. (Y.) wenzianensis* were consistently smaller among the datasets. Within *Tylostotriton* subgenera, mainly *T. (T.) kweichowensis* diverged in cranial size from its consubgenera. Laterally, *T. (T.) taliangensis* diverged from *T. (T.) uyenoii* and *T. (T.) yangi* in cranial size, whereas the centroid size of *Pleurodeles* was smaller than in *T. (T.) kweichowensis* in lateral view, but larger than in *T. (Y.) lizhenchangii* and *T. (Y.) wenzianensis* in dorsal head morphology.

Only mating mode was associated with the cranial shape in three of four datasets accounting for phylogeny (Table 4). In lateral morphology, species using an amplexus exhibited a smaller eye diameter, the eye being also slightly posteriorly shifted. The cranial roof was flatter in those species, whereas the posterior end of the dorsolateral bony ridges was elevated (Figure 7a,b). Species using a mating dance for copulation in general exhibited a bulkier cranium. The eye diameter was enlarged and anteriorly shifted. The cranial roof was elevated and the dorsolateral bony ridges were inclined. Furthermore, the occiput was shorter in those species. In dorsal view, main shape differences comprised more slender dorsolateral bony ridges and posteriorly moved pterygoid tips, occiput and midcranial suture among frontals and parietals in amplexant species (Figure 7c,d). In dancing species, the opposite shape changes were observed.

**TABLE 2** Test for phylogenetic signal in 2D morphometrics cranial datasets of Pleurodelini, genus *Tylostotriton* and the two subgenera *Yaotriton* and *Tylostotriton*. Significant *p*-values are given in bold

Pleurodelini	K	p
Head lateral	.363	<b>.0039</b>
Head dorsal	.428	<b>.0048</b>
Skull lateral	.51	<b>.0035</b>
Skull dorsal	.52	<b>&lt;.0001</b>
Genus <i>Tylostotriton</i>		
Head lateral	.55	<b>.035</b>
Head dorsal	.58	<b>.0073</b>
Skull lateral	.46	.2
Skull dorsal	.69	<b>&lt;.0001</b>
Subgenus <i>Tylostotriton</i>		
Head lateral	.59	<b>.026</b>
Head dorsal	.73	<b>.025</b>
Skull lateral	.48	.38
Skull dorsal	.69	<b>.013</b>
Subgenus <i>Yaotriton</i>		
Head lateral	.68	.5
Head dorsal	.64	.63
Skull lateral	.68	.37
Skull dorsal	.7	.36

## 4 | DISCUSSION

### 4.1 | Morphology and phylogeny

We investigated the cranial morphology of ribbed and crocodile newts in an integrative approach including their external head and skull morphology. To shed more light on the evolutionary history of pleurodeline newts, well-preserved fossil specimens of the closely related genus *Chelotriton* were included in our multivariate analyses of cranial morphology in relation to selected reproductive traits and distribution across biomes.

General shape changes in external and osteological morphology were similar. Correlations of soft and hard tissue morphometrics were shown already in another basal salamandrid salamander (Pogoda and Kupfer, 2018). However, osteology provides many more possibilities for placing precise landmarks, likely representing a better basis for evolutionary research. Ribbed newts were well separated from crocodile newts in various morphospaces previously confirmed by cranial three-dimensional morphometric analysis (Ivanović and Arntzen, 2017). Cranial shape differentiation coincides with the spatial distribution patterns, Mediterranean *Pleurodeles* being differentiated from the Asian *Echinotriton* and *Tylostotriton*. Nevertheless, mean shapes of the latter genera were different in all except but one of the datasets. To increase support for these results, it is suggested to add more specimens of *E. chinhaiensis* and *E. maxiquadratus*. Dubois and Raffaelli (2009) described the subgenus *Yaotriton* including the uniformly 'black'-coloured crocodile newts of the *asperrimus* group. Later, another two subgenera were distinguished by Fei et al. (2012): i.e. for *T. kweichowensis* the monophyletic subgenus *Qiantriton* and for *T. taliangensis* and *T. pseudoverrucosus* *Liangshantriton* were suggested. The latter is recognized by some authors (Gong et al., 2018) but this would classify *Tylostotriton* as nonmonophyletic and hence is not supported by the majority of the community (Frost, 2018). We showed that *T. (T.) kweichowensis* and *T. (T.) taliangensis* show some degree of morphological distinctiveness from the other members of the subgenus *Tylostotriton*. This may explain the phylogenetic signal within *Tylostotriton* subgenera in terms of dorsal morphology, as those two species are phylogenetically at the base of the ancestral line. Overall, *T. kweichowensis* and *T. taliangensis* do not occupy a separate morphospace and thus are still treated as members of *Tylostotriton* subgenera. A clear separation of *Yaotriton* and *Tylostotriton* subgenera (*verrucosus* group) is only achieved when species mean shapes are considered. In lateral view, species of *Yaotriton* have a flatter cranium and a steeper occiput with a shorter maxilla, compared with *Tylostotriton* subgenera. In dorsal view, *Yaotriton* exhibits wider dorsolateral ridges, a shorter snout, maxilla and smaller orbits than members of the subgenus *Tylostotriton*. *Echinotriton* resembles more the species of *Yaotriton* than *Tylostotriton* subgenera, including a generally shared appearance of *Yaotriton* and *Echinotriton*: the latter exhibit only few orange highlighted body structures, e.g. tail edges, digits and parotoid tips, whereas species of *Tylostotriton* subgenera are often more colorful (Nussbaum and Brodie, 1982; Hernandez, 2016). The particular color patterns likely

**TABLE 3** Procrustes ANOVA of 2D morphometric shape data and centroid size (CS) of crania of pleurodeline newts tested for species and genus. Significant *p*-values are given in bold

	Model	df	F	<i>p</i>
Head lateral	shape ~ species	19	7.6	<b>&lt;.0001</b>
	shape ~ genus	2	11.7	<b>&lt;.0001</b>
	log(CS) ~ species	19	14.0	<b>&lt;.0001</b>
	log(CS) ~ genus	2	.3	.78
Head dorsal	shape ~ species	22	5.7	<b>&lt;.0001</b>
	shape ~ genus	2	12.9	<b>&lt;.0001</b>
	log(CS) ~ species	22	1.7	<b>&lt;.0001</b>
	log(CS) ~ genus	2	6.4	<b>.0021</b>
Skull lateral	shape ~ species	18	6.4	<b>&lt;.0001</b>
	shape ~ genus	2	14.2	<b>&lt;.0001</b>
	log(CS) ~ species	18	11.8	<b>&lt;.0001</b>
	log(CS) ~ genus	2	.04	.96
Skull dorsal	shape ~ species	18	7.7	<b>&lt;.0001</b>
	shape ~ genus	2	13.5	<b>&lt;.0001</b>
	log(CS) ~ species	18	11.4	<b>&lt;.0001</b>
	log(CS) ~ genus	2	.8	.45
Skull dorsal incl. <i>Chelotriton</i>	shape ~ species	19	8.6	<b>&lt;.0001</b>
	shape ~ genus	3	15.5	<b>&lt;.0001</b>
	log(CS) ~ species	19	24.0	<b>&lt;.0001</b>
	log(CS) ~ genus	3	34.6	<b>&lt;.0001</b>

correlate with a peculiar defence behavior, the 'unkenreflex', which is known only from *Echinotriton* and *Yaotriton* (Brodie et al., 1984; Sparreboom et al., 2001; Gong and Mu, 2008). Other antipredator postures were described for *T. (T.) verrucosus* (Brodie et al., 1984). However, to draw final conclusions about phylogenetic relationships on a larger scale, more data would be required.

The phylogenetic signal is strongest among *Tylostotriton*, whereas within its subgenera we detected only an influence of phylogeny within subgenus *Tylostotriton*. In the phylomorphospace, the closely related *T. (T.) anguliceps*, *T. (T.) uyenoii*, *T. (T.) podichthys* and *T. (T.) panwaensis* and also *T. (T.) himalayanus*, *T. (T.) shanorum*, *T. (T.) kachinorum* and *T. (T.) kweichowensis* plot together, whereas *T. (T.) podichthys/panwaensis* is sister to *T. (T.) verrucosus/shanjing* (Figures 4 and 5). The latter apparently diverged in the opposite direction of the phylomorphospace. The missing phylogenetic signal in lateral morphology is likely attributable to the low number of taxa included in the analysis. *Tylostotriton (T.) verrucosus* and *T. (T.) shanjing* are two sister taxa, whereas the status of *T. (T.) shanjing* is still under debate (Zhang et al., 2007; Zhao et al., 2012). Although the genetic divergence is quite low among the two taxa (e.g. Phimmachak et al., 2015a; Wang et al., 2018; Grismer et al., 2019), most authors accept *T. (T.) shanjing* as a valid taxon (e.g. Stuart et al., 2010; Nishikawa et al., 2013) and the description of *T. (T.) pulcherrimus* adds to this confusion. Even though it was synonymized with *T. (T.) shanjing* by Nishikawa et al. (2013), other authors accept *T. (T.) pulcherrimus* as a valid taxon (Grismer et al.,

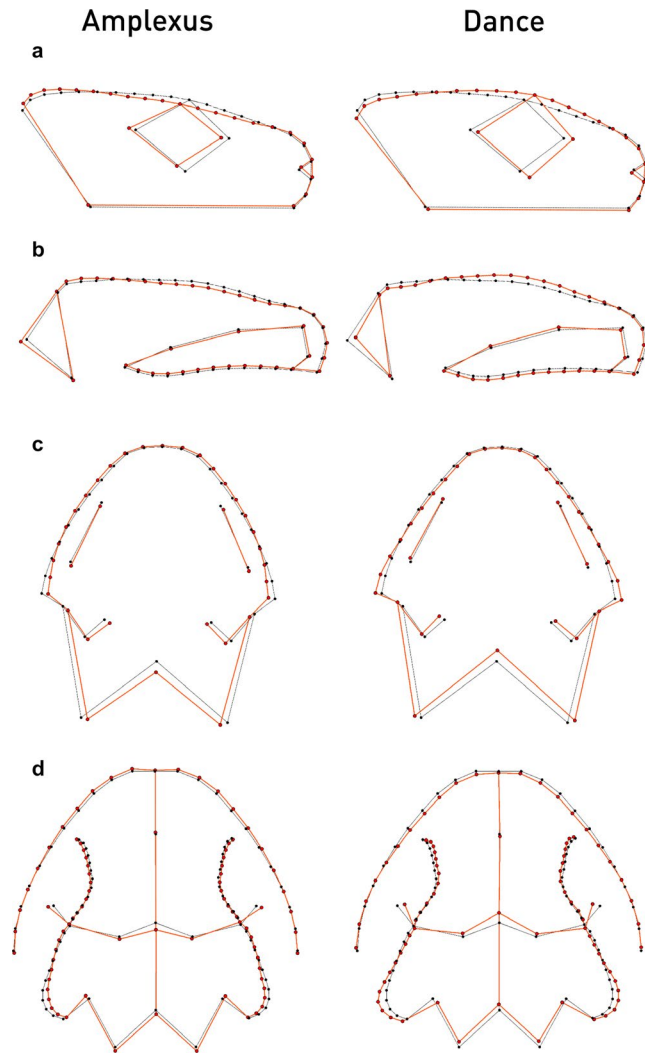
**TABLE 4** Procrustes ANOVA in a phylogenetic framework of 2D morphometric shape datasets of crania of pleurodeline newts tested for ecological traits. Significant *p*-values are given in bold

	Model	df	F	<i>p</i>
Head lateral	shape ~ mating mode	1	3.61	<b>.044</b>
	shape ~ mating habitat	1	1.61	.199
	shape ~ oviposition site	1	1.74	.17
	shape ~ climate	3	.65	.67
Head dorsal	shape ~ mating mode	1	.96	.39
	shape ~ mating habitat	1	.46	.76
	shape ~ oviposition site	1	1.22	.27
	shape ~ climate	3	1.48	<b>.012</b>
Skull lateral	shape ~ mating mode	1	8.41	<b>.011</b>
	shape ~ mating habitat	1	.34	.075
	shape ~ oviposition site	1	1.12	.3
	shape ~ climate	3	.92	.44
Skull dorsal	shape ~ mating mode	1	3.58	<b>.014</b>
	shape ~ mating habitat	1	.61	.72
	shape ~ oviposition site	1	1.3	.25
	shape ~ climate	3	1.03	.38
Skull dorsal incl. <i>Chelotriton</i> <sup>a</sup>	shape ~ mating mode	1	2.67	<b>.041</b>
	shape ~ mating habitat	1	.41	.88
	shape ~ oviposition site	1	1.29	.26
	shape ~ climate	3	.96	.5

<sup>a</sup>Note that *Chelotriton*-shape *per se* is not included in the models of LH traits, as no information on these is available for this genus. The models instead concern data processing (GPA alignment) of the remaining shapes prior analysis.

2019; Zaw et al., 2019). Unfortunately, data for *T. (T.) pulcherrimus* were not available for this study. *Tylostotriton (T.) shanjing* is distinguished from *T. (T.) verrucosus* mainly on differences in coloration (Nussbaum et al., 1995). In the phylomorphospace, *T. (T.) verrucosus* and *T. (T.) shanjing* are neither closer nor further apart from each other in comparison with other sister taxa within *Tylostotriton*. Recently, Grismer et al. (2019) detected no morphometric differences between *T. (T.) verrucosus* and *T. (T.) shanjing*. In contrast, our pairwise comparisons indicated a more distinct morphology, especially in the lateral view of skulls (Table S9). As several populations, formerly assigned to *T. (T.) verrucosus* or *T. (T.) shanjing*, have been described as new species in recent years, more detailed fieldwork is needed, especially in northern Indochina, to identify new taxa and distribution boundaries.

*Chelotriton* from Enspel resembles more closely *Yaotriton* and *Echinotriton* than subgenus *Tylostotriton* in the morphospace of PC1 and PC2. On other PC components up to PC9 (accounting for > 95% of variance), Enspel-*Chelotriton* arrived mostly closer to *Echinotriton* (not shown). As pairwise comparisons revealed a strong cranial disparity throughout extant pleurodeline newts, the phylogenetic position of *Chelotriton* from Randeck is far from clear. In morphospace, it falls between *Tylostotriton* and *Pleurodeles* but on other PC



**FIGURE 7** Shape change of cranial morphology in different datasets of 2D morphometric shape data of pleurodeline newts corresponding to different mating modes. (a) Lateral head morphology, (b) lateral skull morphology, (c) dorsal head morphology, (d) dorsal skull morphology. Black wireframe corresponds to the mean shape, red wireframe represents the shape of either amplexant species (left column) or dancing species (right column). Shape changes are magnified by a factor of two

components, Randeck-*Chelotriton* arrived closer to *Tylotriton* subgenera than to *Pleurodeles* (not shown), indicative of the newt fossil remains of the two deposits belonging to different species (already assumed by Schoch et al., 2015). Obvious morphological differences are visible among *Chelotriton* specimens, e.g. a shorter maxilla and missing quadrate spines in the specimen from Randeck crater lake (Schoch et al., 2015). Analysis of different skull datasets, both including and excluding *Chelotriton*, showed weak differences in the morphospace of extant relatives and also revealed that *Chelotriton* does not add much additional variation to the morphospace. Thus, the analyses of fossil newts likely allow some general conclusions to be drawn. Nevertheless, one must always keep in mind that taphonomic processes could severely alter the shape of fossil crania. Most notably, the dorsolateral ridges are displaced distally, presumably

altered by taphonomy. Further, morphological traits for landmark acquisition are frequently altered or destroyed, and estimating those by algorithms never can reproduce the full truth. *Chelotriton* specimens cluster well together in the morphospace and specimens with estimated missing landmarks are not clustered in a specific region of it. Hence, we assume that the estimation of the missing landmarks has not led to significant alteration of the data. More detailed morphological comparisons among *Chelotriton* specimens remain to be made, and the four currently known species within the genus await validation (Marjanović and Witzmann, 2015). Nevertheless, we have shown that *Chelotriton* represents the largest bodied members within pleurodeline newts, although extant members deviate only little in their cranial size. *Echinotriton andersoni* from the Amami archipelago is thought to be smaller in body size than the populations from Okinawa (Utsunomiya et al., 1978; Hernandez, 2016). Our study does not support the same pattern in cranial size, although this might be due to the small sample size of Amami specimens.

## 4.2 | Morphology and ecology

Data on the general ecology are still scarce for crocodile newts (see also Kieren et al., 2018), although several studies deal with the reproductive ecology of *Echinotriton* (Utsunomiya et al., 1978; Xie et al., 2000; Sparreboom et al., 2001; Utsunomiya and Matsui, 2002; Igawa et al., 2013). Only little information is available for *Tylotriton*, often only from anecdotal observations (e.g. Gong and Mu, 2008; Phimmachak et al., 2015b). Although the mating mode and habitat of *E. maxiquadratus* are unknown, we could infer from phylogeny that its mating is terrestrial. Although various crocodile newt species are kept as pets for quite a long time, the origin and species affiliation of captive newts is often uncertain (Mudrack, 1972; Fleck, 2010a; 2010b). Various observations of the mating mode or habitat in *T. (T.) verrucosus* in captivity are available (Rehberg, 1986; Sparreboom, 1999; Jungnickel, 2007), but there is only circumstantial evidence that these observations are all attributable to currently described *T. (T.) verrucosus* or to other morphologically similar species. From published figures, it might be assumed that some observations rather deal with *T. (T.) shanjing* than *T. (T.) verrucosus* (Rehberg, 1986) leading to confusion. Whereas we report terrestrial mating for *T. (Y.) wenxianensis* based on Gong and Mu (2008) and Pasmans et al. (2017), Kieren et al. (2018) list aquatic mating for the species without any source of information. However, Gong and Mu (2008) observed clutch deposition under water, contrary to Pasmans et al. (2017). More studies on the ecology of crocodile newts are urgently needed to understand how reproductive strategies vary interspecifically and even between populations.

Despite the fragmentariness of data, some ecological signals are visible. A correlation between morphology and ecology is obvious for oviposition site, as *Echinotriton* and *Yaotriton* deposit their clutches on land, whereas members of *Tylotriton* subgenera deposit them in water bodies. For *T. (T.) podichthys*, terrestrial clutch deposition has been observed (Phimmachak et al., 2015b). Nevertheless, it is not apparent whether in this case cranial shape similarity among

*Echinotriton* and *Yaotriton* is due to ecology or is constrained by phylogeny. In the latter case, *Tylototriton* subgenera would have re-evolved aquatic oviposition, as *Pleurodeles* as the stem group also deposits clutches in water (Figure 3). Only the mating mode is correlated with cranial shape, simultaneously accounting for phylogeny, the different character states being randomly distributed within *Tylototriton*. How the different shapes might contribute to the different modes is yet speculative. In lateral view, amplexant species show a flatter shaped cranium. In dorsal view, those species exhibit a narrower head shape. This shape might be more streamlined, leading to lower resistance during swimming or crawling.

No cranial adaptations towards specific climate zones were detectable among extant crocodile newts and fossil *Chelotriton*, although the climate was markedly different when the deposits of Enspel and Randeck were formed. The mean annual temperature (MAT) was higher in central Europe than nowadays (Böhme, 2003; Uhl and Herrmann, 2010). The Randeck Maar dates back to the Mid-Miocene climatic optimum, with a MAT of about 24°C (Böhme, 2003), whereas Enspel is about 10 Ma older than Randeck, with a MAT of between 15 and 17°C (Uhl and Herrmann, 2010), similar to the Mediterranean nowadays.

*Chelotriton* as a whole rather represents a subtropical to tropical distributed genus (Böhme, 2003; Uhl and Herrmann, 2010) as are the known distribution ranges of most extant crocodile newts. The relative long timespan between the two deposits investigated here, different climates and morphological disparity support the idea that different species were involved herein and in other European deposits. Westphal (1980) noticed that *Chelotriton* is rarely found in lake deposits and argued that *Chelotriton* was more terrestrial than other urodeles such as *Tylototriton*. This would coincide with our findings that Enspel-*Chelotriton* more closely resembles *Echinotriton* and *Yaotriton*, which mate and lay their clutches terrestrially and spend less time in aquatic habitats. A more extended study including more material of *Chelotriton* from other deposits would be helpful to resolve intergeneric relationships of fossil in relation to extant newt species.

## 5 | CONCLUSIONS

The different datasets (external morphology and osteology) were mostly congruent in their results, osteological datasets leading to a better separation of taxonomic units such as in *Pleurodeles*. With skulls, there are more possibilities to place accurate landmarks along bones and their sutures and the repeatability is higher compared with landmarks placed on soft tissues.

Cranial morphology of crocodile newts provides a congruent phylogenetic signal separating the subgenera. As we had no access to specimens of *T. pseudoverrucosus*, we could not draw a conclusion about the morphological distinctness and validity of *Liangshantriton* and rather follow the opinion placing *T. talianensis* in *Tylototriton* subgenera. Within subgenera, phylogeny plays a minor role in the evolution of cranial shape in crocodile newts. Among reproductive traits, oviposition site was evidently

correlated to phylogeny. This also supports the morphological similarity of *Echinotriton* and subgenus *Yaotriton*, both of which deposit clutches on land. It is not apparent in this case whether cranial shape represents an adaptation to ecology or rather is constrained by phylogeny. Mating mode was the only trait associated with cranial shape, simultaneously correcting for phylogeny. Climate zone had no effect on cranial shape of pleurodeline newts, confirming their quite conservative morphology (Hernandez et al., 2018). Fossil remains were partly distorted and not fully preserved, so that retrodeformation was applied and some landmarks were virtually reconstructed. Nevertheless, the analysis of data with and without fossils revealed a similar amount of morphological variation, promoting cranial shape conservatism in crocodile newts. But fossil *Chelotriton* showed a larger disparity in cranial shape and size in comparison with extant species, underpinning that *Chelotriton* represents a separate lineage of pleurodeline newts rather than a grade towards extant species groups (Schoch et al., 2015). *Chelotriton* from the deposits of Enspel and Randeck probably represent different species and cannot be assigned to *C. paradoxus* simultaneously. As skull morphology of fossil *Chelotriton* from Enspel closely resembles that of *Echinotriton* and *Yaotriton*, we conclude a more terrestrial ecology of the fossil pleurodeline newt. Further studies on the ecology of crocodile newts are urgently needed for two reasons: to better understand how ecology affects evolution of morphology and for conservation purposes, as crocodile newts are highly threatened by various factors driving them close to extinction in the near future (Rowley et al., 2010; et al.2016).

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## AUTHOR CONTRIBUTIONS

P.P. – study design, specimen loans, CT-scanning, data collection, data analysis, data interpretation, drafting manuscript. M.Z. – CT-scanning, data collection. T.B. – CT-scanning. R.R.S. – study design, specimen loans. A.K. – study design, specimen loans, data interpretation, drafting manuscript. All authors edited and approved the manuscript draft. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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## Supporting Information

Table S1: Specimens of pleurodelin salamanders examined for cranial morphometrics analyses in external and osteological morphology. Holo- and paratype catalogue numbers are given in bold.

Species	Catalogue no.	External morphology	Osteology	Comment
<i>Echinotriton andersoni</i> - Okinawa	CAS22130	X		
<i>Echinotriton andersoni</i> - Okinawa	CAS22148	X		
<i>Echinotriton andersoni</i> - Okinawa	CAS22153	X		
<i>Echinotriton andersoni</i> - Okinawa	CAS22154	X	X	
<i>Echinotriton andersoni</i> - Okinawa	CAS22185	X		
<i>Echinotriton andersoni</i> - Okinawa	CAS22266	X	X	
<i>Echinotriton andersoni</i> - Okinawa	CAS22269		X	
<i>Echinotriton andersoni</i> - Okinawa	CAS22286	X	X	
<i>Echinotriton andersoni</i> - Okinawa	CAS22301	X	X	
<i>Echinotriton andersoni</i> - Amami	SMF70901	X		
<i>Echinotriton andersoni</i> - Amami	SMF86880	X	X	
<i>Echinotriton andersoni</i> - Amami	SMF86881	X	X	
<i>Echinotriton andersoni</i> - Amami	SMF86882	X	X	
<i>Echinotriton maxiquadratus</i>	<b>SY20131101ENT</b>	X		from Literature
<i>Tylototriton anguliceps</i>	<b>HNUE A.I.110</b>	X		from Literature
<i>Tylototriton anguliceps</i>	<b>HNUE A.I.1.109</b>	X		from Literature
<i>Tylototriton anguliceps</i>	NCSM82952	X	X	
<i>Tylototriton anguliceps</i>	NCSM82953	X	X	
<i>Tylototriton anguliceps</i>	<b>TBU PAE.671</b>	X		from Literature
<i>Tylototriton asperrimus</i>	SMNS14610	X	X	
<i>Tylototriton asperrimus</i>	ZFMK82728	X	X	
<i>Tylototriton asperrimus</i>	ZFMK82730	X	X	
<i>Tylototriton asperrimus</i>	ZFMK82735	X	X	
<i>Tylototriton asperrimus</i>	ZFMK85178	X	X	
<i>Tylototriton asperrimus</i>	ZFMK85179	X	X	
<i>Tylototriton asperrimus</i>	<b>ZMB34090</b>		X	
<i>Tylototriton broadoridgus</i>	<b>HNUL840513527</b>	X		from Literature
<i>Tylototriton hainanensis</i>	NCSM78989	X	X	
<i>Tylototriton himalayanus</i>	MNHN1976.623		X	
<i>Tylototriton himalayanus</i>	MNHN1976.626	X	X	
<i>Tylototriton himalayanus</i>	MNHN1976.643	X		
<i>Tylototriton himalayanus</i>	MNHN1976.651	X		
<i>Tylototriton himalayanus</i>	MNHN1976.669		X	
<i>Tylototriton himalayanus</i>	MNHN1976.670	X	X	
<i>Tylototriton himalayanus</i>	MNHN1976.678	X	X	
<i>Tylototriton himalayanus</i>	MNHN1976.685	X		
<i>Tylototriton himalayanus</i>	MNHN1976.688	X		
<i>Tylototriton himalayanus</i>	MNHN1976.689	X	X	

<i>Tylostotriton himalayanus</i>	MNHN1976.690	X		
<i>Tylostotriton himalayanus</i>	MNHN1976.692	X	X	
<i>Tylostotriton himalayanus</i>	MNHN1976.693		X	
<i>Tylostotriton himalayanus</i>	SMF1135	X	X	
<i>Tylostotriton himalayanus</i>	ZMB10026	X		
<i>Tylostotriton kachinorum</i>	<b>ZDUM0101</b>	X		from Literature
<i>Tylostotriton kachinorum</i>	<b>ZDUM0102</b>	X		from Literature
<i>Tylostotriton kachinorum</i>	<b>ZDUM0103</b>	X		from Literature
<i>Tylostotriton kachinorum</i>	<b>ZDUM0104</b>	X		from Literature
<i>Tylostotriton kachinorum</i>	<b>ZDUM0105</b>	X		from Literature
<i>Tylostotriton kachinorum</i>	<b>ZMMUA5953</b>	X		from Literature
<i>Tylostotriton kachinorum</i>	<b>ZMMUA5954</b>	X		from Literature
<i>Tylostotriton kweichowensis</i>	MTKD30364	X		
<i>Tylostotriton kweichowensis</i>	MTKD30369	X		
<i>Tylostotriton kweichowensis</i>	MTKD30372	X		
<i>Tylostotriton kweichowensis</i>	MTKD31097	X		
<i>Tylostotriton kweichowensis</i>	MTKD38093	X		
<i>Tylostotriton kweichowensis</i>	USNM95518	X	X	
<i>Tylostotriton kweichowensis</i>	USNM95520	X	X	
<i>Tylostotriton kweichowensis</i>	USNM95524	X	X	
<i>Tylostotriton kweichowensis</i>	USNM95562	X	X	
<i>Tylostotriton kweichowensis</i>	USNM95563	X	X	
<i>Tylostotriton liuyangensis</i>	<b>HNUL11053108</b>	X		from Literature
<i>Tylostotriton lizhenchangi</i>	MTKD47802	X	X	
<i>Tylostotriton lizhenchangi</i>	MTKD47859	X	X	
<i>Tylostotriton lizhenchangi</i>	MTKD48158	X		
<i>Tylostotriton ngarsuensis</i>	<b>LUSHC13763</b>	X		from Literature
<i>Tylostotriton ngarsuensis</i>	<b>LUSHC13764</b>	X		from Literature
<i>Tylostotriton notialis</i>	<b>FMNH271121</b>	X		from Literature
<i>Tylostotriton notialis</i>	NCSM80315	X	X	
<i>Tylostotriton panhai</i>	NCSM82954	X	X	
<i>Tylostotriton panhai</i>	NCSM82955	X	X	
<i>Tylostotriton panhai</i>	NCSM82956	X	X	
<i>Tylostotriton panwaensis</i>	CAS245290	X	X	
<i>Tylostotriton panwaensis</i>	<b>CAS245418</b>	X	X	
<i>Tylostotriton panwaensis</i>	<b>CAS245426</b>	X	X	
<i>Tylostotriton podichthys</i>	NCSM86520	X	X	
<i>Tylostotriton podichthys</i>	ZFMK95521	X	X	
<i>Tylostotriton podichthys</i>	ZFMK95522	X	X	
<i>Tylostotriton shanorum</i>	<b>CAS230940</b>	X		from Literature
<i>Tylostotriton shanorum</i>	ZFMK83199	X	X	
<i>Tylostotriton shanorum</i>	ZFMK83200	X		
<i>Tylostotriton shanorum</i>	ZFMK83202	X	X	
<i>Tylostotriton shanorum</i>	ZFMK83203	X	X	
<i>Tylostotriton shanorum</i>	ZFMK83204	X	X	
<i>Tylostotriton shanjing</i>	CAS215118	X	X	
<i>Tylostotriton shanjing</i>	CAS215119	X	X	

<i>Tylostotriton shanjing</i>	CAS215120	X	X	
<i>Tylostotriton shanjing</i>	CAS242484	X		
<i>Tylostotriton shanjing</i>	CAS242518	X		
<i>Tylostotriton shanjing</i>	CAS242535	X	X	
<i>Tylostotriton shanjing</i>	ZFMK83208	X	X	
<i>Tylostotriton shanjing</i>	ZFMK83209	X		
<i>Tylostotriton shanjing</i>	ZMB73596	X	X	
<i>Tylostotriton shanjing</i>	ZMB73597	X	X	
<i>Tylostotriton shanjing</i>	ZMB73598	X	X	
<i>Tylostotriton shanjing</i>	ZMB73599	X		
<i>Tylostotriton shanjing</i>	ZMB73600	X	X	
<i>Tylostotriton taliangensis</i>	MTKD36799	X		
<i>Tylostotriton taliangensis</i>	MTKD37682	X	X	
<i>Tylostotriton taliangensis</i>	MTKD37790		X	
<i>Tylostotriton taliangensis</i>	MTKD38088		X	
<i>Tylostotriton taliangensis</i>	NHMMW39889.12	X	X	
<i>Tylostotriton taliangensis</i>	NHMMW39889.16	X	X	
<i>Tylostotriton taliangensis</i>	NHMMW39889.18	X	X	
<i>Tylostotriton taliangensis</i>	SMNS14609	X		
<i>Tylostotriton taliangensis</i>	ZFMK83110	X	X	
<i>Tylostotriton taliangensis</i>	ZFMK93752	X	X	
<i>Tylostotriton taliangensis</i>	ZFMK93760	X	X	
<i>Tylostotriton taliangensis</i>	ZFMK93762	X	X	
<i>Tylostotriton uyanoi</i>	<b>KUHE19147</b>	X		from Literature
<i>Tylostotriton uyanoi</i>	MNHN1987.3725	X	X	
<i>Tylostotriton uyanoi</i>	MNHN1987.3731	X	X	
<i>Tylostotriton uyanoi</i>	MNHN1987.3734	X	X	
<i>Tylostotriton uyanoi</i>	MNHN1987.3739	X		
<i>Tylostotriton uyanoi</i>	MNHN1987.3742		X	
<i>Tylostotriton uyanoi</i>	MNHN1987.3749	X		
<i>Tylostotriton uyanoi</i>	MNHN1987.3750	X	X	
<i>Tylostotriton uyanoi</i>	MNHN1987.3752	X		
<i>Tylostotriton uyanoi</i>	MNHN1987.3758		X	
<i>Tylostotriton uyanoi</i>	MNHN1987.3759	X		
<i>Tylostotriton uyanoi</i>	MNHN1987.3761	X		
<i>Tylostotriton uyanoi</i>	MNHN1987.3765	X	X	
<i>Tylostotriton uyanoi</i>	MNHN1987.3768	X	X	
<i>Tylostotriton uyanoi</i>	SMNS15132	X	X	
<i>Tylostotriton verrucosus</i>	CAS215065	X	X	
<i>Tylostotriton verrucosus</i>	CAS215068	X	X	
<i>Tylostotriton verrucosus</i>	CAS215071	X	X	
<i>Tylostotriton verrucosus</i>	CAS215072	X	X	
<i>Tylostotriton verrucosus</i>	CAS215075	X	X	
<i>Tylostotriton verrucosus</i>	CAS215077	X	X	
<i>Tylostotriton verrucosus</i>	CAS234480	X	X	
<i>Tylostotriton verrucosus</i>	CAS245445	X	X	
<i>Tylostotriton verrucosus</i>	CAS245449	X		

<i>Tylostotriton verrucosus</i>	KIZ20130605?	X		from Literature
<i>Tylostotriton verrucosus</i>	MNHN1887.223	X	X	
<i>Tylostotriton verrucosus</i>	MNHN1893.529	X	X	
<i>Tylostotriton verrucosus</i>	MNHN1893.530	X		
<i>Tylostotriton verrucosus</i>	NHMMW8608.1	X	X	
<i>Tylostotriton verrucosus</i>	NHMMW8608.2	X	X	
<i>Tylostotriton verrucosus</i>	SMF1134	X	X	
<i>Tylostotriton verrucosus</i>	SMNS1598.1	X	X	
<i>Tylostotriton vietnamensis</i>	NHMMW8607.1	X		
<i>Tylostotriton vietnamensis</i>	NHMMW8607.2	X	X	
<i>Tylostotriton vietnamensis</i>	SMF1200	X		
<i>Tylostotriton vietnamensis</i>	SMF83417	X	X	
<i>Tylostotriton vietnamensis</i>	SMF83418	X	X	
<i>Tylostotriton vietnamensis</i>	SMF83419	X	X	
<i>Tylostotriton vietnamensis</i>	SMF83420	X	X	
<i>Tylostotriton vietnamensis</i>	SMF83421	X	X	
<i>Tylostotriton vietnamensis</i>	ZFMK86339	X	X	
<i>Tylostotriton vietnamensis</i>	ZFMK92049	X		
<i>Tylostotriton vietnamensis</i>	ZFMK95519	X	X	
<i>Tylostotriton wenxianensis</i>	SMNS15012	X	X	
<i>Tylostotriton wenxianensis</i>	SMNS15013	X	X	
<i>Tylostotriton wenxianensis</i>	ZFMK83738	X		
<i>Tylostotriton wenxianensis</i>	ZFMK83739	X	X	
<i>Tylostotriton wenxianensis</i>	ZFMK83740	X	X	
<i>Tylostotriton wenxianensis</i>	ZFMK83741	X	X	
<i>Tylostotriton wenxianensis</i>	ZFMK83744	X		
<i>Tylostotriton yangi</i>	no voucher	X		Live specimen
<i>Tylostotriton yangi</i>	no voucher	X		Live specimen
<i>Tylostotriton zieglerei</i>	ROM35327	X	X	
<i>Tylostotriton zieglerei</i>	ROM35328	X		
<i>Tylostotriton zieglerei</i>	ROM35330	X	X	
<i>Tylostotriton zieglerei</i>	ROM35333	X		
<i>Tylostotriton zieglerei</i>	ROM35334	X	X	
<i>Tylostotriton zieglerei</i>	ROM35338	X		
<i>Tylostotriton zieglerei</i>	ROM35339	X	X	
<i>Tylostotriton zieglerei</i>	ROM35342	X		
<i>Tylostotriton zieglerei</i>	ROM35343		X	
<i>Tylostotriton zieglerei</i>	ROM35344	X		
<i>Tylostotriton zieglerei</i>	ROM35346	X		
<i>Pleurodeles waltl</i>	SMNS13466	X	X	
<i>Pleurodeles waltl</i>	SMNS1563	X	X	
<i>Pleurodeles waltl</i>	SMNS1564	X	X	
<i>Pleurodeles waltl</i>	ZSM5505-2005	X	X	
<i>Pleurodeles waltl</i>	ZSM5506-2005	X	X	
<i>Chelotriton</i> - Enspel	PW_1998-5058		X	
<i>Chelotriton</i> - Enspel	PW_1999-5000		X	
<i>Chelotriton</i> - Enspel	PW_2001-5002		X	

<i>Chelotriton</i> - Enspel	PW_2001-5010	X
<i>Chelotriton</i> - Enspel	PW_2006-5031A	X
<i>Chelotriton</i> - Enspel	PW_2010-5000	X
<i>Chelotriton</i> - Enspel	PW_2015-5163	X
<i>Chelotriton</i> - Randeck	SMNS80672	X

Table S2: 2D landmark definitions on external and osteological cranial images for geometric morphometrics analyses in pleurodeline newts.

<b>Head Dorsal View</b>	
1	Tip of snout
2-3	Medial canthus
4-5	Lateral canthus
6-7	Widest point of maxillaries
8-9	Distal point of dorso-lateral ridges
10-11	Posterior point of dorso-lateral ridges
12-13	Proximal point of dorso-lateral ridge in the posterior part
14-15	Posterior point of parotoids
16	Anterior point of vertebral ridge
17-36	Semilandmarks along snout
<b>Head Lateral View</b>	
1	Anterior naris edge
2	Anterior point of oral fissure
3	Posterior point of oral fissure
4	Medial canthus
5	Lateral canthus
6	Ventral edge of eye
7	Posterior point of dorso-lateral ridge
8-27	Semilandmarks along cranial roof
<b>Skull Dorsal View</b>	
1	Suture among premaxilla
2	Anterior suture of frontals
3	Posterior suture of frontals
4	Posterior suture of parietals
5-6	Tip of occipital condyle
7-8	Distal point of suture among frontals and parietal
9-10	Tip of pterygoid
11-12	Proximal tip of dorsolateral ridge at posterior part
13-14	Posterior end of squamosal
15-16	Distal point of processus alaris frontalis
17-18	Posterior point of prefrontal
19-20	Anterior orbit edge
21-22	Posterior point of maxilla (connection with quadratum)
23-42	Semilandmarks along maxilla and premaxilla
43-62	Semilandmarks along distal edge of squamosal



63-72

Semilandmarks along distal edge of frontal

73-82

Semilandmarks along distal edge of prefrontal

**Skull Lateral view**

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1	Posterior point of occipital condyle
2	Posterior point of squamosal
3	Posterior point of quadratojugale
4	Tip of maxillary
5	Dorsal point of maxillary in posterior half
6	Ventral point of prefrontal
7	Suture among maxillary and nasale
8	Suture among maxillary and premaxillary at naris
9	Suture among maxillary and premaxillary at tooth row
10	Anterior point of premaxillary at tooth row
11-30	Semilandmarks along cranial roof
31-40	Semilandmarks along tooth bearing edge of maxillary

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Fig. S1: Some exemplary fossil crania in dorsal view before and after retrodeformation using algorithmic symmetrisation.

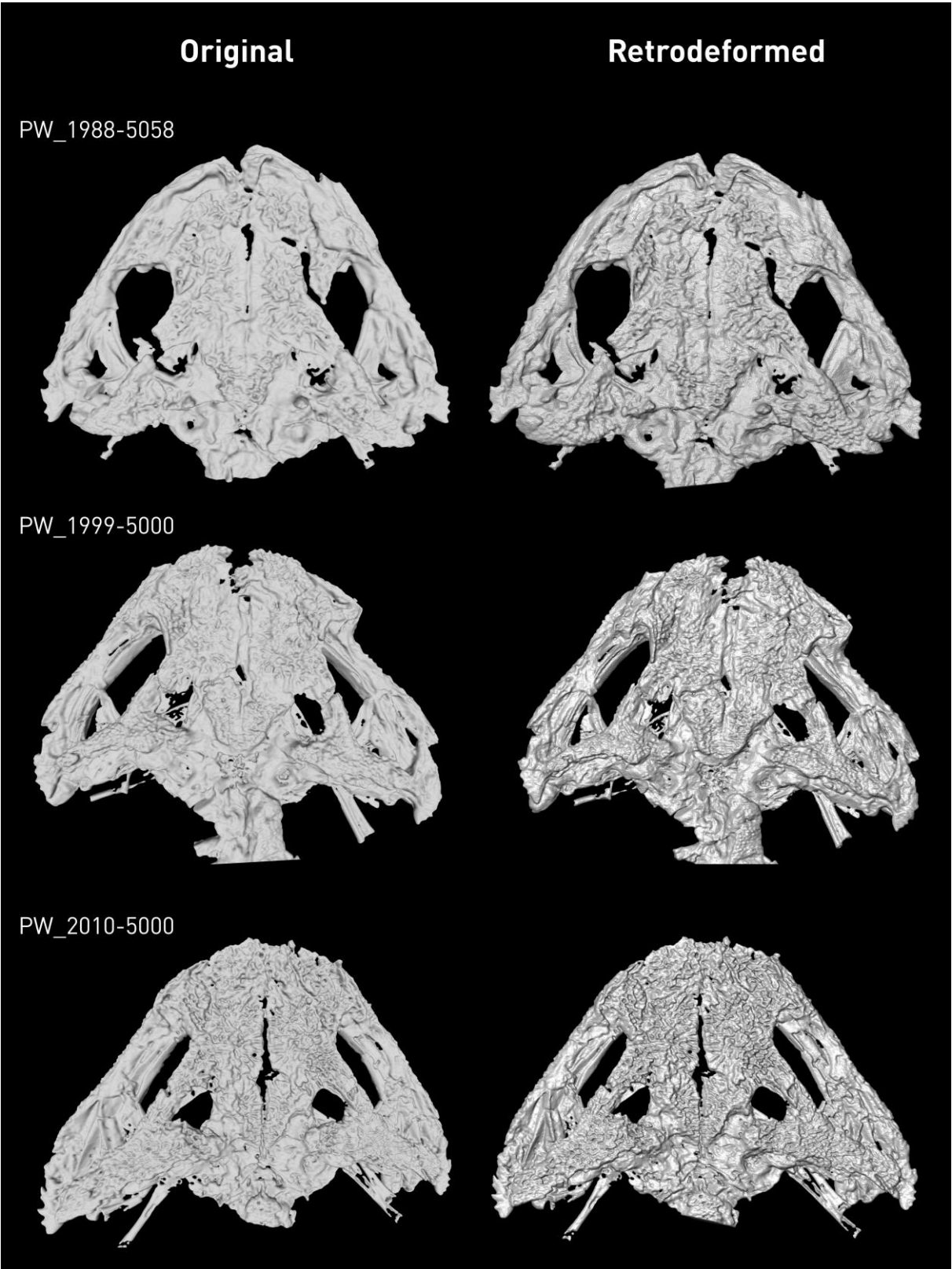


Fig. S2: PCA-morphospace along PC2 and PC4 of GPA-aligned, allometry free shapes of external cranial morphology in dorsal view of pleurodeline newts. Triangles correspond to the members of the subgenus *Yaotriton*, ellipses to subgenus *Tylototriton*, diamonds to *Echinotriton* and square to *Pleurodeles*. For colour code see figure 3.

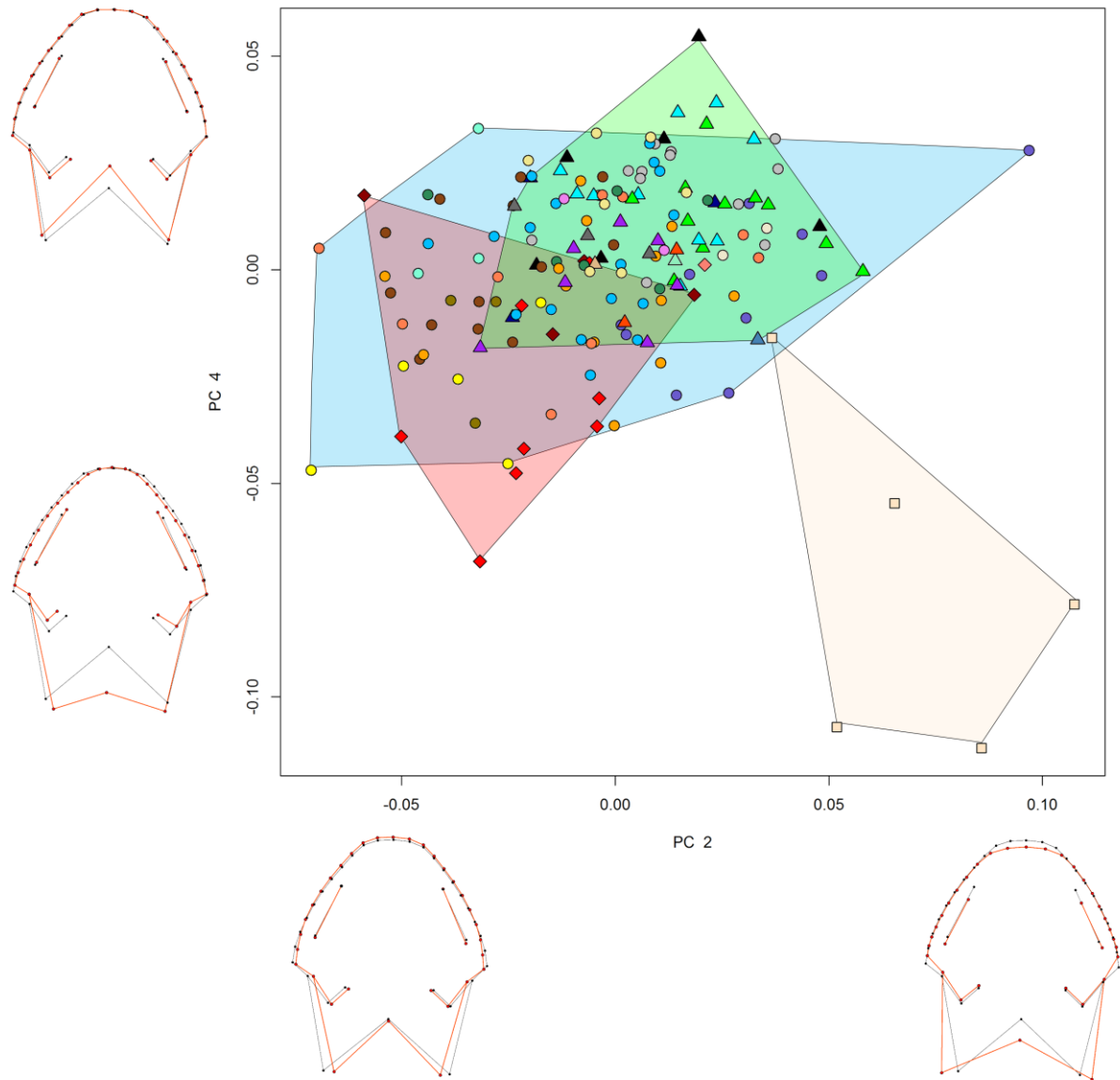
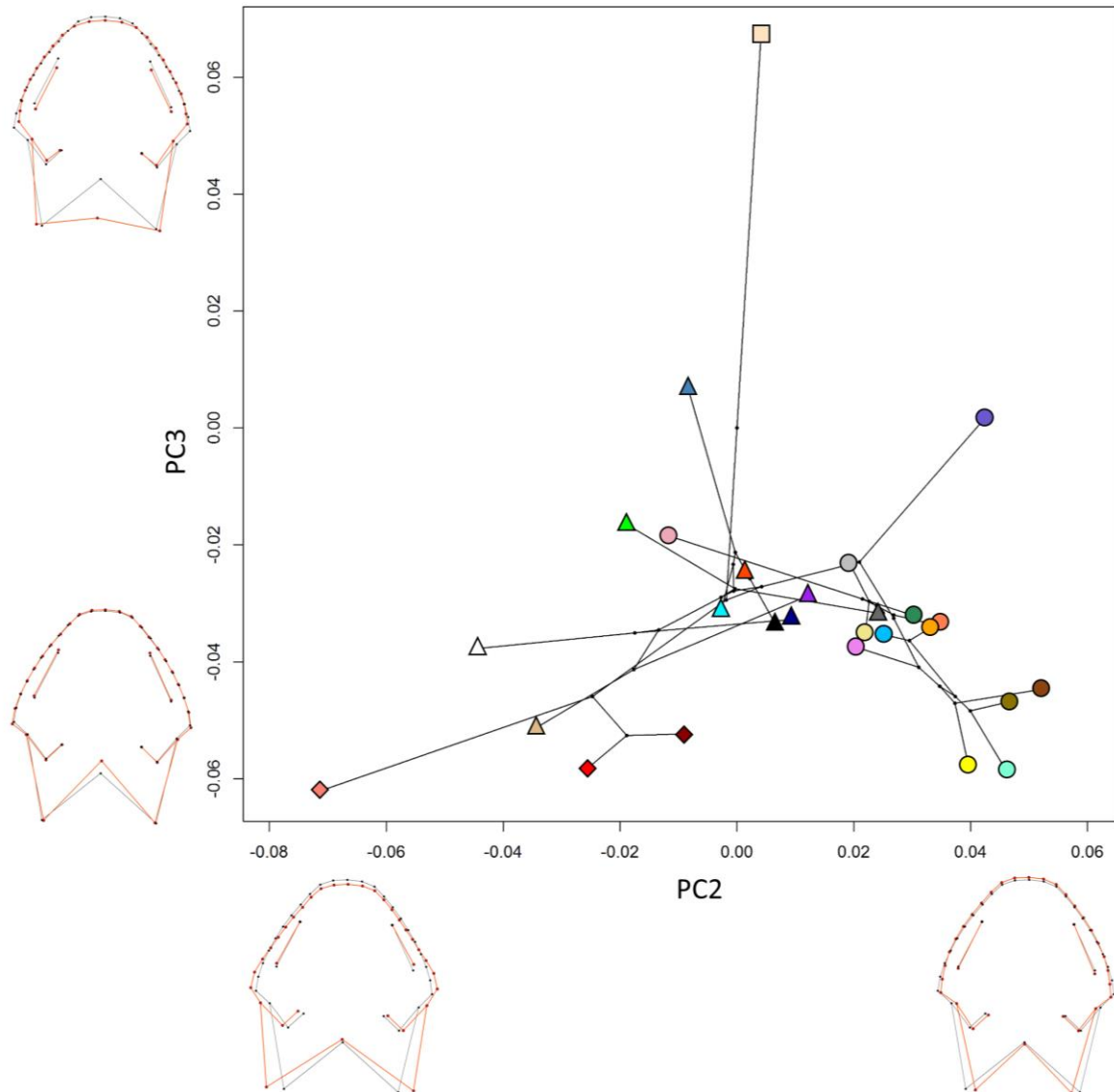


Fig. S3: PCA morphospace of species mean shapes along PC2 and PC3 of GPA-aligned, allometry free shapes of external cranial morphology in dorsal view of pleurodeline newts. Triangles correspond to the members of the subgenus *Yaotriton*, ellipses to subgenus *Tylototriton*, diamonds to *Echinotriton* and square to *Pleurodeles*. For colour code see figure 3.



Running title: Sexual dimorphism in crocodile newts

**Clasp and dance: Mating mode promotes variable sexual size and shape dimorphism trajectories in crocodile newts (Caudata: Salamandridae)**

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## Abstract

Sexual dimorphism (SD) is a main source of intraspecific morphological variation, however sexual shape dimorphism (SShD) was long time neglected in evolutionary research. Especially in cold-blooded animal groups only subtle shape differences are expressed between males and females and the selective forces behind it are poorly understood. Crocodile newts of the genera *Echinotriton* and *Tylototriton* are highly polymorphic in their reproductive ecology and hence, are a highly suitable model system to investigate potential evolutionary forces leading to SShD differences. We applied 3D geometric morphometrics to the cranial and humerus morphology of nine species of crocodile newts to investigate patterns of SShD in relation to the different mating modes. Trajectories of shape differences between males and females differ in both, cranium and humerus but mating mode does explain differences in SShD trajectories between species only in cranial morphology. Nevertheless, cranial morphology shape differed between the amplexing and circle dancing species. Hence, other selective forces must act here. Variable interspecific allometric trajectories are a potential source of shape differences whereas these trajectories are quite stable for the sexes irrespective of the species.

Keywords: *Tylototriton* – geometric morphometrics – *Echinotriton* – reproductive biology – salamander – ecology – selection – osteology

## Introduction

Sexual dimorphism (SD) subscribing marked differences between sexes such as in morphology is a common phenomenon in the animal kingdom (e.g., FAIRBAIRN et al. 2007). Different theories try to explain the causal relationship between selection and morphology, namely i. fecundity (e.g., KUPFER et al. 2004), ii. sexual selection (SHINE 1979) and iii. ecological niche partitioning (HEDRICK & TEMELES 1989, SHETTY & SHINE 2002). In context with body architecture, SD can be expressed as size dimorphism (SSD) or shape dimorphism (SShD) underlying different selection processes (e.g., SCHWARZKOPF 2005, KUPFER 2007, POGODA & KUPFER 2018). While SSD is better studied and known in many vertebrates (e.g., COX et al. 2007, LINDENFORS et al. 2007), SShD needs much more attention as it substantial influence species ecology (e.g., SHETTY & SHINE 2002, ALCORN et al. 2013, POGODA & KUPFER 2018, POGODA et al. 2020).

To understand the processes leading to different morphologies between males and females, comparative studies are needed allowing conclusions from species ecology to its

morphology. Amphibians are a vertebrate group with heavily female-biased SSD (e.g., SHINE 1979, KUPFER 2007). Nevertheless, only approximately 61% of known salamander species exhibit female-biased SSD, while about 19% exhibit a male-biased SSD (KUPFER 2007, AMAT 2019). Although less diverse in terms of species numbers, urodeles evolved various reproductive modes and behaviours including diverse life history strategies (SPARREBOOM 2014, KIEREN et al. 2018). As Salamandridae or true salamanders includes most of the variability in reproductive biology known from urodeles (SPARREBOOM 2014, FROST 2018), making the group well-suited for the investigation of SSD and SShD.

Among Salamandridae monophyletic crocodile newts evolved a variety of reproductive modes and strategies (SALVADOR & GARCÍA-PARÍS 1999, HERNANDEZ 2016, KIEREN et al. 2018). For mating either a ventral amplexus or a circular mating dance is performed either taking place in aquatic or terrestrial habitats. Also female crocodile newts deposit egg clutches either in water or on land (IGAWA et al. 2013, PHIMMACHAK et al. 2015b, PASMANS et al. 2017, GONG et al. 2018). These differences in terms of mating and reproductive ecology can even be observed within one genus *Tylototriton* comprising most crocodile newt species. While some of the traits correlate with phylogeny others do not, both patterns leading to coevolution of similar cranial shapes among crocodile newts (POGODA et al. 2020). There is only little known about SD in crocodile newts except for i.e., the study by SEGLIE et al. (2010) on *T. himalayanus* (as *T. verrucosus*), knowledge on SSD is restricted to body size and the cloaca of *Tylototriton* (see FEI & YE 2016, HERNANDEZ 2016) whereas the presence of SSD and SShD is largely unknown for other body parts. A variety of SSD and SShD patterns such as longer and wider trunks in females and longer limbs and crania in males are known in many other salamandrids (e.g., MALMGREN & THOLLESSON 1999, ROMANO et al. 2009, AMAT et al. 2015, REINHARD & KUPFER 2015, REINHARD et al. 2015, ALTUNIŞIK 2017). Consequently, it is likely that also a variety of dimorphic traits is undetected so far in crocodile newts. Especially osteology probably harbours many dimorphic characters yet unknown, as most studies concentrate on the external morphology of salamanders (IVANOVIĆ & KALEZIĆ 2012, POGODA & KUPFER 2018). Increasingly cost-efficient micro Computer Tomography ( $\mu$ CT) becomes available in many institutions, leading to an enormous increase using this technique in morphology studies (BROECKHOVEN & DU PLESSIS 2018). In a terrestrial salamandrid salamander, it was shown that the same patterns of SSD can be detected in the osteology as in the external morphology (POGODA & KUPFER 2018). Further, excluding soft tissue leads to an enormous increase of morphological structures which can be used in studying especially SShD otherwise covered. In ventral

amplecting *Pleurodeles*, the sister taxon to the crocodile newt genera *Tylototriton* and *Echinotriton*, differentially shaped humeri between sexes are known (e.g., HERRE 1952). The common ancestry of ribbed and crocodile newts and the interspecifically different reproductive strategies may imply variable SD patterns tightly linked to ecology. Understanding SSD and SShD patterns in context with phylogeny and ecology will aid understanding the evolutionary biology of salamanders.

The aim of our study was to investigate SD in crocodile newts in a comprehensive way, linking patterns to the different reproductive ecologies of species. Our focus was laid on the cranium and fore limb morphology. For limb morphology, we hypothesize that species which apply an amplexus during mating have a more pronounced SSD of their fore limbs than species mating without physical contact and that the different mating patterns lead also to differences in SShD. Male cranial morphology was different among amplexant and dancing species (POGODA et al. 2020). Thus, we assumed different interspecific patterns of SShD of crocodile newt cranial morphology applying different mating modes. We used  $\mu$ CT scans of crania and humeri of crocodile newts and employed 3D geometric morphometrics (GM) to test our hypotheses.

### **Material & Methods**

For resolving SSD and SShD, we investigated 227 crocodile newt specimens of the genera *Echinotriton* and *Tylototriton* housed in natural history collections (Supplementary Table S1) including the following: *E. andersoni* from Okinawa Island, *Tylototriton asperrimus*, *T. himalayanus*, *T. kweichowensis*, *T. shanjing*, *T. shanorum*, *T. taliangensis*, *T. uyenoii* and *T. verrucosus*. The selected species represent all major clades of crocodile newts comprising the different mating modes i.e., showing a circle dance or applying an amplexus (POGODA et al. 2020). *Tylototriton asperrimus* represent the only member of the subgenus *Yaotriton* with a sufficient sample size of both sexes whereas unfortunately not enough female specimens of other species were available in natural history collections due to a heavy male biased field sampling during the breeding season. To access osteology for SD analyses, specimens were  $\mu$ CT-scanned. CT-scans were carried out either with a Bruker SkyScan1272 with the software NRecon (Bruker CT) for reconstructions or within the X-ray imaging laboratory at the Institute for Photon Science and Synchrotron Radiation, Karlsruhe Institute of Technology (KIT) employing a microfocus x-ray tube (XWT-225, X-RAY WorX, Garbsen, Germany) and a flat panel detector (XRD 1621 CN14 ES, PerkinElmer, Waltham, USA) in combination with a custom designed mechanical sample manipulator. For the CT scans made at KIT, Octopus 8.6 (Inside Matters, Gent, Belgium) was used to perform the tomographic



reconstruction. The scan resolution was either 20.1 (SkyScan) or 21.3  $\mu\text{m}$  (KIT-custom build scanner).

To catch the entire shape variation of the cranium 45 three-dimensional (3D) landmarks were digitized and for the analysis of the humerus shape six fixed landmarks and 50 semi-landmarks in three curves were digitized (Fig.1). Prior to landmark digitization, potential error in setting landmarks was validated by digitizing one specimen five times and five additional specimens of the same species to compare consistent placement by Procrustes distance of the respective mean shapes. Landmark digitization was carried out by one author with the software Checkpoint v.2019.03.04.1102 (Stratovan Ltd.). Geometric morphometrics was performed in R version 3.6.3 (R DEVELOPMENT CORE TEAM 2019) using the packages geomorph v.3.2.1, RRPP v. 0.5.2 and Morpho 2.8 (SCHLAGER 2017, COLLYER & ADAMS 2018, ADAMS et al. 2019). Complete landmark configurations are a prerequisite for GM analyses. Hence, missing landmarks (e.g., due to anomalies or injuries) were first estimated by thin plate spline approach implemented in the function 'estimate.missing'. Semi-landmarks in the humerus dataset were equally spaced along the digitized curve. Variation due to location, rotation and scale was removed by a generalized Procrustes alignment (GPA) using the function 'gpagen' (ROHLF & SLICE 1990). In the humeri dataset, semi-landmarks were simultaneously slid using minimized bending energy (BOOKSTEIN 1997a, PEREZ et al. 2006). As asymmetry was not in the scope of this study, bilateral landmarks in cranial landmark configuration was symmetrized by averaging left and right landmark pairs. Skulls and humeri were analysed further in the same approach. A principal component analysis (PCA) on Procrustes coordinates was performed and plotted to investigate general shape variation. To account for size, we used logarithm of centroid size (CS), which represents a measure of size in GM (BOOKSTEIN 1997b, ZELDITCH et al. 2012).

A full factorial model design including species and mating mode was precluded by the model system as each species comprises only a single mating mode. Thus, several Procrustes ANOVAs had to be performed to investigate all potential sources of morphological variation. First, a Procrustes ANOVA as implemented in the function 'procD.lm' with size, species and sex including all interactions was performed. Allometry between sexes was not different, indicated by non-significant interaction between sex and CS. Thus, we explored allometric shape change by another Procrustes ANOVA including CS and sex only. To test our hypothesis that the mating mode affects the pattern of SD, we first ran a model design including only sex, mating mode and its interaction, and second a model including these factors plus CS as covariate. To explore different patterns of SShD, we performed a trajectory

analysis to visualize shape change directions between species and performed a group mean prediction with 95%-confidence intervals for males and females in each species, implemented in the function ‘predict.lm.rpp’ in the RRPP package. Sexual size dimorphism patterns between species and mating modes were estimated by a Procrustes ANOVA of species and sex on CS and, in a second one, sex and mating mode as variables. The function ‘pairwise’ was used to reveal which groups were different. According to the model, a grouping variable of sex with species or mating mode was used. Significance testing was performed using Residual Randomization by 10.000 random permutations (COLLYER et al. 2015, COLLYER & ADAMS 2018). Shape changes were visualized as TPS-grids by warping the mean shape by thin-plate spline approach with the function ‘plotRefToTarget’.

## Results

### *Size-shape correlations*

The first two principal component (PC) axes of humerus shape explained 34.5% (PC1) and 13.7% (PC2) of the observed shape variation (Fig.2). *Echinotriton andersoni* occupied a slightly different morphospace than *Tylototriton*, although there was some overlap especially with *T. uyenoi* and *T. kweichowensis*. Generally shape changes on the first PC corresponded to humerus thickness, representing thick humeri for negative PC1-scores and thin humeri for positive PC1-scores. The second PC corresponded to the thickness of the middle part of the humerus and the orientation of the Crista dorsalis humeri. According to that, *Echinotriton* exhibits more robust humerus shape compared to *Tylototriton*, *T. taliangensis* exhibiting the most gracile humerus. PC1 explained 20.1% and PC2 11.6% of the observed shape variation (Fig.3) of the PCA of cranial shape. *Echinotriton* was well separated from all other species of the genus *Tylototriton*, but *T. asperrimus* showed the highest similarity to *Echinotriton*. Among *Tylototriton* spp., *T. kweichowensis* occupied the most distinct morphospace (Fig.3). *Echinotriton*-skull shape (positive PC1 scores) showed a robust skull with a strong maxillary connection to the quadrate and pterygoid. The snout was more pointed and with lower and anteriorly ranging nasals than in all *Tylototriton*. The second PC axis corresponds to the height of the fronto-squamosal arch in relation to the skull roof, the posterior extent of the squamosal and the occiput width.

Procrustes ANOVA revealed a strong allometric effect both in humerus and cranial shape (Tab.1). The allometric trajectories differed interspecifically (Fig.4) but intersexually (Fig.5), indicated by a significant interaction of size and species but not size and sex (Tab.1). In allometric trajectories of the humerus, *T. uyenoi* and *T. shanorum* exhibited a different direction (Fig.4a) while in cranial shape, especially *T. asperrimus* showed a different pattern

(Fig.4b). Accounting for sexual allometry only, generally large specimens exhibited a thinner humerus (Fig. 5a) whereas in the cranium the dorso-lateral ridge became more elaborate and the connection of the maxillary with the pterygoid and quadrate turned more pronounced and the quadrate shifted more posteriorly (Fig. 5b).

### *Sexual dimorphism*

The Procrustes ANOVA revealed different interspecific patterns of sexual dimorphism, indicated by the significant interaction of species and sex (Tab.1). In order to test our hypothesis that the mating mode might explain different shapes, we carried out additional Procrustes ANOVAs on shape including mating mode as explanatory variable. The mating mode explained a significant amount of variation, with and without log(CS) as covariate, in the humerus and cranium (Tab.2). In general the species applying a circle dance had more robust and thicker humeri including an elaborated crista dorsalis and a higher crista ventralis compared to species employing a ventral amplexus (Fig.6). The cranium of circle dancers was wider at its occiput, exhibited a shorter frontal arch, less connection between the maxillary bone and the quadrate and pterygoid, a higher snout tip, longer vomerine tooth rows and more distal internal nares. The interaction term of mating mode with sex was significant in cranial shape when accounting for size as covariate (Tab.2 b), indicating different SShD-trajectories between the two different mating modes among identical size classes (Tab.2 b, d). We found no indication for different SShD-trajectories between mating modes in humerus shape.

As the analysis indicated different SD-trajectories, we performed a trajectory analysis with sex as grouping factor to figure out and illustrate shape changes in different trajectories across male and females between species (Fig.7, 8). The cranium showed more diverse SD-trajectories than humerus shape did. *Echinotriton andersoni* did not differ markedly from *Tylototriton* spp. In the latter, cranial SD-trajectories showed contrary directions between some species (Fig.8). Pairwise species-comparison revealed only one pair of species (*T. asperrimus* : *T. taliangensis*) with an alpha-level below 5% and six species pairs below 10% for humerus SShD patterns and two species pairs below 5% and 10% (*T.himalayanus*: *T. kweichowensis* and *T. shanjing* : *T. verrucosus*), respectively in SShD patterns of cranium shape (Supplementary Tab. S2). To illustrate the different male to female SD-trajectories we plotted TPS-grids for amplexing *T. himalayanus* and circle dancing *T. kweichowensis*, both species deviating strongly in SD-trajectories (Fig.7, 8). In *T. himalayanus*, the humerus turned thinner in the middle part while the distal end was more twisted in females. Further, the crista ventralis was slightly more pronounced in females. In *T. kweichowensis* especially the crista dorsalis appeared more elaborate in females (Fig. 7). Male to female shape changes in

cranium morphology of *T. himalayanus* included an elaborated squamosal bony ridge, a posterior shift of the quadrate, a stronger connection of the maxillary with quadrate and pterygoid, lower nostrils and a shorter frontal arch. In *T. kweichowensis* cranial shape changes between sexes were much less pronounced and comprised a posterior shift of the quadrate and a slightly posteriorly shift of the palatal fissure between the vomers. Shape changes from the mean shape to male and female shape, respectively, were similar in the humerus, but differed between males and females in their extent, whereas cranial shape changes to the mean deviated between sexes of *T. himalayanus* but not in *T. kweichowensis* (Fig. 9).

Procrustes ANOVA on humerus and cranium log(CS) of species and sex revealed interspecific but also intersexual differences in size (Tab.3 a, b). Further, SSD differed between species indicated by a significant interaction of species and sex (Tab.3 a, b) . Analysis of the effect of mating mode on size yielded no general size differences in the humerus between dancing and amplexing species but the interaction of sex and mating mode was close to significance level (Tab.3 c) which would indicate differences of SSD patterns between mating modes. Cranial size and SSD-patterns do differ between mating modes (Tab.3 d). Pairwise comparisons showed that male and females of amplexing species differ ( $Z=2.04$ ,  $p=0.019$ ) in cranial size while this is not the case for circle dancers ( $Z=-1.76$ ,  $p=0.96$ ). For the humerus, the same pattern applies but the effect size between amplexing males and females is only close to significance ( $Z=1.53$ ,  $p=0.066$ ).

## Discussion

We applied 3D geometric morphometrics on humerus and cranial osteology in order to reveal patterns of SD in crocodile newts, a group of largely understudied Asian salamandrid salamanders with polymorphic reproductive strategies. We are aware that our sample size of some of the studied species and/or sex was relatively small, mainly attributable to the overwhelming male bias of crocodile newts housed in natural history collections sometimes with a male/female ratio of about 30/1 but GM was proved in being well capable of revealing even quite subtle shape differences (e.g., BLANCO & GODFREY 2006, ABDEL-RAHMAN et al. 2009, ARENDT 2010, IVANOVIĆ & KALEZIĆ 2012, POGODA & KUPFER 2020). Our study does add new insights into the evolutionary forces leading to morphological differences between the sexes in urodeles.

We hypothesized crocodile newts evolved different patterns of SSD and SShD in relation to their variable reproductive ecologies and mating behaviours. Crocodile newt humeri exhibited SSD and SShD also differing interspecifically. Humerus shape, but not size, differed between amplexing and dancing species but sexual shape changes between males

and females did not differ between mating modes whereas this appears to be the case for sexual size differences. Post-hoc analysis indicated that amplexant species exhibit size differences between males and females while dancing species showed no SSD. The analysis of cranial morphology revealed SSD and SShD and the intersexual shape and size changes differed interspecifically. The mating mode correlated well with different cranial shape and size and different trajectories of SSD and SShD. Again, the sexes of amplexing species differed in size while dancing species did not. Thus, we confirmed our hypothesis that the different reproductive ecologies of crocodile newts lead (at least partly) to different evolutionary trajectories of SD.

Pleurodeline newts comprise a diverse monophyletic group of salamandrid salamanders (e.g., VEITH et al. 2018, WANG et al. 2018) thus, being important in the reconstruction and understanding of the evolutionary processes forming SD among Salamandridae (POGODA & KUPFER 2018). So far, SD was rarely investigated in crocodile newts (SEGLIE et al. 2010, PHIMMACHAK et al. 2015b). Such studies are regularly descriptive and limited to a few body measurements such as snout-vent length, cloacal size and some others (e.g., KHATIWADA et al. 2015, FEI & YE 2016, HERNANDEZ & HOU 2018) often with low sample sizes. There is even less known about SD in the osteology of urodeles in general (IVANOVIĆ & KALEZIĆ 2012, POGODA & KUPFER 2018). Researchers just started exploring this field of morphology research accessed mainly by modern non-invasive CT technology. Sexual shape dimorphisms in the cranium of other newts and salamanders comprise differences in vomer length, quadrate position, occipital region size, skull width and snout shape (IVANOVIĆ et al. 2008, IVANOVIĆ & KALEZIĆ 2012, ALARCÓN-RÍOS et al. 2017, POGODA & KUPFER 2020). These shape changes were also present in crocodile newts but they additionally differ in the extent of the squamosal bone.

In salamandrids, it has been shown that allometric trajectories differ between taxonomic units allowing higher flexibility for shape evolution (IVANOVIĆ et al. 2007, CVIJANOVIĆ et al. 2014, IVANOVIĆ & ARNTZEN 2017). Even within populations of a species, differences in allometries can be traced (IVANOVIĆ & KALEZIĆ 2012). Most of the pleurodeline newt species showed similar allometric trajectories but some were able to evolve into a different direction. Although there was a high flexibility in allometries across different genera, similar allometric shape changes were observed throughout e.g., the pterygoid, quadratum and the decrease of the occipital region (IVANOVIĆ et al. 2012, IVANOVIĆ & KALEZIĆ 2012). Apparently the anterior skull shape was therefore more flexible. We found similar allometric trajectories between males and females in all species. In some populations

of selected European newts, allometries differ between males and females (IVANOVIĆ et al. 2008) whereas in the most basal true salamander this is not the case (POGODA & KUPFER 2020). In general put on record, if selection acts on size in one sex, this can lead to shape differences due to allometric shape changes during growth (IVANOVIĆ & KALEZIĆ 2012, POGODA & KUPFER 2020). Complex interactions of different allometric trajectories between species and sexes make it complicated to pinpoint individual selective mechanisms. The different allometric trajectories between species likely lead at least partly to differences in shape as well as size differences in cranium and humerus occur in the different newt species. Thus, SShD-patterns can vary based on the allometric shape changes and the degree of SSD in a species. Further, some body parts in urodeles are rather less strongly ossified right after metamorphosis e.g., including the cranium, metacarpals and –tarsals but they ossify during ontogenetic growth. This could explain further shape variation in larger crania. Crocodile newts exhibit elaborated bony ridges and ornamentation on the cranial skeleton which may increase with age and size, leading to more elaborated squamosal bony ridge at the posterior part. Increasing connection with size of the maxillary with the quadratum and pterygoid can likely be attributed also to the ongoing ossification during life.

Different shape changes from males to females between populations were already indicated in other salamandrids like *Lissotriton* (IVANOVIĆ & KALEZIĆ 2012), *Ichthyosaura* (IVANOVIĆ et al. 2009), *Salamandra* (ALARCÓN-RÍOS et al. 2017) and *Salamandrina* (ROMANO et al. 2009, POGODA & KUPFER 2020). Different ecological selective forces each population of a species endures at its specific site, likely play a major role on the maintenance of subtle shape differences between species (KALEZIĆ et al. 1992, SCHÄUBLE 2004, ANGELINI et al. 2015). Crocodile newts inhabit a large distribution area from Nepal to Japan, including a variety of habitats from lowland rainforest to temperate, mountainous grasslands (HERNANDEZ 2016, WANG et al. 2018) forcing specific adaptations to those contrasting environments. On the other hand, this group of salamanders showed high conservatism in their macro-ecological differentiation (HERNANDEZ et al. 2018) likely leading to little shape differentiation between species as well (POGODA et al. 2020).

Sexual selection and sex roles during reproduction are a main force forming body shapes (see DARWIN 1871 and also FAIRBAIRN et al. 2007). Previously, we found already that the mating mode partly explains shape variation in crocodile newts (POGODA et al. 2020). While the mating mode is explaining the different SSD and SShD-trajectories of cranial morphology between species, it does not apply to the humerus, although they differ in shape between mating modes. As both sexes of amplexing species respond differently in cranial

shape changes if referenced accordingly to the mean cranial shape but not in the humerus (Fig. 9) differences of SD-trajectories of the cranium but not humerus morphology might be explained. Further, as the cranium is much more complex build it provides more possibilities for shape variation than the humerus does. Only amplexing species exhibit SSD in the body parts investigated herein. Often it is tried to explain cranial shape differences with differences in food niches (SHINE 1989, SHETTY & SHINE 2002, IVANOVIĆ & KALEZIĆ 2012, REINHARD & KUPFER 2015) however our knowledge on food niche differences in salamanders is incomplete and a definite association of cranial shape differences with food niches is yet untested. No intersexual differences in food allocation were found in *T. podichthys* the only studied crocodile newt in terms of trophic ecology (PHIMMACHAK et al. 2015b). Size differences and slender humeri shape likely facilitate clasping (compare to *Pleurodeles*) while more robust and ossified fore limbs provide better standing during circular dancing. This is contradictory to patterns found in European newts applying also a stereotypic courtship behaviour. Among European newts males regularly bear longer fore limbs (e.g., MALMGREN & THOLLESON 1999, ÇIÇEK et al. 2011, REINHARD & KUPFER 2015). We demonstrated that different selective forces are acting differently on the crocodile newt taxa inducing interspecifically different allometric trajectories while the trajectories were constant between the sexes.

We conclude that morphological adaptations between species take place in correlation with and probably adaptation to their occupied environments which include large variation from the tropics to temperate climate zones while the selective forces stay more or less constant in the entire group as sex roles do not diverge, although some aspects of reproduction and courtship adapted. The interaction of variable allometric trajectories of species and sexes might partly explain the interspecific differences in SD patterns observed. Additionally, ecological parameters might influence strength and direction of SD across populations. The mating mode is to some degree an explanatory variable of the interspecific cranial shape variation in the SD-trajectories. The humerus differed with mating mode but other selective forces shape SD-trajectories of this specific body part. However, to understand evolutionary processes future studies especially on the ecology of the enigmatic crocodile newts are needed. Unfortunately no information on ecology from the many recently described species is available and observations in captivity are often based on individuals of uncertain genetic identity. It will become more crucial to focus more on the intraspecific morphological differences applying an integrative approach for future taxonomic research on crocodile newts. Especially the subgenus *Yaotriton* includes genetically distinct lineages not accessed

accurately so far (e.g., WANG et al. 2018, BERNARDES et al. 2020) but these appear very conservative in morphological differentiation (see POGODA et al. 2020). Numerous species were just recently identified based on molecular data leading to an enormous increase in species numbers over the last decade (STUART et al. 2010, SHEN et al. 2012, NISHIKAWA et al. 2013a, NISHIKAWA et al. 2013b, HOU et al. 2014, NISHIKAWA et al. 2014, YANG et al. 2014, KHATIWADA et al. 2015, LE et al. 2015, PHIMMACHAK et al. 2015a, QIAN et al. 2017, GRISMER et al. 2018, GRISMER et al. 2019, ZAW et al. 2019, BERNARDES et al. 2020). However morphological distinct characters are hard to identify and often appear somehow descriptive summarising differences of particular body part proportions based on a few specimens without any accounting for SD. Many of the diagnostic characters differentiating species are likely not valid if a larger sample size is considered and an integrative morphological approach is employed.

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### **Author contributions**

P.P – study design, specimen loans, CT-scanning, data collection, data analysis, data interpretation, drafting manuscript. M.Z. – CT-scanning, data collection. T. B. – CT-scanning. A.K. – study design, specimen loans. All authors edited and approved the manuscript draft.



## Tables

Table 1: Results of a Procrustes ANOVA on 3D morphometric shape data of the humerus (a) and cranium (b) of crocodile newts (*Echinotriton* and *Tylototriton*) to test for size-shape allometric relationship and shape differences between species and sex. Significant p-values are given in bold.

	Df	SS	F	Z	P
<b>(a) Humerus shape</b>					
Size	1	0.052	12.2422	5.1659	<b>&lt;0.0001</b>
Species	8	0.52131	15.3404	13.493	<b>&lt;0.0001</b>
Sex	1	0.052	12.2419	5.9927	<b>&lt;0.0001</b>
Size × Species	8	0.06435	1.8935	3.6992	<b>0.0002</b>
Size × Sex	1	0.0054	1.2712	0.7827	0.2173
Species × Sex	8	0.0515	1.5155	2.6089	<b>0.0058</b>
Size × Species × Sex	8	0.04296	1.2642	1.3734	0.0835
Residuals	190	0.80709			
<b>(b) Cranium shape</b>					
Size	1	0.07857	24.2181	8.9468	<b>&lt;0.0001</b>
Species	8	0.47169	18.1735	21.2585	<b>&lt;0.0001</b>
Sex	1	0.00659	2.0323	2.6806	<b>0.0037</b>
Size × Species	8	0.04496	1.7323	5.0003	<b>&lt;0.0001</b>
Size × Sex	1	0.00314	0.9666	0.0356	0.4806
Species × Sex	8	0.03238	1.2476	2.0453	<b>0.0226</b>
Size × Species × Sex	8	0.03127	1.2049	1.5578	0.0603
Residuals	191	0.61967			

Table 2: Results of Procrustes ANOVA on 3D morphometric shape data of the humerus (a,b) and cranium (c,d) of crocodile newts (*Echinoscriton* and *Tylotriton*) to test mating mode as a source of different SShD-patterns between species. Significant p-values are given in bold.

Humerus shape						Cranium shape					
	Df	SS	F	Z	P	Df	SS	F	Z	P	
<b>(a) Shape</b>						<b>(c) Shape</b>					
Sex	1	0.07468	12.0665	5.1554	< <b>0.0001</b>	1	0.02077	3.9271	4.0012	<b>0.0002</b>	
Mating mode	1	0.1398	22.5875	6.4138	< <b>0.0001</b>	1	0.08331	15.7536	7.8498	< <b>0.0001</b>	
Sex × Mode	1	0.00809	1.3074	0.802	0.2134	1	0.00494	0.9348	-0.0401	0.5012	
Residuals	222	1.37403				223	1.17926				
<b>(b) Size as covariate</b>						<b>(d) Size as covariate</b>					
Size	1	0.052	8.8207	4.527	< <b>0.0001</b>	1	0.07857	16.1129	7.8370	< <b>0.0001</b>	
Sex	1	0.08462	14.3539	5.5015	< <b>0.0001</b>	1	0.01238	2.5378	2.855	<b>0.0035</b>	
Mating mode	1	0.13052	22.1391	6.4716	< <b>0.0001</b>	1	0.06952	14.2569	7.7197	< <b>0.0001</b>	
Sex × Mode	1	0.00851	1.4431	1.0257	0.1541	1	0.00901	1.8474	1.9898	<b>0.0292</b>	
Size × Mode	1	0.02488	4.2202	3.2723	<b>0.0003</b>	1	0.03158	6.4757	5.6303	< <b>0.0001</b>	
Size × Sex	1	0.00603	1.0235	0.2734	0.3943	1	0.00819	1.6791	1.6685	0.0531	
Size × Sex × Mode	1	0.00481	0.8154	-0.2323	0.5914	1	0.01111	2.2793	2.556	<b>0.0083</b>	
Residuals	218	1.28523				219	1.06792				

Table 3: Results of a Procrustes ANOVA on 3D morphometric shape data of the humerus (a) and cranium (b) of crocodile newts (*Echinotriton* and *Tylototriton*) to test for SSD and mating mode as a potential selection force for different SSD-patterns between species (c, d). Significant p-values are given in bold.

	Df	SS	F	Z	P
<b>(a) Humerus size</b>					
Species	8	2.0433	38.2325	6.8966	<b>&lt;0.0001</b>
Sex	1	0.193	28.8894	2.0828	<b>&lt;0.0001</b>
Species × Sex	8	0.1218	2.2799	1.7555	<b>0.0228</b>
Residuals	208	1.3895			
<b>(b) Cranium size</b>					
Species	8	2805.8	38.9619	6.9389	<b>&lt;0.0001</b>
Sex	1	813.7	90.3948	2.5681	<b>&lt;0.0001</b>
Species × Sex	8	164.1	2.2787	1.7348	<b>0.0249</b>
Residuals	209	1881.3			
<b>(c) Humerus size with mating mode</b>					
Sex	1	0.1617	10.2769	1.62461	<b>0.0018</b>
Mating mode	1	0.0325	2.0677	0.91544	0.1491
Sex × Mating mode	1	0.0608	3.8643	1.18851	0.053
Residuals	222	3.4927			
<b>(d) Cranium size with mating mode</b>					
Sex	1	1045.9	55.5155	2.3744	<b>&lt;0.0001</b>
Mating mode	1	275.3	14.6103	1.7744	<b>&lt;0.0001</b>
Sex × Mating mode	1	142.5	7.5664	1.4634	<b>0.0064</b>
Residuals	223	4201.2			

## Figures

Figure 1: Definition of three-dimensional fixed landmarks (red) and semi-landmarks (yellow) set on the cranium and humerus of crocodile newts (genera *Tylotriton* and *Echinotriton*) for the geometric morphometrics analysis of SD. Cranium of SMF1134 (male *T. verrucosus*) and humerus of ZSM0830-2012 (female *T. himalayanus*) derived from mCT data.

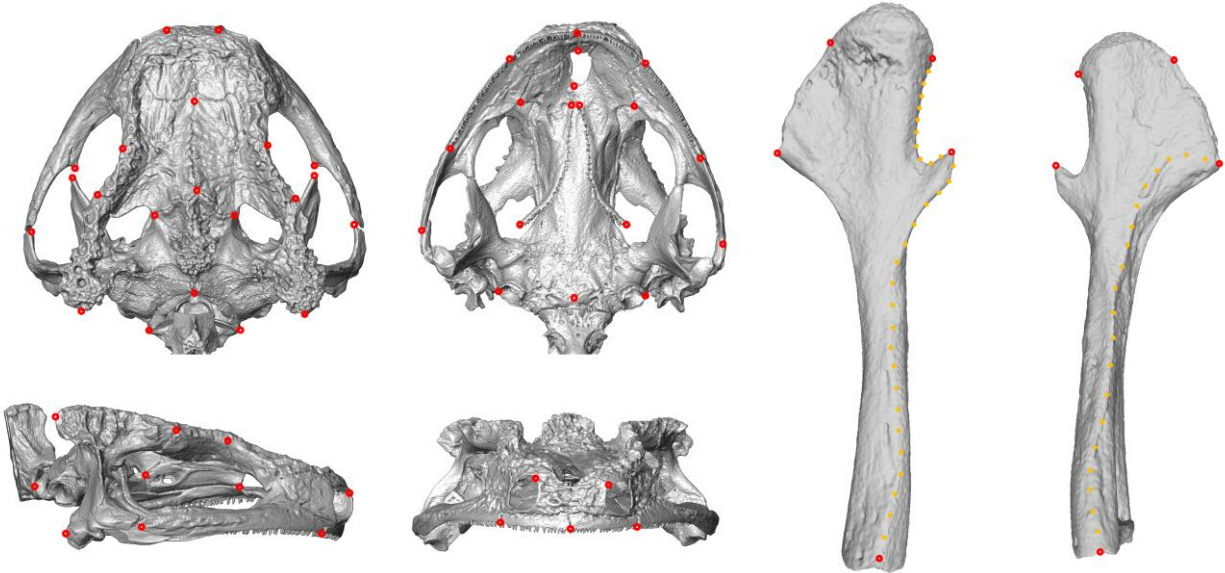


Figure 2: Morphospace of humerus shape of crocodile newts (*Tylototriton* spp. and *Echinotriton andersoni*) build by the first PC axes of a PCA of 56 GPA-aligned 3D landmarks. Shape changes at the minimum and maximum of each axis are presented as TPS-deformed grids. Colour code correspond to settings in subsequent figures.

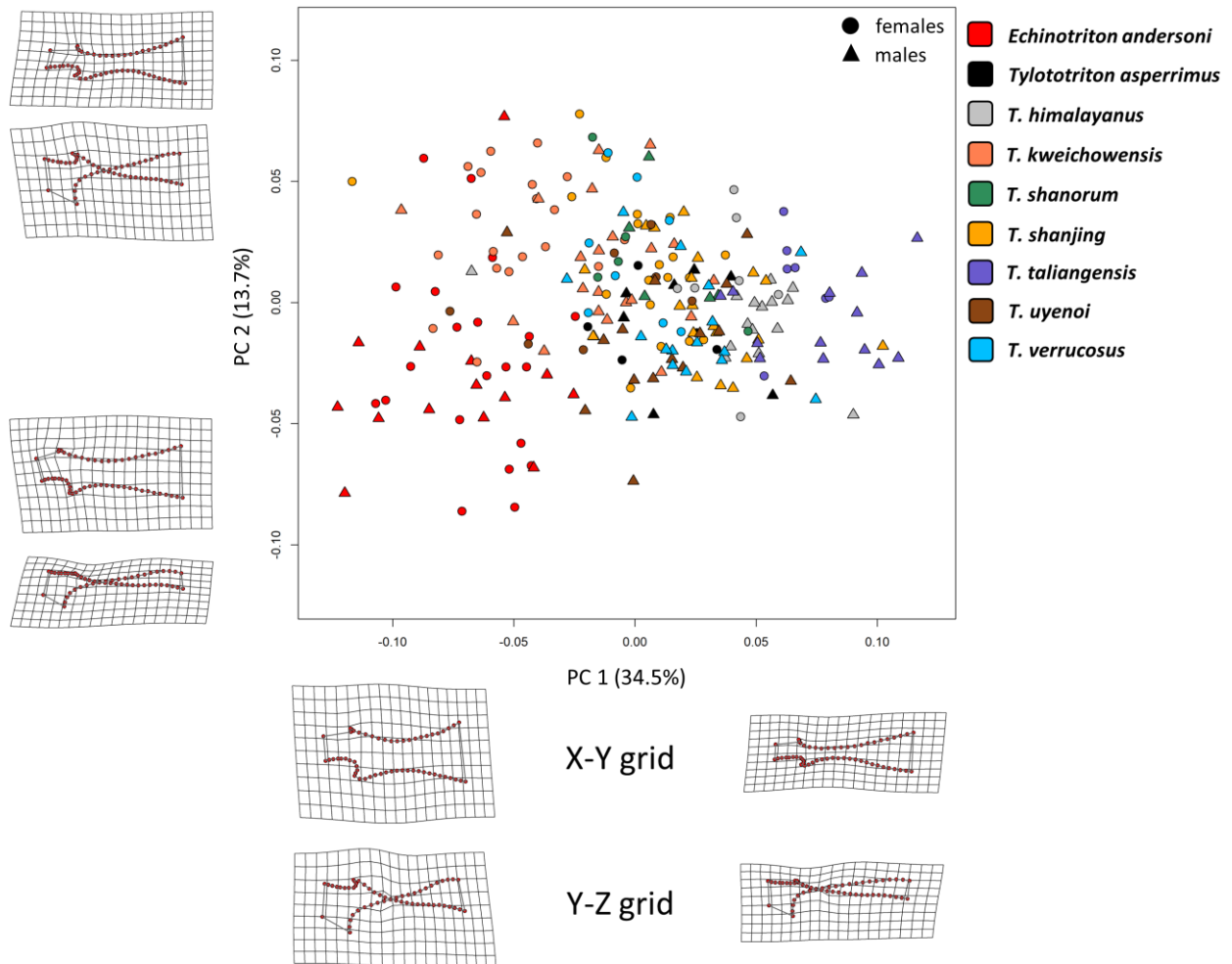


Figure 3: Morphospace of cranial shape of crocodile newts (*Tylototriton* spp. and *Echinotriton andersoni*) build by the first PC axes of a PCA of 45 GPA-aligned 3D landmarks. Shape changes at the minimum and maximum of each axis are presented as TPS-deformed grids from the mean shape. For colour coding see Figure 2.

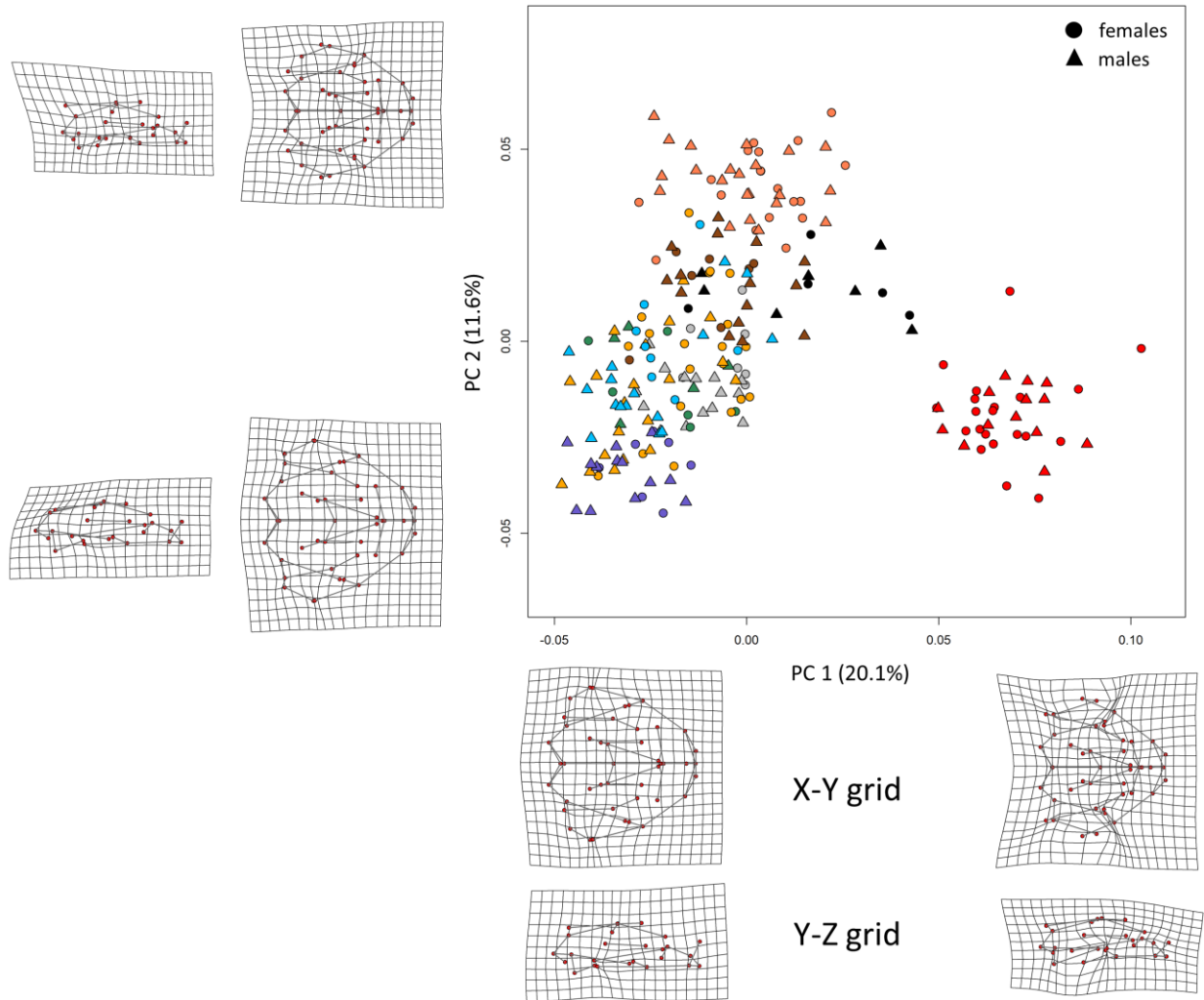


Figure 4: Unique allometry in humerus (a) and cranium (b) shape of nine species of crocodile newts (*Echinotriton andersoni* and *Tylototriton* spp.) estimated by multivariate regression. Shape changes to the mean shape are presented as TPS-deformation grids for the largest (upper) and smallest (lower) fitted value. For colour coding see Figure 2.

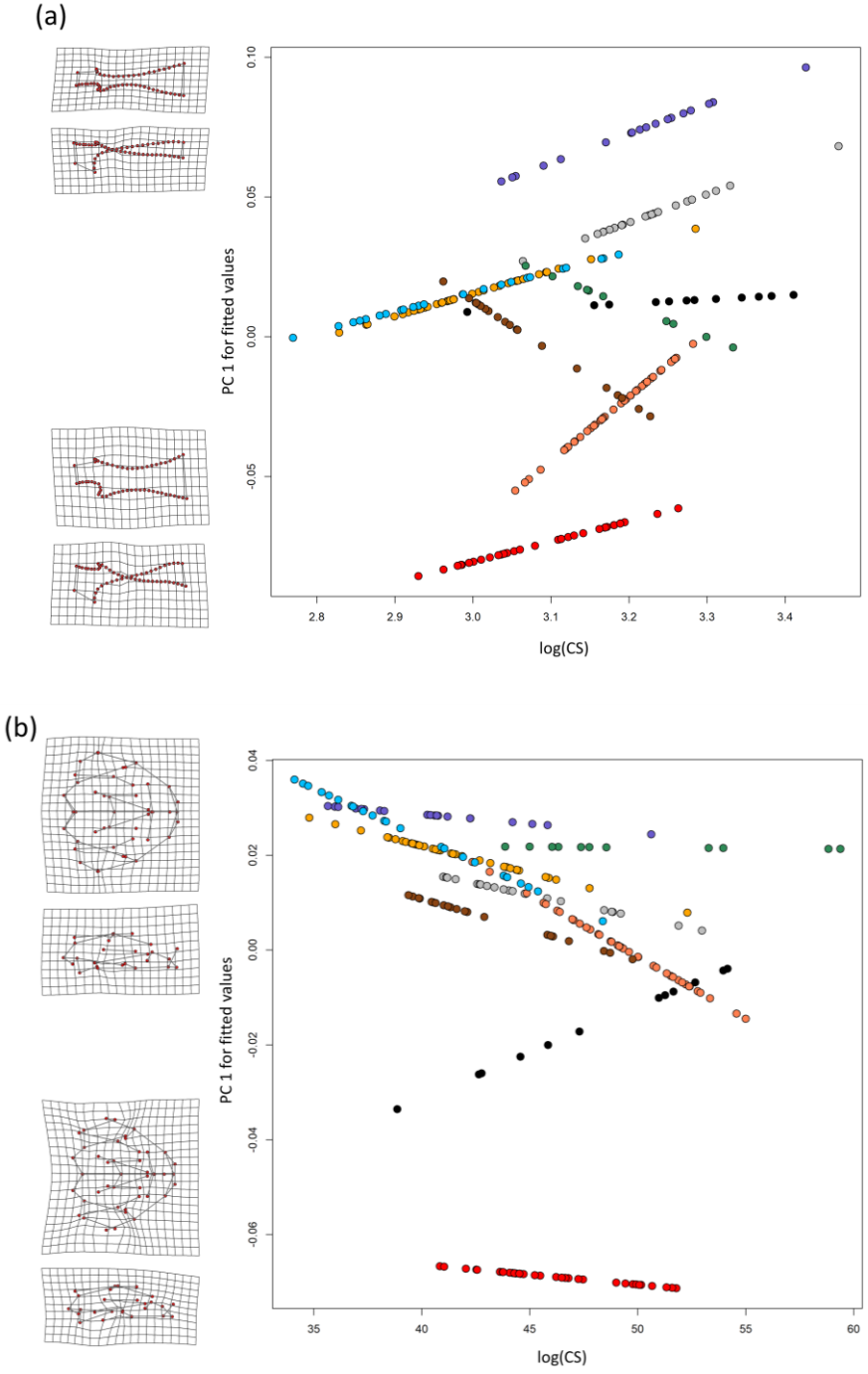


Figure 5: Common allometry in humerus (a) and cranium (b) shape of crocodile newts (*Tylotriton* spp. and *Echinotriton andersoni*) of females and males estimated by multivariate regression. Shape changes to the mean shape are presented as TPS-deformation grids for the largest (upper) and smallest (lower) fitted value. The shape changes for the humerus are magnified by the factor of three. For colour coding see Figure 2.

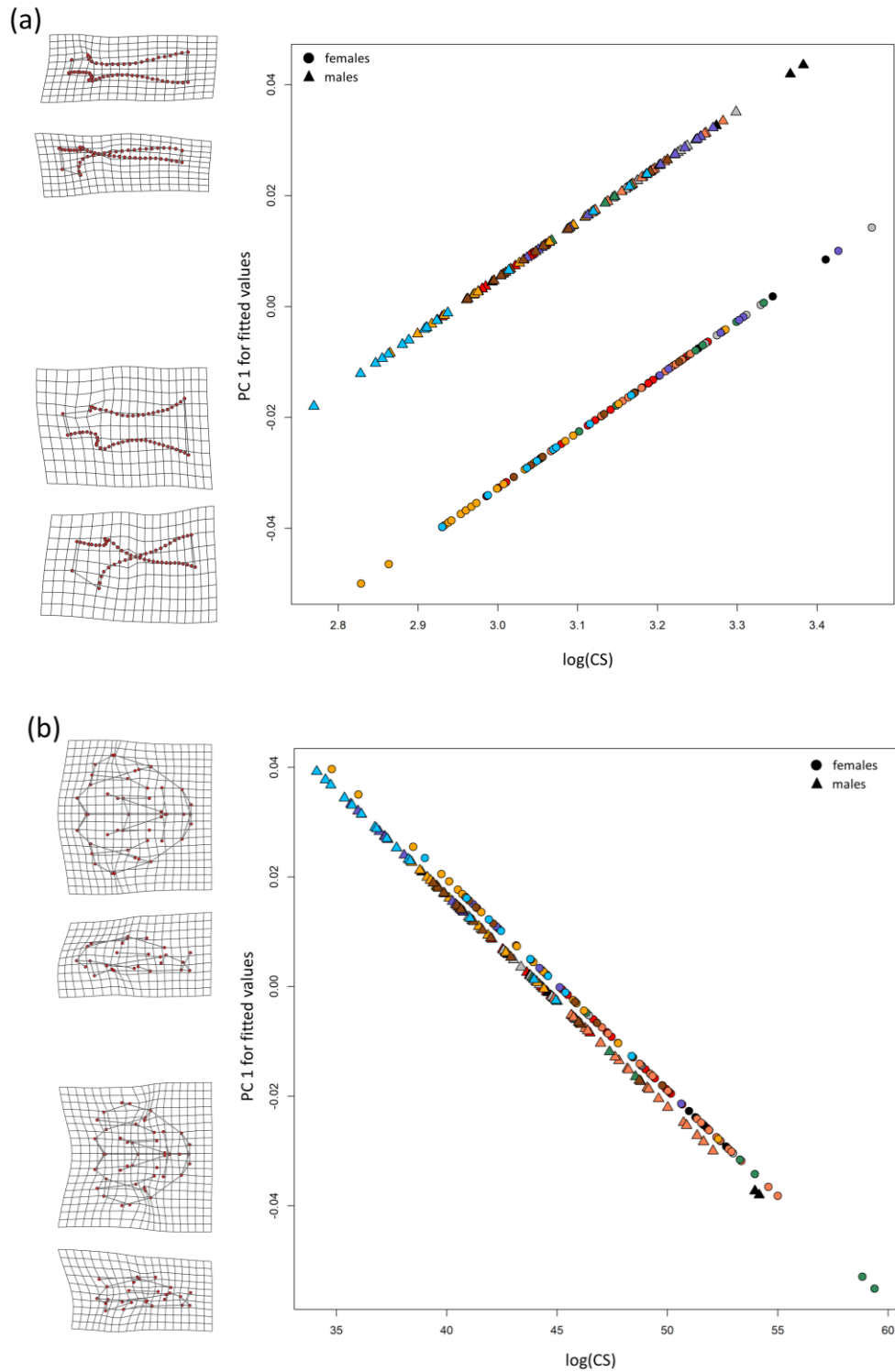




Figure 6: TPS-deformation grids from the mean shape (reference) to the different mating modes (target: circle dance, amplexus) of crocodile newts (*Tylotriton* spp. and *Echinotriton andersoni*) of the humerus (upper rows) and cranial shape (lower rows). The shape changes are magnified by the factor of three.

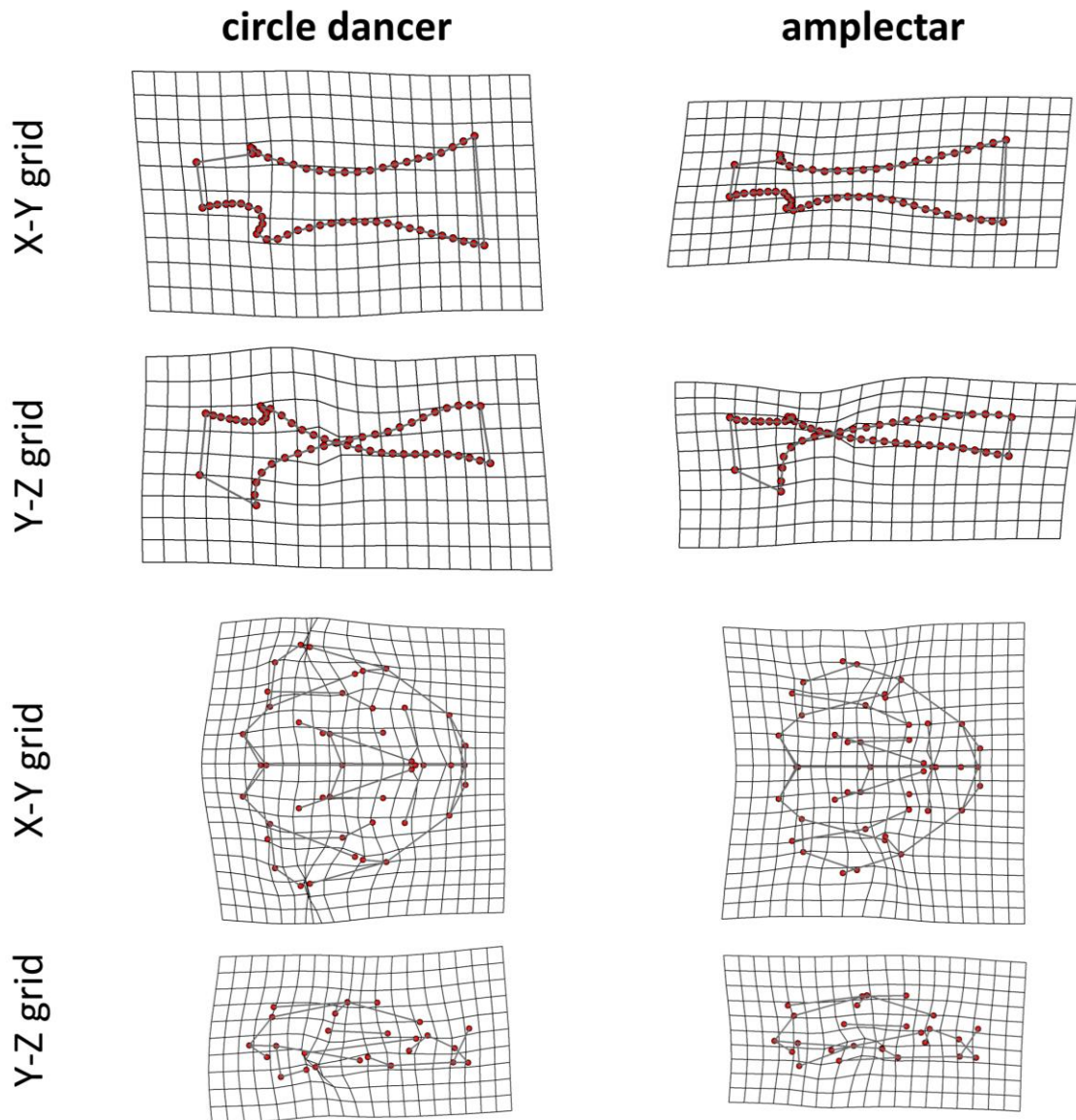


Figure 7: Trajectory analysis of SD in humerus shape of crocodile newt (*Tylototriton* spp. and *Echinotriton andersoni*) for whole data set (upper left) and for mean shape predictions for each sex and species (upper right). TPS-deformation grids of two exemplary species with different trajectories are illustrated in the lower rows. Those trajectories are marked in the upper graphs by an arrow indicating the direction from male to female. Shape changes for those are shown from male (reference) to females (target). The shape changes are magnified by the factor of two. For colour coding see Figure 2.

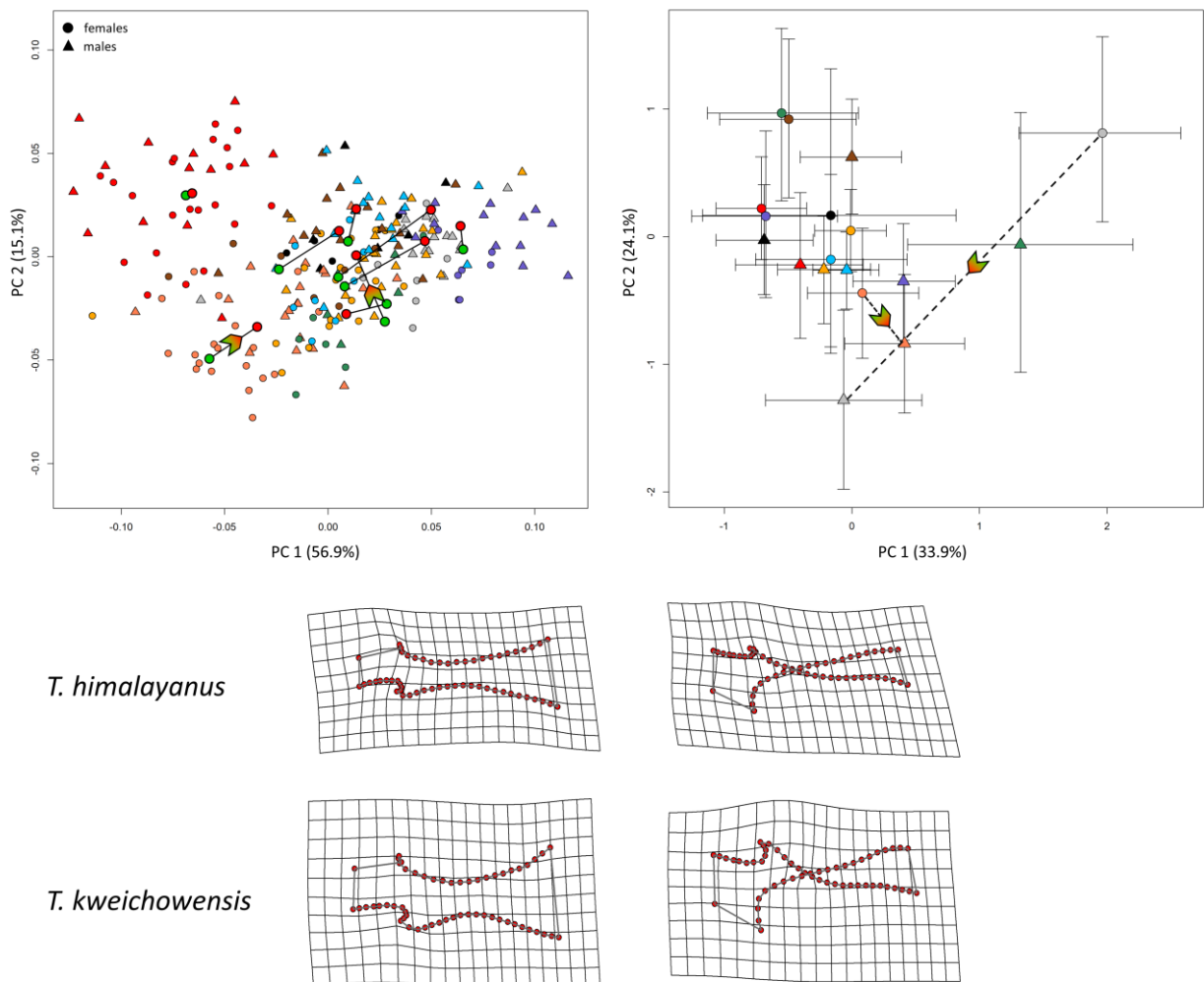


Figure 8: Trajectory analysis of SD in cranial shape of crocodile newts (*Tylototriton* spp. and *Echinotriton andersoni*) for whole data set (upper left) and for mean shape predictions for each sex and species (upper right). In the lower row TPS-deformation grids of two exemplary species with different trajectories are illustrated. Those trajectories are marked in the upper graphs by an arrow indicating their directions. Shape changes for those are shown from male (reference) to females (target). The shape changes are magnified by the factor of three. For colour coding see Figure 2.

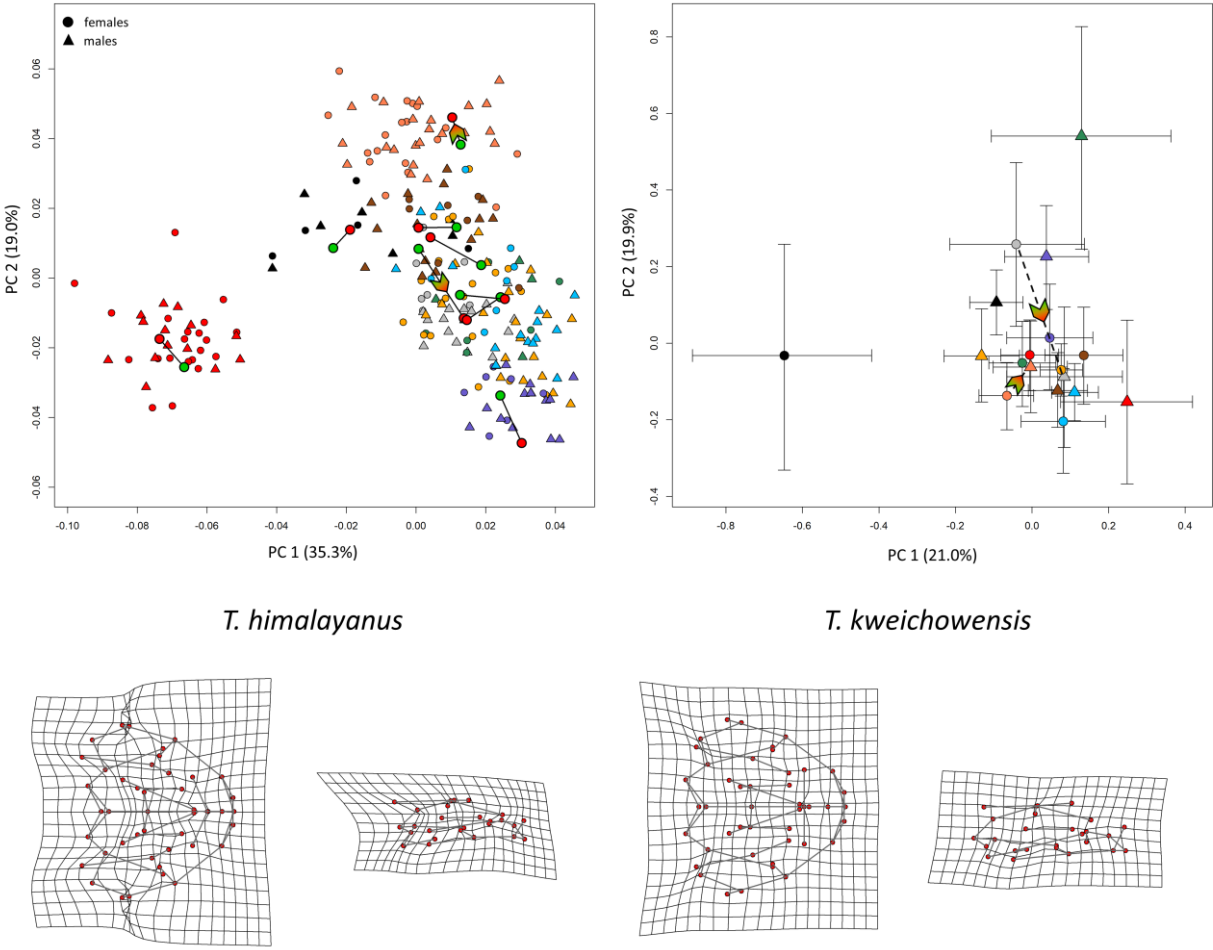
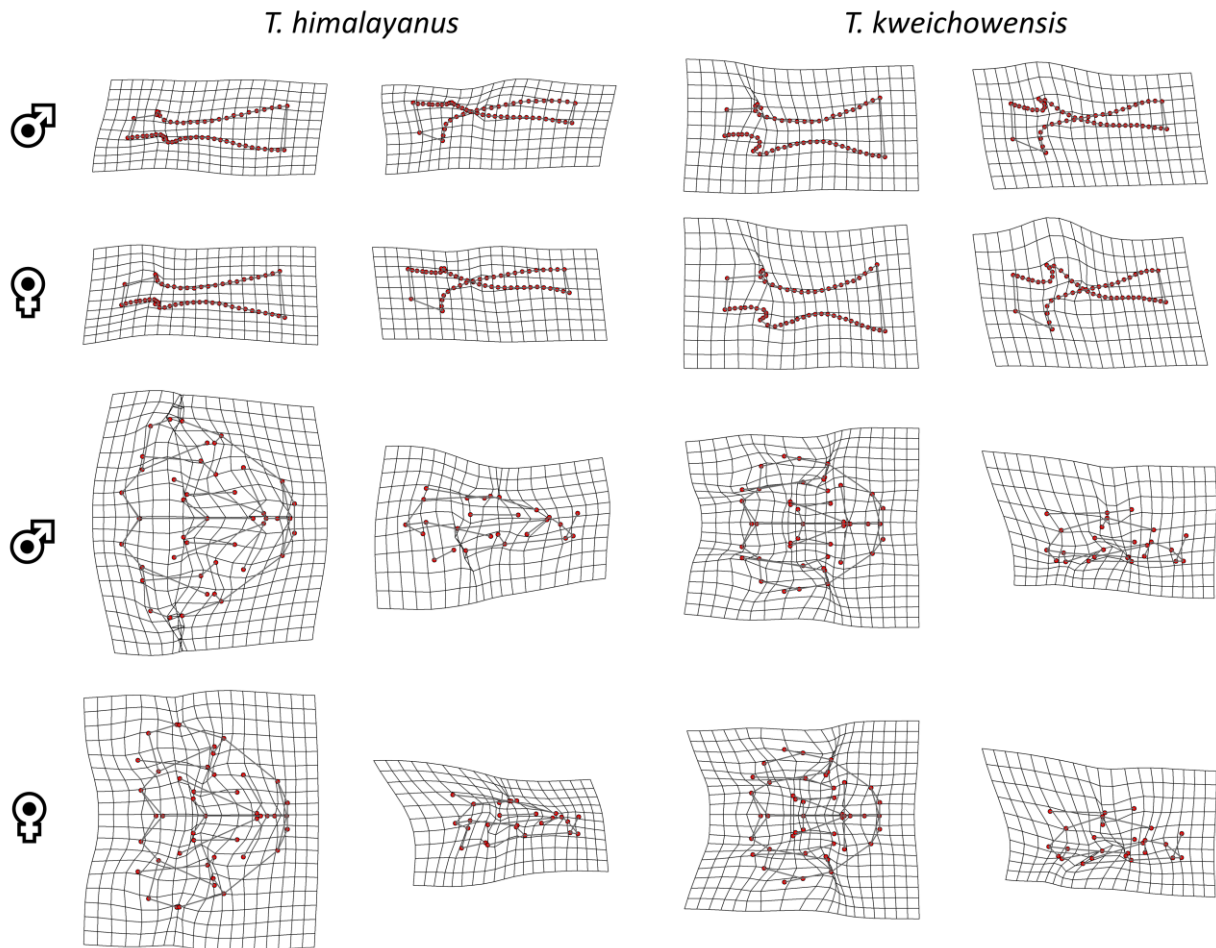


Figure 9: TPS-deformation grids from the mean shape (reference) to male and female shapes (target) of *Tylototriton himalayanus* and *T. kweichowensis* for the humerus (upper rows) and cranium (lower rows). The selected species represent different sexual dimorphism-trajectories and different mating modes. The shape changes are magnified by the factor of two for the humerus and by the factor of three for the cranium.



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## Supplementary Tables

Table S1: Specimens used for the study of SSD and SShD in cranium and humerus of pleurodeline newts of the genera *Echinotriton* and *Tylototriton*.

Collection-Number	Species	Sex			
			SMNS-14610	<i>T. asperrimus</i>	M
CAS-22261	<i>E. andersoni</i>	F	ZFMK-85179	<i>T. asperrimus</i>	M
CAS-22277	<i>E. andersoni</i>	F	ZFMK-82735	<i>T. asperrimus</i>	M
CAS-22280	<i>E. andersoni</i>	F	ZFMK-82730	<i>T. asperrimus</i>	M
CAS-22285	<i>E. andersoni</i>	F	ZFMK-82728	<i>T. asperrimus</i>	M
CAS-22304	<i>E. andersoni</i>	F	ZFMK-85178	<i>T. asperrimus</i>	M
CAS-22292	<i>E. andersoni</i>	F	MNHN-1976.637	<i>T. himalayanus</i>	F
SMF-1045	<i>E. andersoni</i>	F	MNHN-1976.686	<i>T. himalayanus</i>	F
SMNS-14617	<i>E. andersoni</i>	F	ZSM-0830-2012	<i>T. himalayanus</i>	F
CAS-22250	<i>E. andersoni</i>	F	ZSM-0841-2012	<i>T. himalayanus</i>	F
CAS-22308	<i>E. andersoni</i>	F	MNHN-1976.684	<i>T. himalayanus</i>	F
CAS-22302	<i>E. andersoni</i>	F	MNHN-1976.619	<i>T. himalayanus</i>	F
CAS-22257	<i>E. andersoni</i>	F	ZFMK-37458	<i>T. himalayanus</i>	F
CAS-22263	<i>E. andersoni</i>	F	MNHN-1976.621	<i>T. himalayanus</i>	M
CAS-22162	<i>E. andersoni</i>	F	MNHN-1976.623	<i>T. himalayanus</i>	M
CAS-22163	<i>E. andersoni</i>	F	MNHN-1976.625	<i>T. himalayanus</i>	M
CAS-22182	<i>E. andersoni</i>	F	MNHN-1976.626	<i>T. himalayanus</i>	M
CAS-22179	<i>E. andersoni</i>	F	MNHN-1976.629	<i>T. himalayanus</i>	M
CAS-22143	<i>E. andersoni</i>	F	MNHN-1976.632	<i>T. himalayanus</i>	M
CAS-22145	<i>E. andersoni</i>	F	MNHN-1976.669	<i>T. himalayanus</i>	M
CAS-22152	<i>E. andersoni</i>	F	MNHN-1976.67	<i>T. himalayanus</i>	M
CAS-22269	<i>E. andersoni</i>	M	MNHN-1976.673	<i>T. himalayanus</i>	M
CAS-22286	<i>E. andersoni</i>	M	MNHN-1976.677	<i>T. himalayanus</i>	M
CAS-22289	<i>E. andersoni</i>	M	MNHN-1976.678	<i>T. himalayanus</i>	M
CAS-22131	<i>E. andersoni</i>	M	MNHN-1976.68	<i>T. himalayanus</i>	M
CAS-22133	<i>E. andersoni</i>	M	MNHN-1976.689	<i>T. himalayanus</i>	M
CAS-22154	<i>E. andersoni</i>	M	MNHN-1976.693	<i>T. himalayanus</i>	M
CAS-22185	<i>E. andersoni</i>	M	MNHN-1976.692	<i>T. himalayanus</i>	M
CAS-22258	<i>E. andersoni</i>	M	SMNS-14616	<i>T. kweichowensis</i>	F
CAS-22266	<i>E. andersoni</i>	M	NHMW-39893	<i>T. kweichowensis</i>	F
CAS-22301	<i>E. andersoni</i>	M	MTKD-38092	<i>T. kweichowensis</i>	F
SMF-1044	<i>E. andersoni</i>	M	MTKD-31175	<i>T. kweichowensis</i>	F
CAS-22148	<i>E. andersoni</i>	M	MTKD-32938	<i>T. kweichowensis</i>	F
CAS-22153	<i>E. andersoni</i>	M	MTKD-31174	<i>T. kweichowensis</i>	F
CAS-22136	<i>E. andersoni</i>	M	MTKD-30365	<i>T. kweichowensis</i>	F
CAS-22130	<i>E. andersoni</i>	M	MTKD-38091	<i>T. kweichowensis</i>	F
ZFMK-86338	<i>T. asperrimus</i>	F	MTKD-31094	<i>T. kweichowensis</i>	F
ZFMK-83719	<i>T. asperrimus</i>	F	MTKD-38090	<i>T. kweichowensis</i>	F
ZFMK-86337	<i>T. asperrimus</i>	F	MTKD-31095	<i>T. kweichowensis</i>	F
ZFMK-85177	<i>T. asperrimus</i>	F	MTKD-30363	<i>T. kweichowensis</i>	F
ZFMK-89124	<i>T. asperrimus</i>	F	MTKD-37789	<i>T. kweichowensis</i>	F
ZMB-34090	<i>T. asperrimus</i>	M	MTKD-30370	<i>T. kweichowensis</i>	F

MTKD-32937	<i>T. kweichowensis</i>	F	ZMB-73599	<i>T. shanjing</i>	M
MTKD-30298	<i>T. kweichowensis</i>	F	ZMB-73600	<i>T. shanjing</i>	M
ZFMK-90815	<i>T. kweichowensis</i>	F	ZFMK-83208	<i>T. shanjing</i>	M
MTKD-30366	<i>T. kweichowensis</i>	F	CAS-215118	<i>T. shanjing</i>	M
USNM-95563	<i>T. kweichowensis</i>	M	CAS-215119	<i>T. shanjing</i>	M
USNM-95660	<i>T. kweichowensis</i>	M	CAS-215120	<i>T. shanjing</i>	M
USNM-95520	<i>T. kweichowensis</i>	M	CAS-242535	<i>T. shanjing</i>	M
USNM-95562	<i>T. kweichowensis</i>	M	ZSM-2417-2006	<i>T. shanjing</i>	M
USNM-95518	<i>T. kweichowensis</i>	M	NHMW-33610:2	<i>T. shanjing</i>	M
USNM-95522	<i>T. kweichowensis</i>	M	NHMW-40252:3	<i>T. shanjing</i>	M
USNM-95558	<i>T. kweichowensis</i>	M	CAS-242517	<i>T. shanjing</i>	M
USNM-95524	<i>T. kweichowensis</i>	M	CAS-242518	<i>T. shanjing</i>	M
MTKD-30368	<i>T. kweichowensis</i>	M	CAS-215121	<i>T. shanjing</i>	M
MTKD-30372	<i>T. kweichowensis</i>	M	CAS-215122	<i>T. shanjing</i>	M
MTKD-30369	<i>T. kweichowensis</i>	M	SMF-71169	<i>T. shanjing</i>	M
MTKD-30373	<i>T. kweichowensis</i>	M	SMF-71168	<i>T. shanjing</i>	M
MTKD-30364	<i>T. kweichowensis</i>	M	CAS-215133	<i>T. shanjing</i>	M
MTKD-38093	<i>T. kweichowensis</i>	M	CAS-242483	<i>T. shanjing</i>	M
MTKD-30371	<i>T. kweichowensis</i>	M	CAS-215128	<i>T. shanjing</i>	M
MTKD-30299	<i>T. kweichowensis</i>	M	SMNS-15005	<i>T. shanorum</i>	F
MTKD-30300	<i>T. kweichowensis</i>	M	ZFMK-83201	<i>T. shanorum</i>	F
MTKD-38089	<i>T. kweichowensis</i>	M	ZFMK-83197	<i>T. shanorum</i>	F
MTKD-31097	<i>T. kweichowensis</i>	M	ZFMK-83198	<i>T. shanorum</i>	F
MTKD-32936	<i>T. kweichowensis</i>	M	ZFMK-83196	<i>T. shanorum</i>	F
MTKD-31098	<i>T. kweichowensis</i>	M	NHMW-39896.2	<i>T. shanorum</i>	F
MTKD-30367	<i>T. kweichowensis</i>	M	ZFMK-83200	<i>T. shanorum</i>	M
MTKD-31096	<i>T. kweichowensis</i>	M	ZFMK-83199	<i>T. shanorum</i>	M
ZFMK-32193	<i>T. shanjing</i>	F	ZFMK-83202	<i>T. shanorum</i>	M
ZFMK-37461	<i>T. shanjing</i>	F	ZFMK-83203	<i>T. shanorum</i>	M
ZFMK-58064	<i>T. shanjing</i>	F	ZFMK-83204	<i>T. shanorum</i>	M
SMF-71171	<i>T. shanjing</i>	F	MTKD-37683	<i>T. taliangensis</i>	F
ZMB-73601	<i>T. shanjing</i>	F	MTKD-37684	<i>T. taliangensis</i>	F
ZFMK-83207	<i>T. shanjing</i>	F	NHMW-39889:13	<i>T. taliangensis</i>	F
ZFMK-83210	<i>T. shanjing</i>	F	ZFMK-83212	<i>T. taliangensis</i>	F
CAS-215129	<i>T. shanjing</i>	F	ZFMK-64080	<i>T. taliangensis</i>	F
CAS-215132	<i>T. shanjing</i>	F	ZFMK-83109	<i>T. taliangensis</i>	F
CAS-242516	<i>T. shanjing</i>	F	CAS-195126	<i>T. taliangensis</i>	F
NHMW-39890:15	<i>T. shanjing</i>	F	MTKD-38088	<i>T. taliangensis</i>	M
NHMW-39890:17	<i>T. shanjing</i>	F	MTKD-37682	<i>T. taliangensis</i>	M
NHMW-39890:3	<i>T. shanjing</i>	F	MTKD-37790	<i>T. taliangensis</i>	M
NHMW-39890:8	<i>T. shanjing</i>	F	NHMW-39889:12	<i>T. taliangensis</i>	M
NHMW-39890:9	<i>T. shanjing</i>	F	NHMW-39889:18	<i>T. taliangensis</i>	M
SMNS-14620	<i>T. shanjing</i>	F	NHMW-39889:20	<i>T. taliangensis</i>	M
CAS-242482	<i>T. shanjing</i>	F	NHMW-39889:21	<i>T. taliangensis</i>	M
ZMB-73596	<i>T. shanjing</i>	M	NHMW-39889:16	<i>T. taliangensis</i>	M
ZMB-73597	<i>T. shanjing</i>	M	ZFMK-83110	<i>T. taliangensis</i>	M
ZMB-73598	<i>T. shanjing</i>	M	ZFMK-93757	<i>T. taliangensis</i>	M

ZFMK-93752	<i>T. taliangensis</i>	M	CAS-215079	<i>T. verrucosus</i>	F
ZFMK-93760	<i>T. taliangensis</i>	M	CAS-215100	<i>T. verrucosus</i>	F
ZFMK-93762	<i>T. taliangensis</i>	M	ZFMK-37880	<i>T. verrucosus</i>	F
MNHN-1987.3738	<i>T. uyenoii</i>	F	MNHN-1887.531	<i>T. verrucosus</i>	F
ZFMK-35799	<i>T. uyenoii</i>	F	CAS-215066	<i>T. verrucosus</i>	F
SMF-70900	<i>T. uyenoii</i>	F	MNHN-1887.222	<i>T. verrucosus</i>	F
MNHN-1987.3764	<i>T. uyenoii</i>	F	CAS-234481	<i>T. verrucosus</i>	F
MNHN-1987.3763	<i>T. uyenoii</i>	F	CAS-215098	<i>T. verrucosus</i>	F
MNHN-1987.374	<i>T. uyenoii</i>	F	CAS-215065	<i>T. verrucosus</i>	M
MNHN-1987.3725	<i>T. uyenoii</i>	M	CAS-215067	<i>T. verrucosus</i>	M
MNHN-1987.373	<i>T. uyenoii</i>	M	CAS-215068	<i>T. verrucosus</i>	M
MNHN-1987.3731	<i>T. uyenoii</i>	M	CAS-215069	<i>T. verrucosus</i>	M
MNHN-1987.3734	<i>T. uyenoii</i>	M	CAS-215071	<i>T. verrucosus</i>	M
MNHN-1987.3742	<i>T. uyenoii</i>	M	CAS-215072	<i>T. verrucosus</i>	M
MNHN-1987.3745	<i>T. uyenoii</i>	M	CAS-215074	<i>T. verrucosus</i>	M
MNHN-1987.3749	<i>T. uyenoii</i>	M	CAS-215075	<i>T. verrucosus</i>	M
MNHN-1987.3750	<i>T. uyenoii</i>	M	CAS-215077	<i>T. verrucosus</i>	M
MNHN-1987.3752	<i>T. uyenoii</i>	M	CAS-245445	<i>T. verrucosus</i>	M
MNHN-1987.3754	<i>T. uyenoii</i>	M	CAS-245457	<i>T. verrucosus</i>	M
MNHN-1987.3765	<i>T. uyenoii</i>	M	CAS-234480	<i>T. verrucosus</i>	M
ZFMK-33292	<i>T. uyenoii</i>	M	SMF-1134	<i>T. verrucosus</i>	M
SMNS-15132	<i>T. uyenoii</i>	M	NHMW-8608:2	<i>T. verrucosus</i>	M
MNHN-1987.3759	<i>T. uyenoii</i>	M	MNHN-1887.223	<i>T. verrucosus</i>	M
MNHN-1987.3768	<i>T. uyenoii</i>	M	NHMW-8608:1	<i>T. verrucosus</i>	M
MNHN-1987.3758	<i>T. uyenoii</i>	M	CAS-215090	<i>T. verrucosus</i>	M

Table S2: Pairwise comparison of trajectories of SShD's in species of crocodile newts (*Echinotriton* and *Tylototriton*) on humerus (a) and cranium shape (b). P-values below 0.1 are given in bold.

Species pairs	r	angle	Z	Pr
<b>(a) Humerus shape</b>				
AND:ASP	-0.46682813	117.8286	1.42714827	<b>0.0746</b>
AND:HIM	-0.0120951	90.69301	-0.00512674	0.5012
AND:KWE	-0.13387113	97.69335	0.73897297	0.2311
AND:SHA	0.05446646	86.87776	0.056926	0.4666
AND:SHJ	0.04006747	87.70369	-0.05172983	0.5213
AND:TAL	0.53610158	57.58135	-1.76256895	0.9657
AND:UYE	0.36209305	68.77121	-0.7127958	0.7529
AND:VER	0.07283752	85.82302	-0.30812571	0.6189
ASP:HIM	0.14873708	81.44625	-0.66479591	0.7401
ASP:KWE	0.25241557	75.3795	-0.84296483	0.7903
ASP:SHA	-0.01299515	90.74459	0.75814302	0.2282
ASP:SHJ	-0.04465795	92.55956	-0.46166446	0.6804
ASP:TAL	-0.52673433	121.78507	1.87861716	<b>0.0283</b>
ASP:UYE	-0.10685649	96.13414	-0.37192933	0.6473
ASP:VER	0.00894002	89.48777	-0.29657821	0.6105
HIM:KWE	-0.19172995	101.05376	1.64203571	<b>0.0565</b>
HIM:SHA	-0.26771584	105.52839	1.47485254	<b>0.0758</b>
HIM:SHJ	-0.20699712	101.94643	1.36848011	0.0971
HIM:TAL	-0.15937028	99.17035	1.17140961	0.1262
HIM:UYE	0.4003387	66.40065	-0.44493701	0.6487
HIM:VER	0.00412744	89.76351	0.71191072	0.2429
KWE:SHA	-0.0088412	90.50657	1.09575509	0.1425
KWE:SHJ	0.61019192	52.39662	-0.74910418	0.7647
KWE:TAL	-0.22281803	102.8746	1.34217213	<b>0.0978</b>
KWE:UYE	0.36216274	68.76692	0.24327653	0.3818
KWE:VER	0.54550481	56.94083	-0.13251308	0.5195
SHA:SHJ	-0.4629657	117.57864	1.42755566	<b>0.0736</b>
SHA:TAL	0.14496272	81.66488	-0.03093918	0.4975
SHA:UYE	-0.32654714	109.05933	1.38385114	<b>0.0873</b>
SHA:VER	-0.19935571	101.49929	1.27296807	0.1081
SHJ:TAL	0.06429223	86.31378	0.16182486	0.4255
SHJ:UYE	0.49968769	60.02066	0.1341753	0.423
SHJ:VER	0.74251775	42.05367	-0.83387732	0.7875
TAL:UYE	0.1775251	79.77436	0.06487828	0.4555
TAL:VER	0.06432208	86.31207	0.41444624	0.3328
UYE:VER	0.63670485	50.45345	-0.81725357	0.787
<b>(b) Cranium shape</b>				
AND:ASP	-0.1261636	97.24796	0.0775665	0.4723
AND:HIM	-0.30251033	107.60844	1.09158763	0.1389
AND:KWE	0.29503507	72.84036	-0.69800575	0.7466
AND:SHA	0.07864737	85.48918	-0.5373217	0.7013
AND:SHJ	-0.37974107	112.31765	1.46829175	<b>0.0683</b>

AND:TAL	-0.1690142	99.73051	0.17923985	0.4358
AND:UYE	0.08458317	85.14794	0.02649171	0.4898
AND:VER	0.19977892	78.47597	-0.78562098	0.7842
ASP:HIM	0.05401751	86.90352	-0.06967234	0.5258
ASP:KWE	-0.03602013	92.06425	0.16934995	0.4337
ASP:SHA	0.05470757	86.86392	-0.39646266	0.6555
ASP:SHJ	0.1635958	80.58433	-1.11524936	0.8643
ASP:TAL	-0.06271244	93.59552	0.50957328	0.3016
ASP:UYE	-0.03213558	91.84155	-0.75919772	0.7778
ASP:VER	-0.06224594	93.56874	-0.38105796	0.6573
HIM:KWE	-0.41805572	114.7119	2.29509464	<b>0.009</b>
HIM:SHA	-0.1168173	96.70846	0.59568357	0.278
HIM:SHJ	0.28526743	73.42516	-0.66344354	0.7422
HIM:TAL	0.52602148	58.26297	-1.85361949	0.9726
HIM:UYE	-0.28356754	106.47324	0.8218822	0.2097
HIM:VER	0.07493355	85.7026	-0.17084595	0.5734
KWE:SHA	-0.11479272	96.59167	0.0104961	0.4976
KWE:SHJ	-0.31540426	108.38522	1.29039027	<b>0.0973</b>
KWE:TAL	-0.20141995	101.62001	0.61955319	0.2755
KWE:UYE	-0.03372357	91.93258	0.761438	0.2293
KWE:VER	-0.07563739	94.33785	0.08850095	0.4692
SHA:SHJ	0.13243547	82.38965	0.22839201	0.4026
SHA:TAL	0.23361424	76.49005	-0.88320676	0.8091
SHA:UYE	-0.01467623	90.84092	-0.33849917	0.6335
SHA:VER	0.19220274	78.91864	-0.73949528	0.7674
SHJ:TAL	0.22580437	76.94982	-0.06604023	0.5206
SHJ:UYE	0.16990833	80.21751	-1.01623994	0.8425
SHJ:VER	-0.15157714	98.71834	2.21616912	<b>0.0151</b>
TAL:UYE	-0.03780691	92.16669	0.38374482	0.3481
TAL:VER	0.20593045	78.11603	-1.0360533	0.8494
UYE:VER	0.02429858	88.60766	0.30094108	0.375