



# 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).

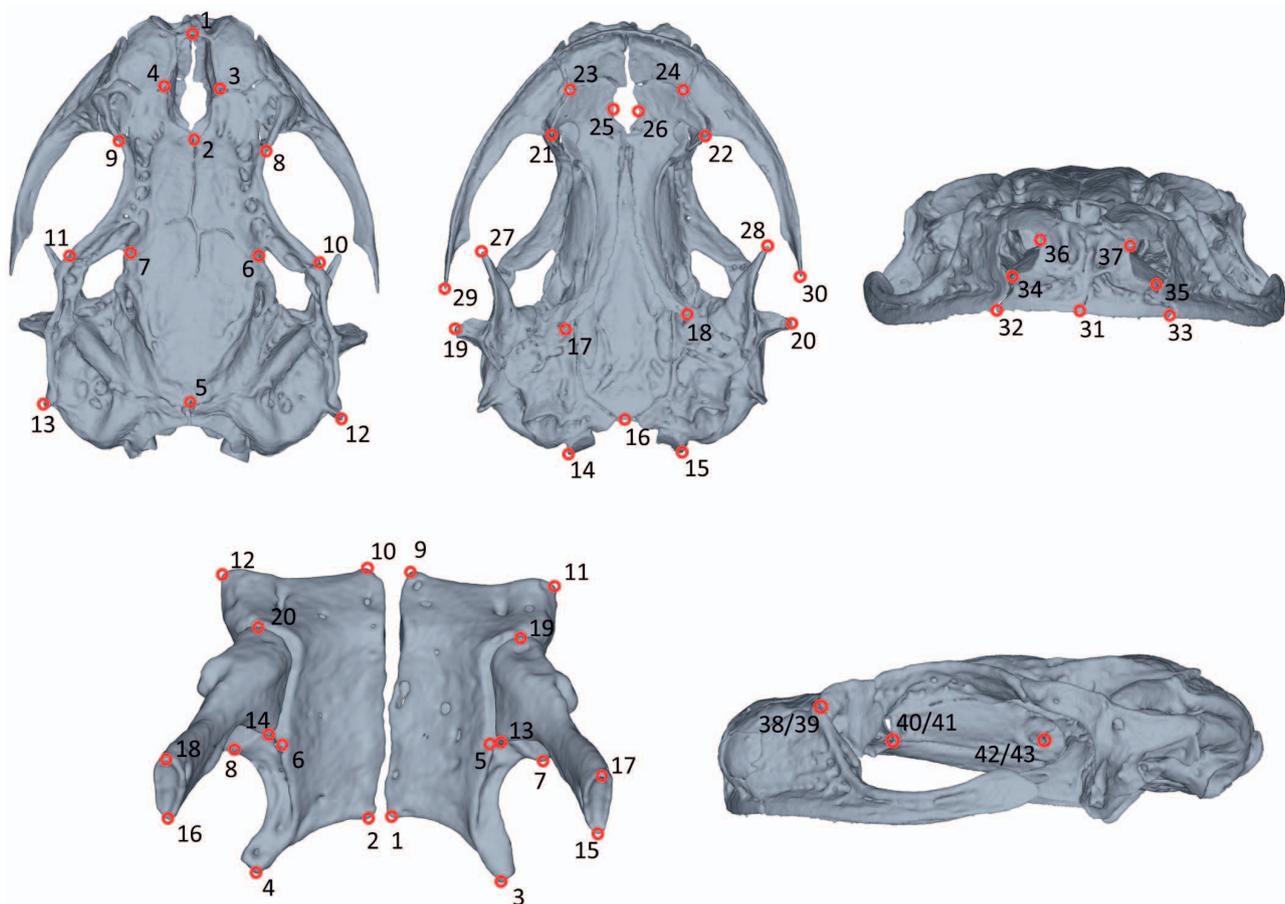


Figure 1. Definition of three-dimensional landmarks (circles) on the cranial and pelvic (lower left) skeleton of Northern spectacled salamanders, *Salamandrina perspicillata*, for the analysis of SShD.

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-

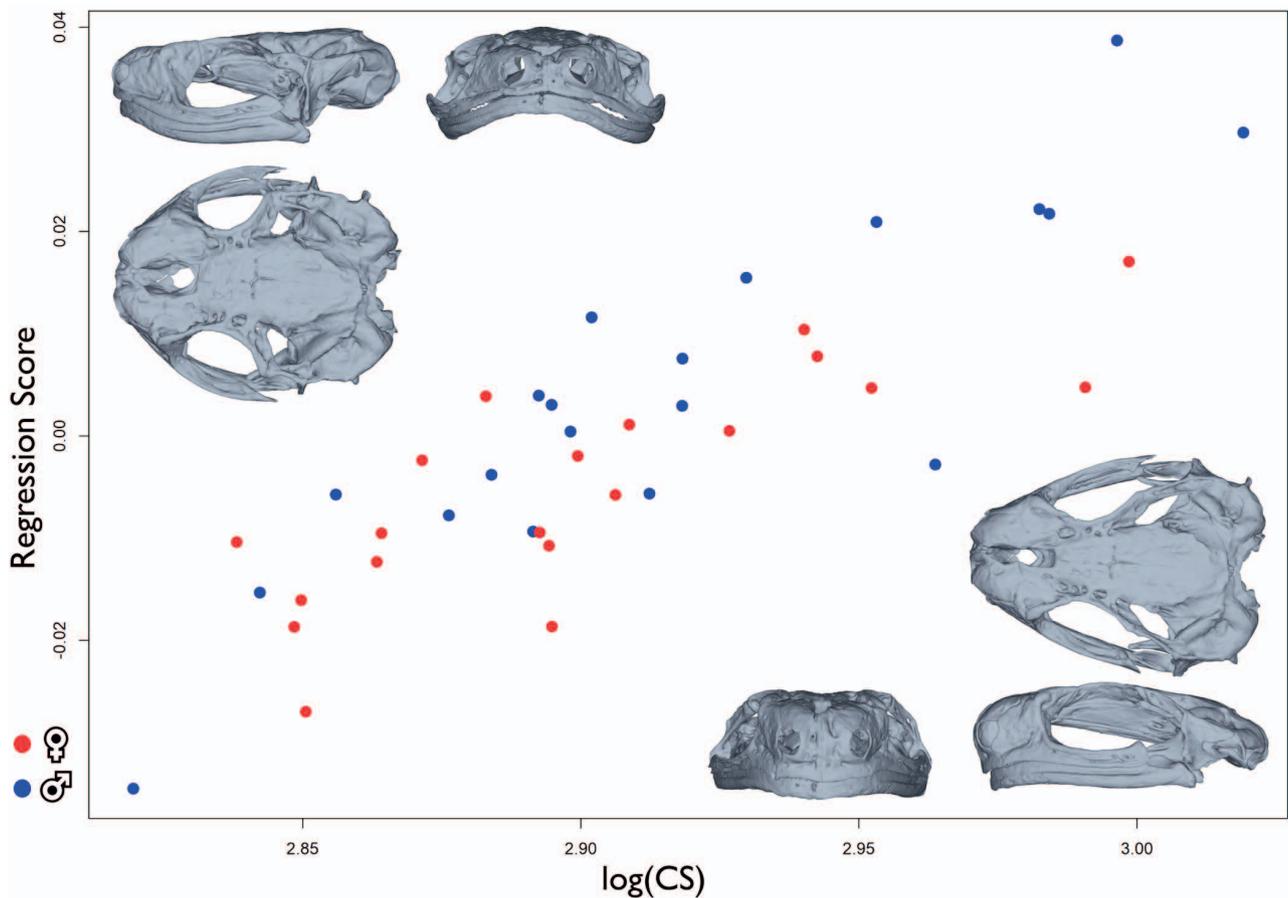


Figure 2. Common allometry estimated by multivariate regression of cranial shape on cranial size (as  $\log(\text{CS})$ ) of Northern spectacled salamanders, *Salamandrina perspicillata*. The shape for the smallest (upper left) and largest (lower right) values of cranial size ( $\log(\text{CS})$ ) were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

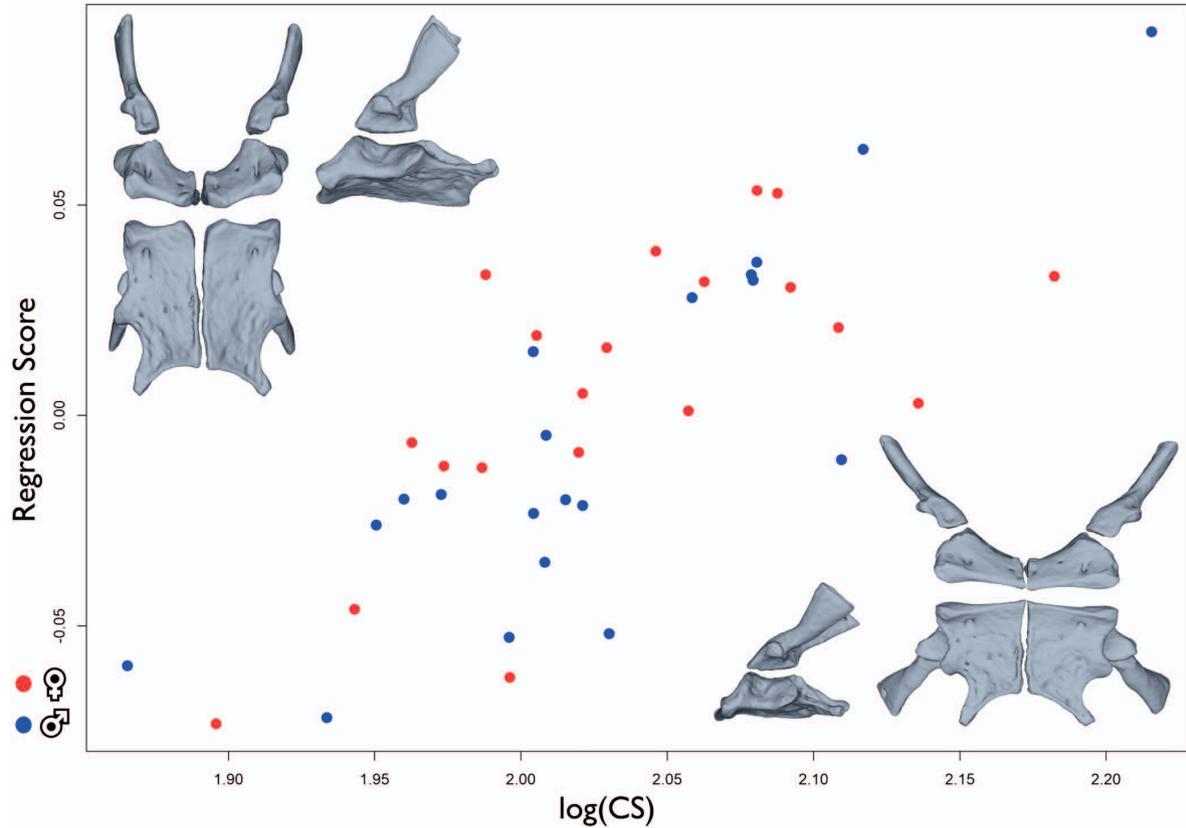


Figure 3. Common allometry estimated by multivariate regression of pelvis shape on pelvis size (as logCS) of Northern spectacled salamanders, *Salamandrina perspicillata*. The shape for the smallest (upper left) and largest (lower right) values of pelvis size (logCS) were visualized from the mean shape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

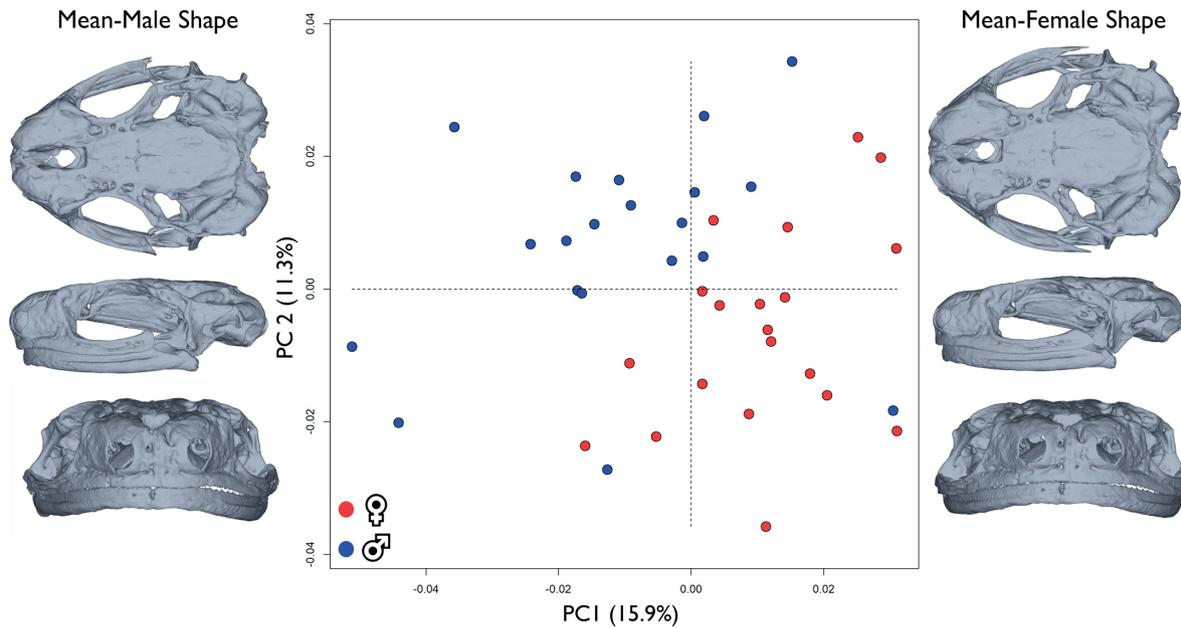


Figure 4. Morphospace for cranial shape data in the Northern spectacled salamander, *Salamandrina perspicillata*, built by the first and second axes of the principal component analysis of 43 three-dimensional landmarks. The mean shape for males (left) and females (right) were visualized from the mean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

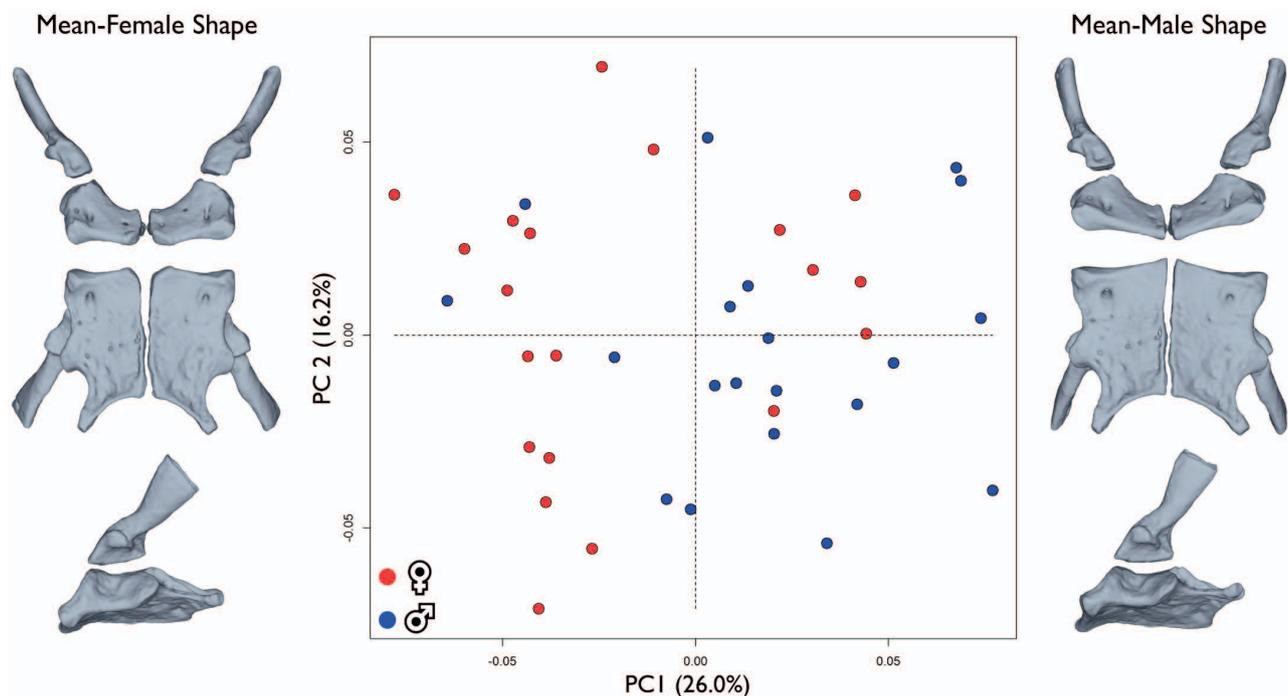


Figure 5. Morphospace for pelvis shape data in the Northern spectacled salamander, *Salamandrina perspicillata*, built by the first and second axes of the principal component analysis of 20 three-dimensional landmarks. The mean shape for males (right) and females (left) were visualized from the mean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