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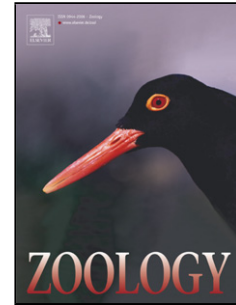
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Geometric morphometric analyses of sexual dimorphism and allometry in two sympatric snakes: *Natrix helvetica* (Natricidae) and *Vipera berus* (Viperidae)

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Highlights

- The grass snake (*Natrix natrix*) and the adder (*Vipera berus*) lives in sympatry throughout most of their range in Great Britain.
- Size sexual dimorphism is recognised in British specimens from both species as a mechanism to reduce intraspecific competition.
- No sexual dimorphism was present in head shape of both snakes.
- The head shape of the grass snake is strongly influenced by allometry so that larger individuals can produce stronger bite force.
- The use of venom in *Vipera berus* explains the lack of allometric patterns in head shape recognised for the British specimens.

ABSTRACT

The non-venomous grass snake (*Natrix helvetica*) and the venomous adder (*Vipera berus*) are two native species that are often found in sympatry in Great Britain and Europe. They occupy partially overlapping ecological niches and prey on small vertebrates, but use different feeding strategies. Here, we investigated the morphologies of grass snakes and adders from Dorset (UK) using two-dimensional geometric morphometrics to assess the degree of sexual dimorphism in size and shape together with the relative impact of allometry and general body dimensions on head shape. Both species showed significant sexual dimorphism in head size, but not in head shape. We found a clear allometric pattern in *N. helvetica*, whereas allometry in *V. berus* was generally less pronounced. Body dimensions were strongly correlated with head shape in the grass snake, but not in the adder. The fact that *V. berus* is venomous appears to explain the lack of allometric patterns and the lack of an association between body dimensions and head shape. The high degree of size dimorphism identified in both species could originate from the advantages of reduced intraspecific competition that are conveyed by a partial differentiation in feeding morphology.

Keywords: allometry; evolution; geometric morphometrics; morphology; snakes

1. Introduction

Allometry is one of the most pervasive components of vertebrate morphological variation and can be interpreted as the impact of size variation on different body parts (Calder, 1996; Klingenberg, 2013a). Different definitions of allometry have been used over the last century. Under the Huxley-Jolicoeur definition, allometry is considered to be the covariation between morphological traits (Jolicoeur, 1963) while Gould and Mosimann defined allometry as the covariation between size and shape (Gould, 1966; Mosimann, 1970; Klingenberg, 2016). The term ‘static allometry’ is used to describe shape variation among individuals of the same age/sex. It is

distinct from both evolutionary allometry (size-shape changes between species) and from ontogenetic allometry (shape differences between age classes) (Klingenberg and Zimmermann, 1992). Static allometry is of great interest for cross-species analyses because variation can be linked to ecological adaptations. For instance, Rodriguez et al. (2015) reported that sexual traits frequently show different allometric patterns between species and also vary among themselves, both in response to the type of selection (i.e., stabilizing or directional) and size-related differences in the net benefit of trait size (Kodric-Brown et al., 2006; Eberhard et al., 2009; Schulte-Hostedde et al., 2011).

Possibly because of their linear body plans, snakes have always been popular subjects for studies of allometry (e.g. Shine, 1994; King, 2002; Feldman and Meiri, 2013). Nevertheless, very few studies to date have applied geometric morphometrics (GMM) techniques to this group (but see, for example, Gentilli et al., 2009). GMM allows more detailed analyses of interactions between size and shape that can be related to other potentially co-evolved biological characteristics (Zelditch et al., 2004). More specifically, it extracts size and shape data from landmark Cartesian coordinates in order to describe the morphology of a biological structure (Adams et al., 2004, 2013; Slice, 2007).

Extant snakes (suborder: Serpentes) comprise over 3600 species and therefore represent 36% of total squamate diversity (Figueroa et al., 2016). They show a wide variety of morphologies and ecologies. Two of the main characteristics associated with the evolution of the snake clade are the loss of ancestral limbs and the extreme elongation of the body. This latter trait followed the decoupling of primaxial and abaxial domains together with an increase in the number of vertebrae (Sarris et al., 2012; Head and Polly, 2015). Furthermore, snakes have evolved a kinetic skull for manipulation and ingestion of large prey items. The snake skull is divided into a “non-trophic” akinetic region associated with the braincase (Camilleri and Shine, 1990), which usually presents closely connected bones, and a “trophic” region involved in the feeding process, which is characterized by an extreme articular elasticity. Non-trophic structures are generally used in

systematics studies because they are less affected by morphological variation related to dietary adaptations (Gloyd and Conant, 1990). Snake mandibles do not present a fused symphysis which allows greater deformability of the feeding apparatus during ingestion of prey that are quite large relative to the snake itself (Cundall and Gans, 1979). Not surprisingly, previous investigations have identified strong associations between maximum prey size and head width and length, a good proxy for skull size and shape (Cundall and Greene, 2000; Meik et al., 2012). However, habitat selection and lifestyle (e.g. fossoriality) also seem to have a major influence on the evolution of snake head size and shape (Voris and Murphy, 2002; Fabre et al., 2016; Da Silva et al., 2018).

Three snakes are native to Great Britain with the two most widespread species being the adder (*Vipera berus*) and the grass snake (*Natrix helvetica*) (Arnold et al., 1978). The latter (family Natricidae) can grow to an average length of 120 cm and is found in a wide variety of habitats, including meadows, dry woods and hedgerows. They are strong swimmers and eat mainly amphibians, especially frogs, that are killed with a single bite and without constriction. Females are the larger sex, as in most other Colubroidea (Shine, 1994; Borczyk, 2007; Baier and Wiedl, 2010). The size dimorphism is thought to cause divergent selection pressures that are reflected in both ecological and morphological traits (Madsen, 1983). Female *N. helvetica* have a comparatively larger head and appear adapted to predate larger prey than males (Borczyk, 2015). In contrast, *V. berus* (family Viperidae) is a much smaller species, growing to an average length of 65 cm (although it can reach 90 cm). It often occupies habitats such as moorlands, heaths and open woodlands (Presst, 1971; Arnold et al., 1978). It is venomous and feeds mainly on small mammals, but also takes birds, amphibians, lizards and invertebrates. Females usually reach larger body sizes than males, which is not entirely expected given that males engage in combat for access to females, with success being dependent on male body size (Andrén and Nilson, 1981; Madsen, 1988).

Here we obtained samples of *N. helvetica* and *V. berus* with the primary aim of comparing intraspecific sexual dimorphism and static allometry between snake species. The nature of our samples, which were collected at similar times within the same area (Dorset, UK), and the fact that

we included venomous and non-venomous species with different feeding strategies, make our study quite unique. We specifically examined the following questions through use of GMM analyses of photographs of the heads of both species: 1) Do *N. helvetica* and *V. berus* exhibit different degrees of sexual dimorphism in size and shape? 2) To what extent do these species show static allometry in the head? 3) What is the degree of covariation between head shape and metrics that quantify relative body dimensions?

2. Materials and Methods

2.1 Specimens, Landmarks and PGMM

All specimens were from the World Museum Liverpool (WML) collection. Adult *Vipera berus* specimens (38 females and 30 males) had been collected from 1958 to 1962, whereas adult *N. helvetica* (39 females and 33 males) had been collected in 1962. All specimens were preserved in alcohol.

Two linear measurements were recorded from each specimen: tail length (TL) and body length (BL), intended as snout-vent length. Following Lawing et al. (2012), the index $\ln BL / \ln TL$ was calculated to describe relative locomotory adaptations in both species. A meta-analysis of North American crotalids demonstrated that this index correlates with degree of arboreality as well as aquatic behaviour (Lawing et al., 2012). Linear measurements, index and catalogue number of the sample are provided in the supplementary material (Table S1).

Photographs of the heads of *N. helvetica* and *V. berus* in dorsal view were taken using a digital SLR camera (Nikon D5300; lens focal length: 18-140mm). The camera was positioned directly above the specimen using a horizontal tripod in order to prevent image perspective alterations (see Muir et al., 2012). The correct alignment of both the camera and the specimen (i.e., camera lens and specimen both parallel to the ground) was checked using a spirit level.

Using the photographs, anatomical landmarks were placed at the junctions of scales for each individual with the software TPSDig (v. 2.21, Rohlf, 2015) (Fig.1). The landmarks that we used are optimal for detecting shape differences between taxa and also cover functionally relevant head components such as relative jaw width and length (Ruane, 2015). Size and shape data were obtained from the Cartesian coordinates of landmarks using Procrustes-based geometric morphometrics (PGMM - Adams et al., 2004, 2013; Cardini, 2013). The procedure for separating shape from size data is known as Procrustes superimposition (Rohlf and Slice, 1990). Size was estimated as centroid size (CS) which is the square root of the sum of squared distances of landmarks from their barycentre. Shape data were obtained in three steps: 1) the standardization of size (division of the landmark Cartesian coordinates of each specimen by its centroid size), 2) the removal of translational variation (barycentres from all specimens are superimposed) and 3) the minimization of rotational differences (least-square minimization of the sum of squared distances of corresponding landmarks in a sample) (Rohlf and Slice, 1990). The asymmetric component of shape data was discarded because it was negligible in our sample (2.3%). Shape differences were visualized using wireframe diagrams, in which straight lines (links) connect the anatomical landmarks in order to represent the structure being measured (Klingenberg, 2013b).

2.2 Measurement Error

In order to assess the repeatability of the chosen landmark configuration, one of the authors (DT) digitized landmarks twice, with a 10 day interval between the first and second replicate measurements (Viscosi and Cardini, 2011; Fruciano, 2016). Differences between these replicates is expected to be negligible, relative to intraspecific variation, and was analysed using the software PAST (v. 2.17c - Hammer et al., 2001). Differences in size estimation were evaluated by calculating product-moment correlation coefficients between CS values obtained from the first and second replicates. Shape differences were compared using a cluster analysis based on Procrustes distances between replicates. If the configuration is repeatable then it is expected that, for size, the correlation

between CS values should be very close to one while, for shape, the two replicates should cluster together within individuals.

2.3 Size and Shape dimorphism

The presence of both interspecific and intraspecific (between-sex) size and shape dimorphisms were tested with two-way (species and sex) Procrustes Analyses of Variance (Procrustes ANOVA: Klingenberg and McIntyre, 1998; Klingenberg et al., 2002). We performed a univariate Procrustes ANOVA on each of the available body dimensions (lnCS, lnTL, lnBL and the index). Shape was analysed using a multivariate Procrustes ANOVA based on Procrustes coordinates as shape dependent variables. All ANOVAs were performed using the R package *geomorph* v. 3.0.3 (Adams and Otárola-Castillo, 2013).

Species-related differences and sexual dimorphism for each of the size variables were visualized using boxplots. An additional estimate of sexual size dimorphism (SSD) was obtained using an SSD index, which was equal to the difference between the mean female size and the mean male size, divided by mean female size (Cardini and Elton, 2008; Porobić et al., 2016). Hence an SSD value of zero indicates no sexual dimorphism.

In order to assess shape variation across all individuals, a Principal Components Analysis (PCA) of Procrustes coordinates (Rohlf, 1993) was performed using MorphoJ (v. 1.06d, Klingenberg, 2011). Due to notable interspecific shape divergence, the PCA was performed using the pooled within-species covariance matrix in order to reduce the distances between the two groups (see Meloro et al., 2014). A sexual shape dimorphism index was then calculated for each species: the Procrustes distances between mean female and mean male measurements within species were divided by the maximum Procrustes distances between males and females. Sexual dimorphism in shape was also evaluated by performing two regressions, one for each species. The dependent variable (i.e., Procrustes coordinates) was regressed onto a dummy variable coding for sex (i.e., 0, 1 variable) (see Cardini and Elton, 2008).

2.4 Static allometry and covariation between size and shape variables

Static allometry was tested by regressing shape coordinates onto four different size variables: 1) the natural logarithm of centroid size (lnCS), 2) the natural logarithm of tail length (lnTL), 3) the natural logarithm of body length (lnBL) and 4) an index related both to TL and BL (index = $\ln\text{BL}/\ln\text{TL}$). Natural logarithms were used because the ranges of sizes were quite large (Klingenberg, 2016). The allometric regressions were first performed within each species (mixed sexes) but then replicated with the sexes separated. Because the same null hypothesis (absence of allometry) was tested multiple times, the usual 5% threshold for statistical significance was corrected using a Bonferroni adjustment (see Tamagnini et al., 2017).

The allometric trajectories from the regressions were compared by computing the angle between pairs of them (Klingenberg and Zaklan, 2000; Klingenberg and Marugán-Lobón, 2013). For each size predictor, we obtained the angle between species (sexes pooled), between sexes within species and between species within sexes. A more complex (three-way) Procrustes ANOVA was additionally calculated in order to summarize the previous analyses: in this model we tested simultaneously the effects of body metrics, sex, species and their mutual interactions.

A two-block Partial Least Squares (PLS) approach was used to determine the degree of covariation between head shape and general body size (here described by lnCS and the other three body dimensions) in both species (see Meloro and Jones, 2012). PLS extracts pairs of vectors that maximise covariation between two blocks of multivariate variables (Rohlf and Corti, 2000). In the present study, six PLS analyses were performed in order to evaluate the covariation between size and shape variables in each species. These analyses were initially carried out across both sexes and then with males and females separate. The degree of covariation in each PLS was evaluated using the correlation between first pair of PLS1 scores and also using the R_V coefficient. The latter is a

similarity coefficient between positive semi-definite matrices (Escoufier, 1973). All analyses were performed using MorphoJ.

3. Results

3.1 Measurement error

The two digitization replicates for CS were very highly correlated ($r = 0.999$). For shape variables, 138 out of 140 (98.5% of the total) replicate measurement pairs taken from the same specimen clustered together.

3.2 Size and shape dimorphism

Species and sex variation was found to be significant for all size variables with the exception of lnTL (univariate Procrustes ANOVAs: see Table 1). A significant interaction between species and sex was also detected for all variables except for lnBL (Table 1), which indicates that degrees of SSD generally differ between the two species.

Boxplots showed greater values in *N. helvetica* than in *V. berus*, for all the size descriptors except for the index (lnBL/lnTL) (Fig. 2). Females were larger than males, with the exception of lnTL for *V. berus*. These findings were confirmed by relatively high SSD indices (Table 2).

Significant shape differences were detected between species but not between sexes (multivariate Procrustes ANOVA: see Table 1). The pooled within-species PCA confirmed strong shape overlap between sexes but not between species. PC1 (27.6% of total variance) stressed greater head width and reductions in the rostral region on negative scores (Fig. 3) but did not discriminate between species or sexes. All *V. berus* specimens showed positive scores on PC2 (14.3% of variance) which were associated with a relative reduction in frontal and parietal scales (Fig. 3). Sexual shape dimorphism was very similar between species (9% in *N. helvetica* and 8% in *V. berus*) confirming the general findings of the PCA (Table 2). Furthermore, regressions of

Procrustes coordinates onto sex (represented as a dummy variable) were not significant for either *N. helvetica* or *V. berus* ($p = 0.108$ and $p = 0.572$, respectively) with sex predicting only a small proportion of the shape variance (2.4% and 1.2%, respectively).

3.3 Allometric regressions and PLS

Results of the 24 allometric regressions performed on nested subsets of the data are shown in Table 3. In general, regressions on sexes-pooled data showed largely congruent results to the ones performed with the sexes separate. *N. helvetica* regressions showed clear allometric patterns, with 11 significant p -values out of 12 (91%). Ten of these p -values (83%) were still significant after application of a Bonferroni adjusted significance level (in the present case equal to $p = 0.0021$). The range of shape variance explained by each size variable in *N. helvetica* was between 10-15% with the exception of two regressions performed using the index $\ln\text{BL}/\ln\text{TL}$ as the shape variation predictor).

Figure 4 shows the pattern of shape change relative to $\ln\text{CS}$ variation in the grass snake. Specimens with low values of $\ln\text{CS}$ have a relatively shorter rounder head, associated with a bigger frontal scale and a relative reduction of head width in the area anterior to the eye. In contrast, individuals with higher $\ln\text{CS}$ values show an arrowhead-like shape, with an increased relative head length. No differences were detected between male and female patterns of allometric shape variation.

Vipera berus regressions were generally not significant, showing an overall lack of allometry in this species: only three p -values were significant out of 12 (25%) and none of these were significant when a Bonferroni correction was applied. Two out of the three cases of significant allometry applied to males only. Similarly, the percentage of shape variance explained by size predictors differed greatly between the two sexes: values for females were always below 3%, while values for males were always greater than 5%. Individual *V. berus* with lower $\ln\text{CS}$ values exhibited wider heads, despite a slight reduction in relative head length (Fig. 5). Scale pattern exhibited relatively

little size-related variation. No between-sex differences in the allometric shape patterns were observed, except for the magnitude of shape variation.

Angles between allometric trajectories are shown in Table 4. Comparisons of *N. helvetica* and *V. berus* allometric trajectories revealed a significant deviation from an angle of 90° in only two cases out of 12 (without Bonferroni correction). Furthermore, angles were greater than 65° in 10 of these cases strongly supporting the hypothesis that the trajectories are not parallel. For comparisons between sexes within each species, angles significantly differed from 90° in six out of eight cases indicating parallelism (Table 4). Figure 6 provides visual representations of allometric trajectories for each size variable across species and sexes for cases in which all specimens are analysed within the same morphospace. A three-way Procrustes ANOVA model confirmed the differences in allometric trajectories between species and sexes (Table 5).

The six PLSs performed in the present study showed clear trends (Table 6). The three PLS performed within *N. helvetica* subsets exhibited highly significant p -values and R_V coefficients that were great than 0.3. In contrast, each PLS performed on *V. berus* showed a non-significant p -value and an R_V coefficient lower than 0.2, indicating a lack of covariation between size and shape matrixes. Correlations between PLS1 scores of shape and size matrix were very high and significant in the grass snake ($r > 0.85$ in all cases), while in the adder none of these correlations were significant ($r < 0.65$ in all cases). The angle between Block1 PLS1 in *N. helvetica* for the comparison of males and females was lower than 50° ($p < 0.05$) which indicates parallel trajectories, i.e., a similar pattern of covariation between shape and body size in both sexes. Shape variation associated with PLS1 in the shape matrix (*N. helvetica*, sexes pooled) is shown in Fig. 7. Positive scores along this axis are associated with an increase in body length (lnBL vs PLS1, $r = 0.66$) relative to tail length (lnTL vs PLS1, $r = 0.60$) and centroid size (lnCS vs PLS1, $r = 0.43$), which is associated with an expansion of head width and rostral area followed by a reduction of the frontal scale and orbital region. Specimens with negative values along this axis had a more rounded head.

4. Discussion

There are notable differences in head size and shape between *N. helvetica* and *V. berus*. In our Dorset samples, the grass snake always showed higher values for measures of relative size than the adder, especially when considering tail length. These differences can be the result of differences in ecological niche between the two species. Despite being sympatric, the grass snake is much more aquatic than the adder and the relatively longer tail appears likely to be an adaptive component related to swimming. Lawing et al. (2012) made similar observations in crotalid snakes. Our results therefore reinforce the hypothesis that relative tail length plays an important adaptive role in snakes.

Differences in size between the grass snake and the adder size might be associated with divergent prey selection which would reduce interspecific competitive interactions within a community. This appears to have been important in shaping snake guilds (MacArthur, 1970; Schoener, 1983; Toft, 1985). There is evidence that interspecific interactions can influence body size in snakes. The North American coachwhip snake (*Coluber flagellum*) appears to reach smaller sizes in areas that the related *Coluber constrictor* is present, relative to similar habitats in which it is the only *Coluber* species (Steen et al. 2012). Presst (1971) found that the commonest *V. berus* food items in low-lying damp meadows in Dorset (UK) were small mammals (shrews, voles and mice). Adders appear to be specialised small mammals feeders in other parts of their European geographical range (Luiselli and Anibaldi, 1991; Forsmann, 1991). Grass snakes in southern England more commonly eat anurans (Gregory and Isaac, 2004). Luiselli (2006) carried out snake community meta-analyses (including adder-grass snake-smooth snake community) and concluded that sympatric species differ considerably in their feeding habits. This corroborates our suggestion that general size differentiation relates to different diets in sympatric *N. helvetica* and *V. berus* although more updated ecological data are required to validate this pattern on a broader geographical scale.

In terms of shape, the very divergent evolutionary histories of the two genera are highlighted by the completely different scale shapes in the “non-trophic” region of the head: in particular both the frontal and the parietal scales occupy a smaller area in *V. berus* relative to *N. helvetica*. A broader head in *N. helvetica* is compatible with anurophagy, as observed previously in the Natricidae (Hampton, 2011). A relative reduction in the width of the anterior region of the *N. helvetica* head is also explained by the need to overcome hydrodynamic constraints, as observed in other aquatic snakes (Segall et al., 2016).

Sexual dimorphism in both species showed a different trend in size compared to shape. SSD analyses confirmed the generally larger size of females (with the only exception being tail length in *V. berus*) which is in agreement with many studies of the Natricidae and Viperidae (Madsen, 1983; Shine, 1994; Gregory, 2004; Maritz and Alexander, 2011; Andjelković et al., 2016). Larger female size is expected to occur in many snake species due to its association with increased fecundity (Shine, 1986, 1989). In addition, developmental studies have shown that testosterone has an inhibitory effect on head growth of male snakes (Shine and Crews, 1988). The degree of SSD differed between the two species for $\ln CS$, $\ln TL$ and the index ($\ln BL/\ln TL$) and each SSD index differed from others between species, indicating a non-homogeneous degree of dimorphism among different body parts. The region of the body mostly affected by SSD seems to be the head (sexual size dimorphism in the head exceeds 10% in both species). These results are similar to the ones available in the literature (see for example Gregory and Isaac, 2004). The increased range of variation in head size between males and females is easy to explain if it arises from divergent feeding habits (gape size affects swallowing performance: Forsman and Lindell, 1993; King, 2002) which reduce the intraspecific competition for food (Vincent and Herrell, 2007). A different trend was observed in the shape dimorphism of both species. Neither multivariate Procrustes ANOVA nor multivariate regression revealed significant sexual dimorphism in the head shape of either species. Andjelković et al. (2016) previously found shape dimorphism in some elements (i.e., braincase, nasal and maxilla) of the grass snake skull, while no significant dimorphism was

detectable in the pterygoid or the quadrate bones, which differed slightly from our findings. The pattern described for *V. berus* mirrors that described for the related *Vipera aspis* which also shows a weak sexual shape dimorphism in the skull (Gentilli et al., 2009).

Allometric regressions detected different pattern between *N. helvetica* and *V. berus*. The grass snake displayed a clear allometric pattern with no substantial differences in the percentage of shape variation explained by body dimensions in males compared to females. All size variables generally explained more than 10% of shape variance. This trend is the same as that observed by Andjelković et al. (2016) and it can be interpreted as arising from the need for a stronger bite force, typical of frog-eating snakes. Nevertheless, as for *V. aspis* (Gentilli et al., 2009), the adder did not exhibit a clear allometric pattern. The absence of allometry can be a consequence of envenomation of prey items, which reduces the need for a strong bite in larger snakes. The primary function of snake venom is to facilitate rapid immobilization of prey, even if the prey manages to escape the initial predator bite (Barlow et al., 2009). However, the trend observed in male adders is slightly different from that in females: in the former all regressions explained more than 5% of shape variance (even when the regressions were non-significant), while in females the percentage was usually less than 3%. Such a discrepancy might be related to the presence of male-male combats for females, frequently observed in the family Viperidae (Madsen, 1988; Senter et al., 2014).

Sexual selection has been linked with static allometry in a multitude of studies (Green, 1992; Tomkins and Simmons, 1996; Emlen and Nijhout, 2000): the resulting allometric pattern might be due to evolution of a large head due to sexual selection (Green, 1992; Bonduriansky and Day, 2003). Static allometry of surface areas (here, head surface) is favoured because it causes a magnification of apparent differences in individual sizes, facilitating the assessment of sexual competitiveness (Wallace, 1987). That is, a wider head shape might be advantageous in bigger adder males because it might be more effective during fighting and/or constitute a signal for male competitors.

Covariation between shape and size matrices was completely lacking in female *V. berus* (and subsignificant in males), which contrasts sharply with *N. helvetica* which showed covariation within all three analytical groupings (i.e., mixed sexes, males and females). Increased venom production in females has been noted in other venomous snakes could potentially play a role in explaining the complete lack of allometry in head shape of female adders (Furtado et al., 2006).

In conclusion, we identified different trends regarding sexual size and shape head dimorphism in *N. helvetica* and *V. berus*. The degree of size difference between the sexes exceeded the shape-related divergence. With regard to size-shape covariation, the grass snake presented a static allometric pattern, while the adder did not (with a partial exception for male adders) supporting the hypothesis that mode of predation is likely to be a primary mechanism that drives phenotypic adaptations in snakes (Vincent et al., 2006).

Declarations of interest:none

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Figure captions

Fig. 1 Landmark configuration on head outlines of the grass snake (*Natrix helvetica*)(top) and the adder (*Vipera berus*)(bottom). Landmarked scales are indicated as frontal (F); and parietal (P).

Scale bar is 1 cm in length.

Fig. 2 Box-whisker plots of $\ln CS$ (a), $\ln TL$ (b), $\ln BL$ (c) and the index (d) across snake species and sexes. Limits on boxes (light males, dark females) correspond to the first and third quartiles, while the internal black line represents the median. Whiskers extend from minimum to maximum values. Circles represent outliers, asterisks are extreme outliers with values more than three times the height of the boxes.

Fig. 3 Scatterplots showing variation on PC1 and PC2, which represent 27.6% and 14.3% of the total variance, respectively. PC scores are from the pooled within-species covariance matrix.

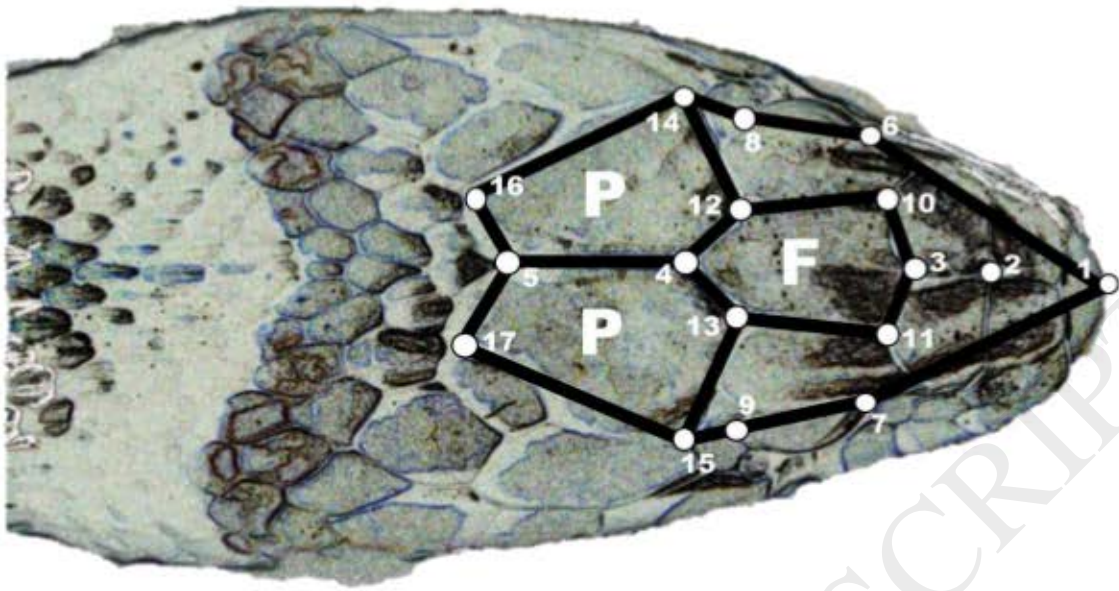
Wireframe visualisations are presented at the extremes on each PC axis, showing relative shape variation (black) and mean configuration (grey).

Fig. 4 Pattern of allometric shape variation in *Natrix helvetica* for (a) mixed-sexes, (b) males, (c) females. Mean shape is visualized in grey, while evaluated shape is visualized in black.

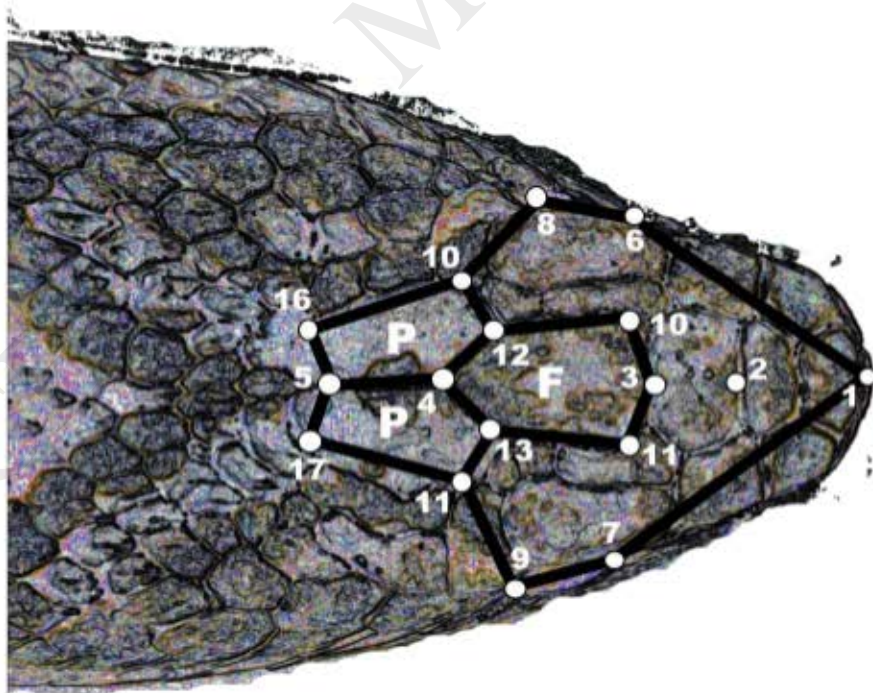
Fig. 5 Pattern of allometric shape variation in *Vipera berus* for (a) mixed-sexes, (b) males, (c) females. Mean shape is visualized in grey, while evaluated shape is visualized in black

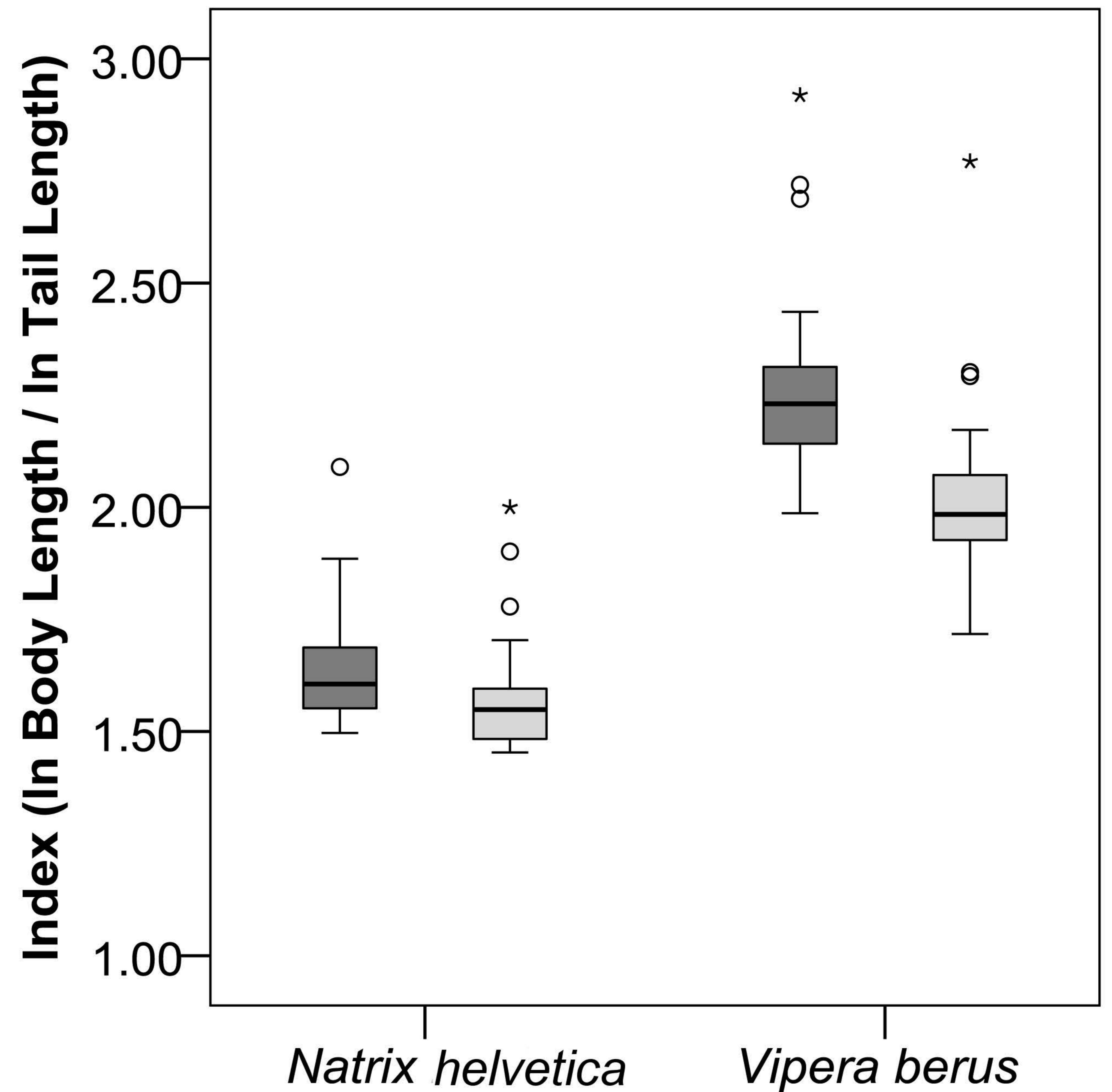
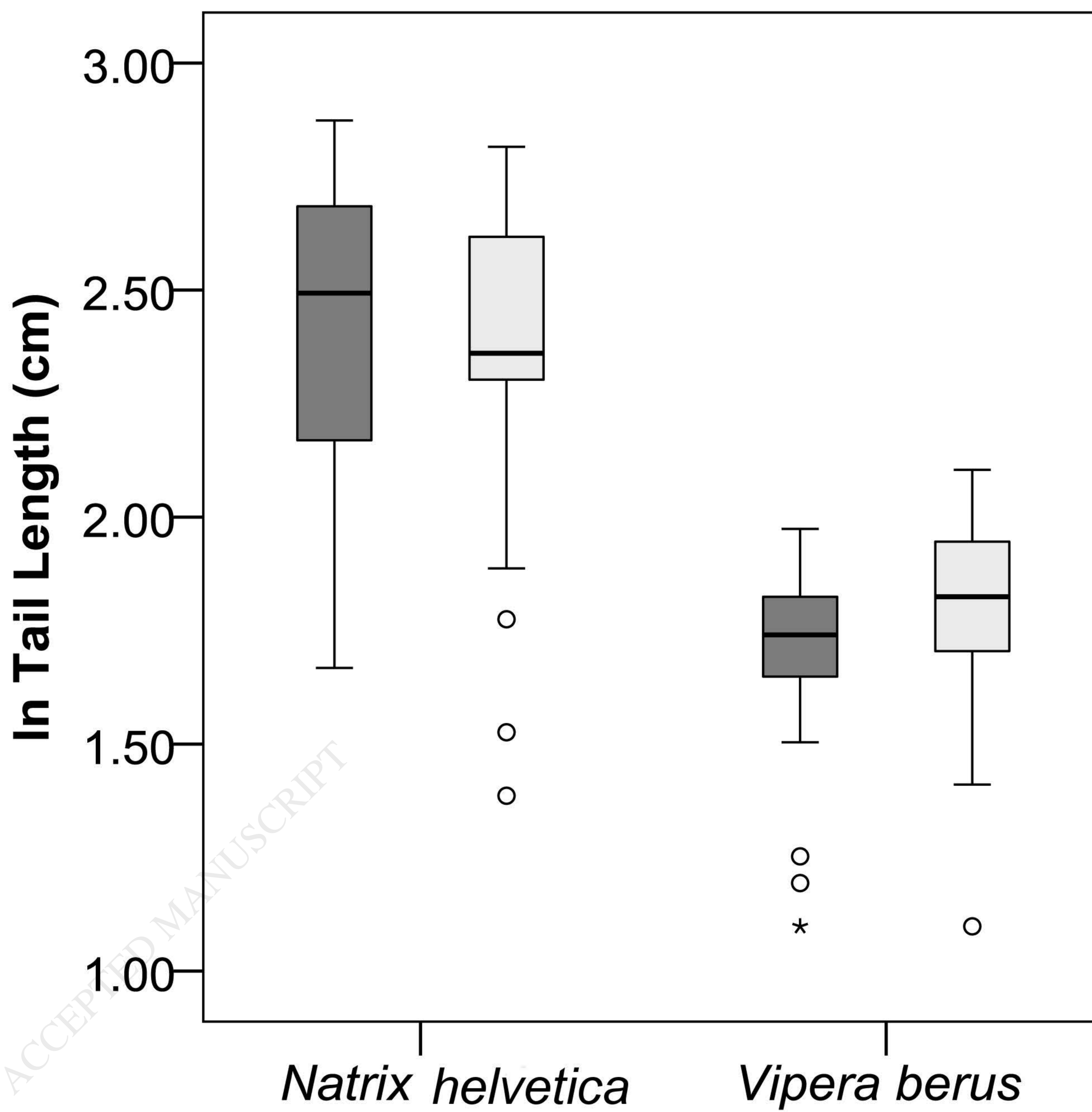
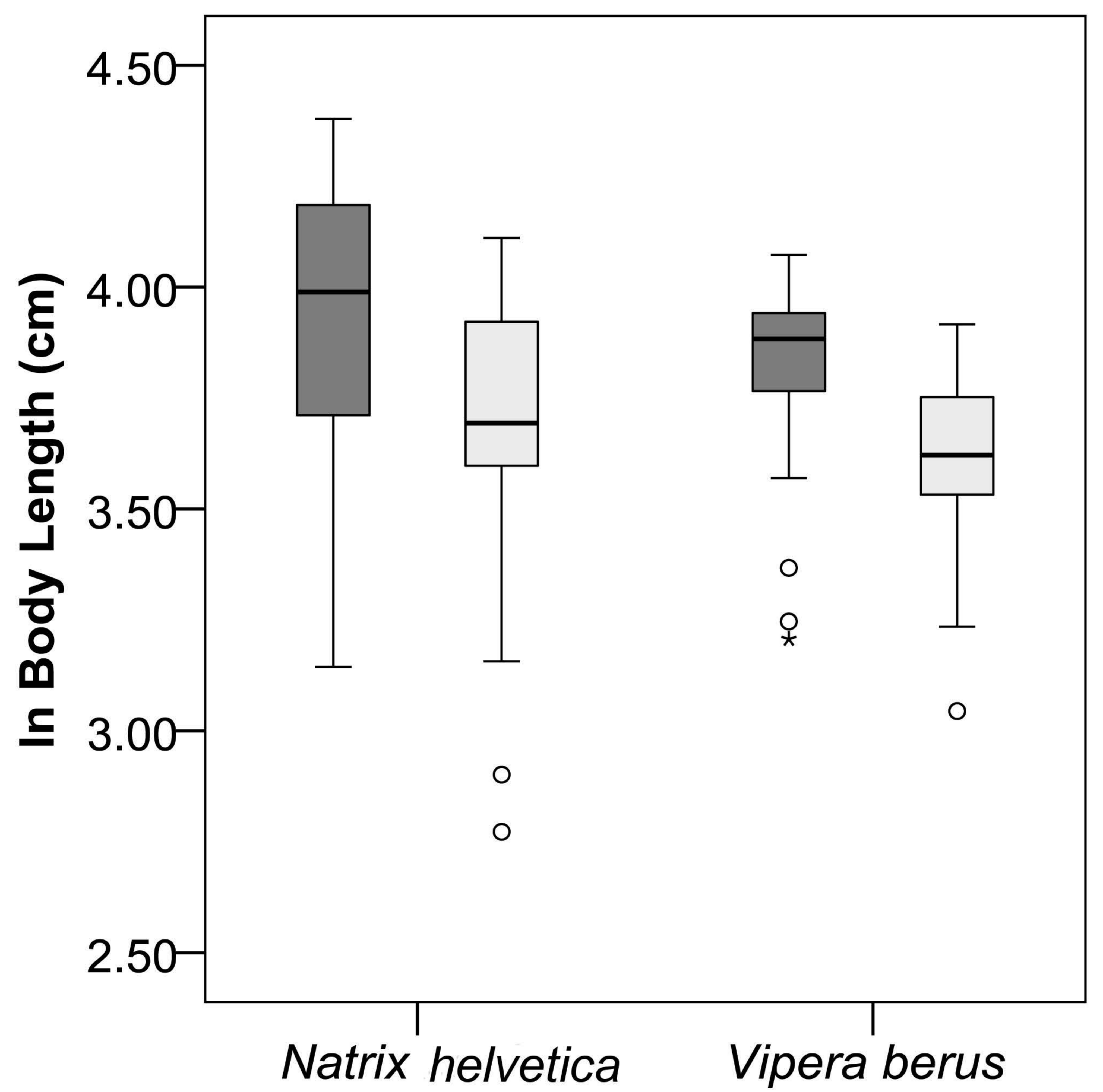
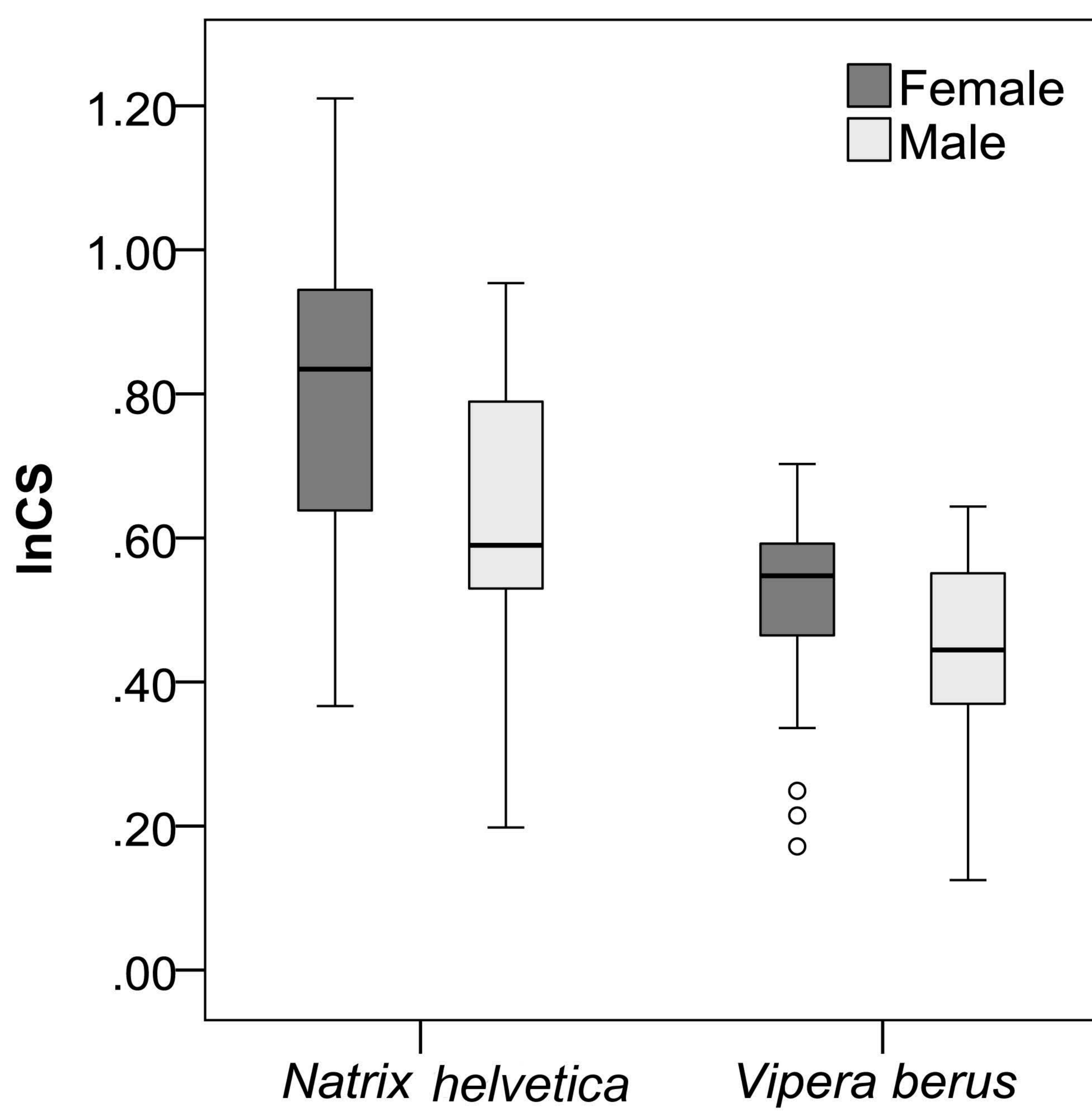
Fig. 6 Visualization of allometric trajectories across species and sexes when (a) $\ln CS$, (b) $\ln B$, (c) $\ln TL$ and (d) the index are used as a size predictor. *V. berus* specimens are shown as triangles, *N. helvetica* are shown as circles. Females are shown in black, males are shown in white.

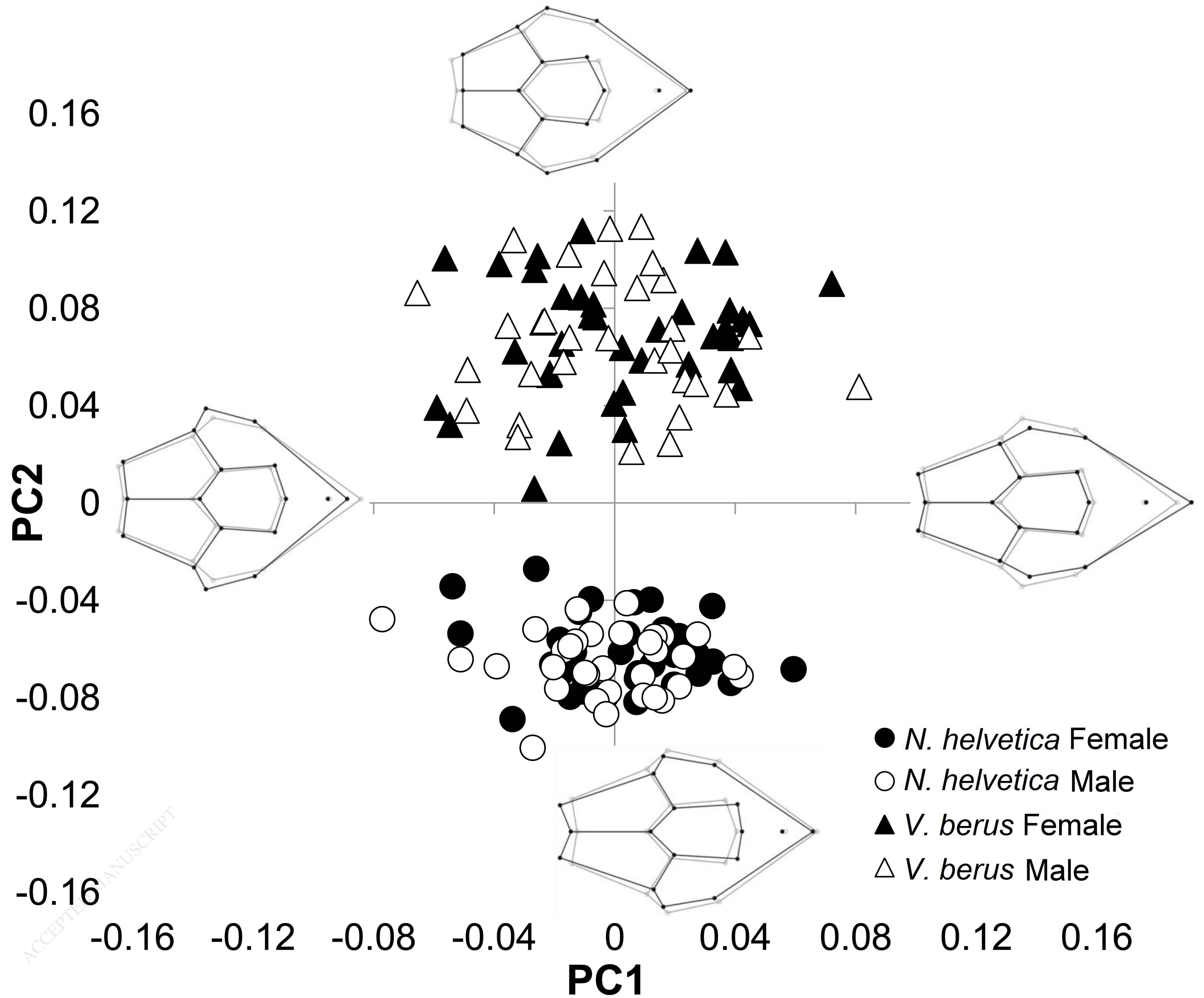
Fig. 7 Pattern of shape variation associated with Block1 PLS1 in pooled-sex *Natrix helvetica* sample. Mean shape is visualized in grey, while evaluated shape is visualized in black.



1cm



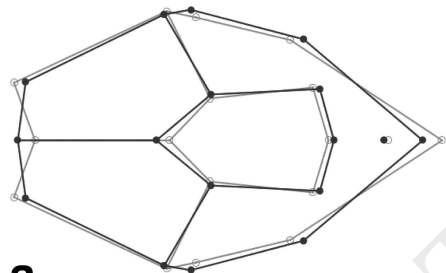




Small size

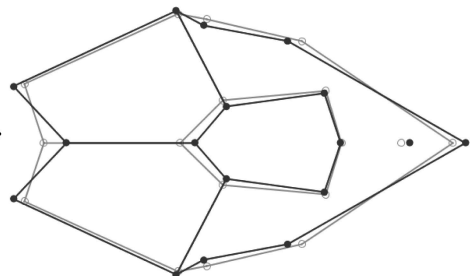
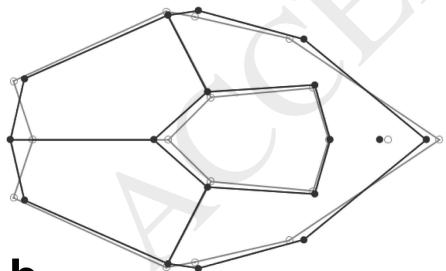
Mixed sexes

Large size



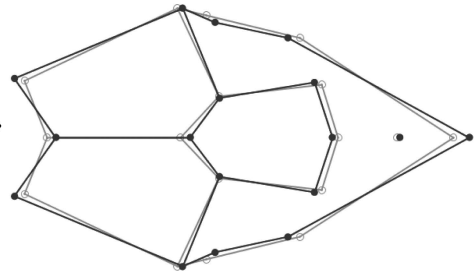
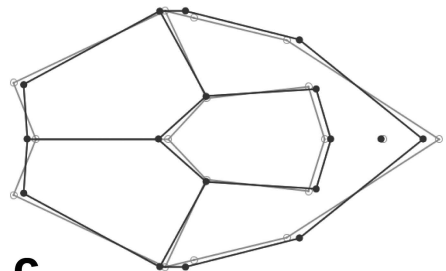
a

Males



b

Females

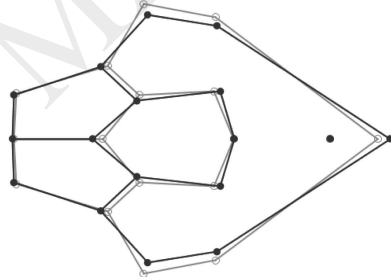
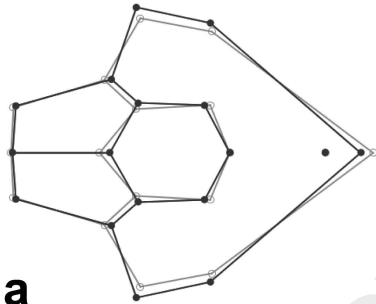


c

Small size

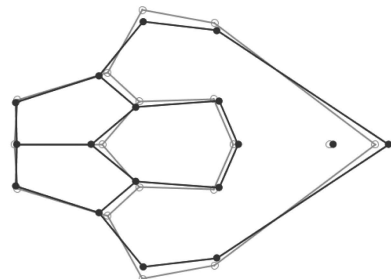
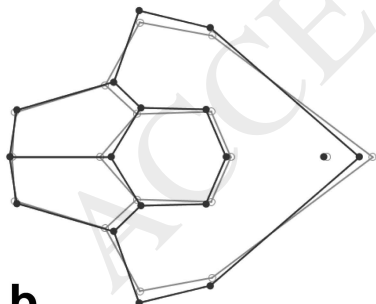
Mixed sexes

Large size



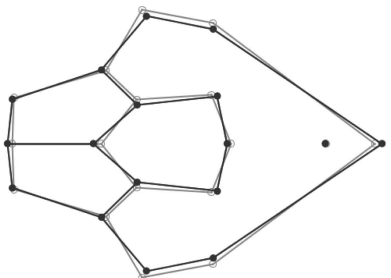
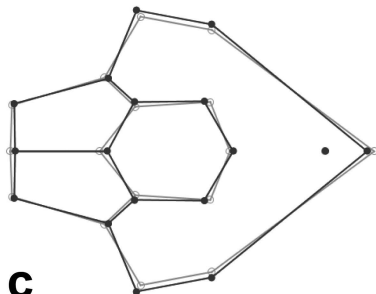
a

Males

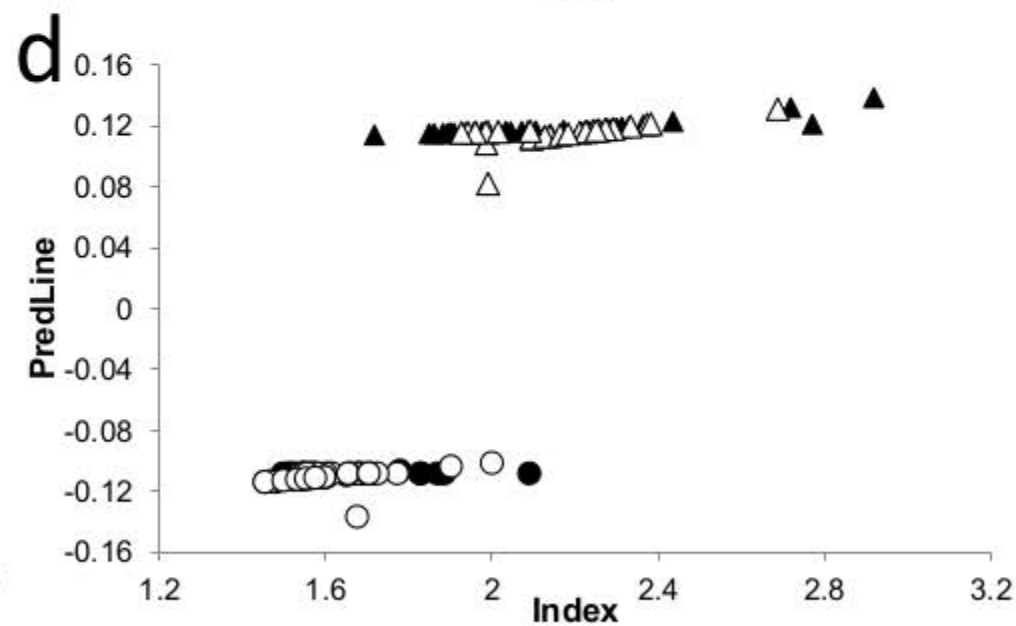
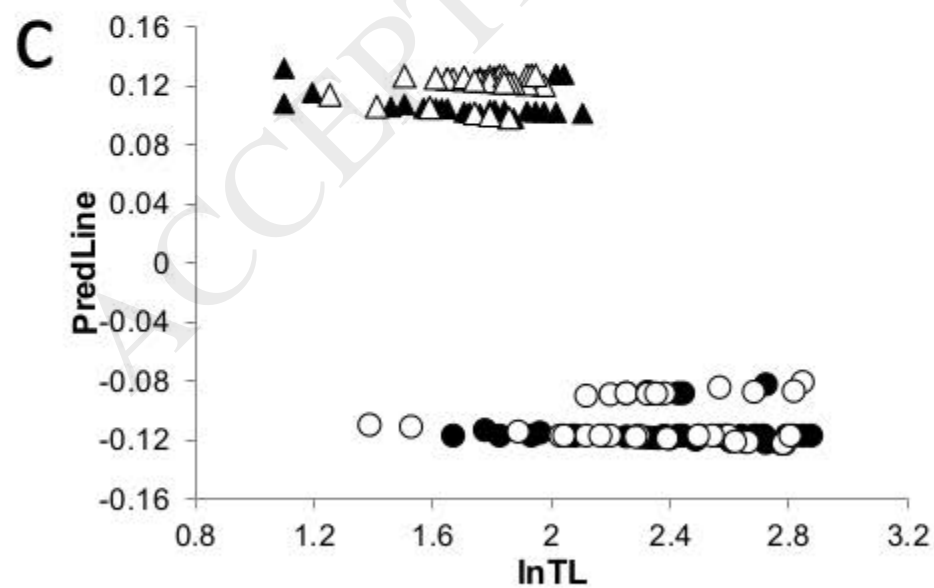
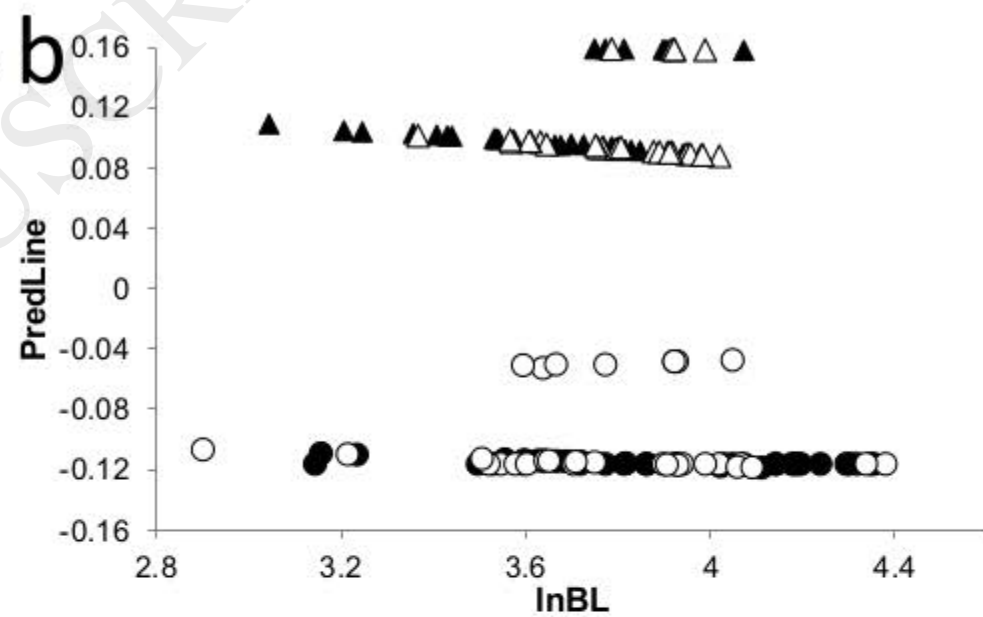
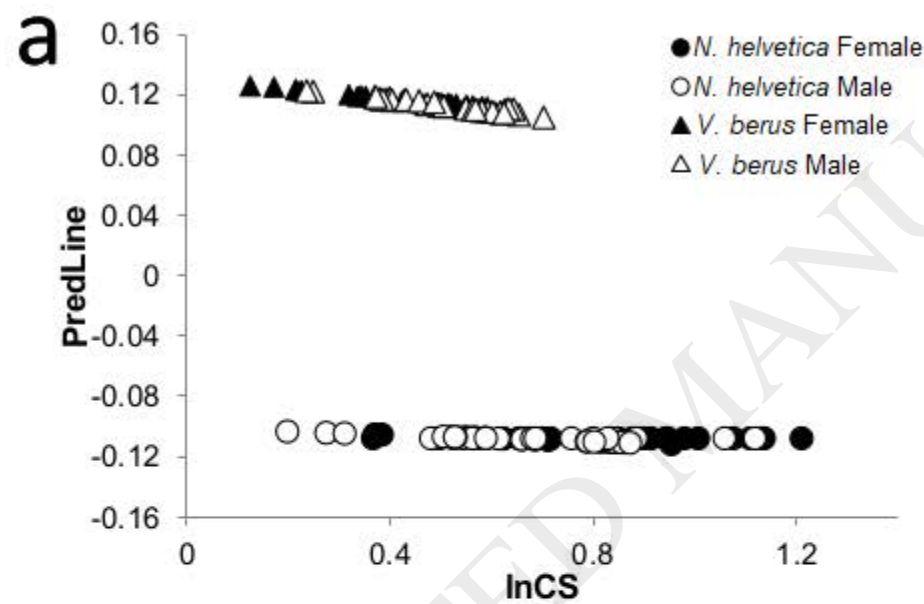


b

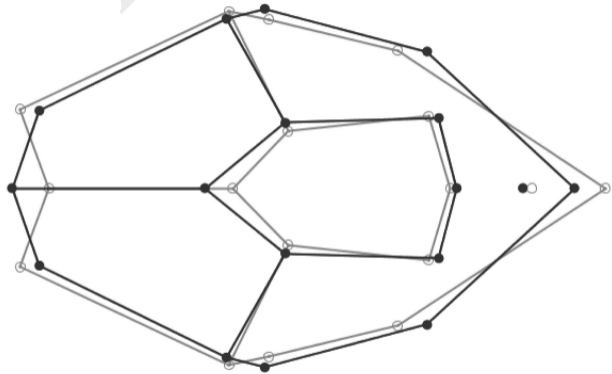
Females



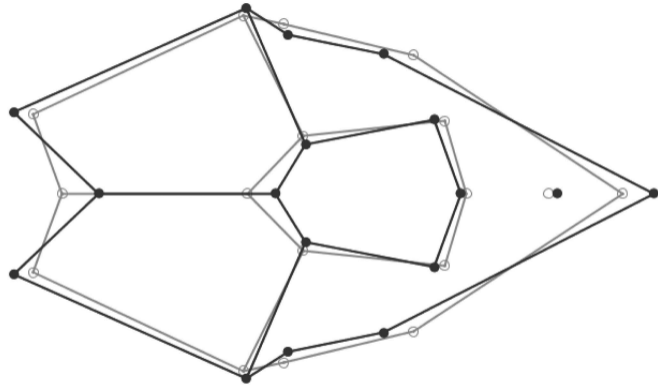
c



Small size



Large size



Tables

Table 1 Univariate (for size) and multivariate (for shape) two-way Procrustes ANOVA to test the impact of species and sex (and their interaction) on head size and shape for 140 *Natrix helvetica* and *Vipera berus* specimens. Significance is highlighted in italics

	Size/Shape variable		<i>df</i>	<i>SS</i>	<i>MS</i>	<i>r</i> ²	<i>F</i>	<i>p</i> -value
Size	lnCS	Sex	1	0.4919	0.49192	0.07458	16.871	<i>0.001</i>
		Species	1	2.022	2.02204	0.30657	69.347	<i>0.001</i>
		Sex:Species	1	0.1161	0.11611	0.0176	3.982	<i>0.013</i>
		Residuals	136	3.9656	0.02916			
		Total	139	6.5956				
	lnTL	Sex	1	0.0377	0.0377	0.00151	0.5086	0.475
		Species	1	14.6939	14.6939	0.58645	198.013	<i>0.001</i>
		Sex:Species	1	0.232	0.232	0.00926	3.1271	<i>0.006</i>
		Residuals	136	10.0921	0.0742			
		Total	139	25.0558				
	lnBL	Sex	1	1.9403	1.94025	0.16717	28.4374	<i>0.001</i>
		Species	1	0.3563	0.35633	0.0307	5.2225	<i>0.014</i>
		Sex:Species	1	0.0311	0.03112	0.00268	0.4561	0.481
		Residuals	136	9.2791	0.06823			
		Total	139	11.6068				
	Index	Sex	1	0.9457	0.9457	0.06359	39.163	<i>0.001</i>
Species		1	10.3769	10.3769	0.69772	429.726	<i>0.001</i>	
Sex:Species		1	0.2659	0.2659	0.01788	11.013	<i>0.001</i>	
Residuals		136	3.2841	0.0241				
Total		139	14.8726					
Shape	Procrustes coordinates	Sex	1	0.00473	0.00473	0.00225	1.6874	0.201
		Species	1	1.71019	1.71019	0.81523	610.079	<i>0.001</i>
		Sex:Species	1	0.00165	0.00165	0.00078	0.5871	<i>0.002</i>

Residuals	136	0.38124	0.0028
Total	139	2.09781	

Table 2 Size and shape sexual dimorphism indexes computed for *Natrix helvetica* and *Vipera berus*. Each index is also expressed as a percentage of size/shape difference between females (bigger) and males (smaller). Average and maximum Procrustes distance between the sexes is also provided as a measure of shape distance

	Sexual size dimorphism (SSD)				Sexual shape dimorphism		
	lnCS	lnTL	lnBL	lnBL/lnTL	Average Proc. Dist.	Max. Proc. Dist.	Percentage
<i>Natrix helvetica</i>	22.4%	2.4%	6.8%	4.3%	0.0134801	0.15185	9%
<i>Vipera berus</i>	12.5%	-6.3%	5.4%	10.9%	0.01426545	0.18316	8%

Table 3 Allometric regressions with percentage of shape variance explained by each body metric and relative p -value. P -values are in italics when significant ($p < 0.05$) and underlined when still significant after a Bonferroni correction for multiple tests

Species	Sex	Size Variable	% predicted	p -value
<i>Natrix helvetica</i>	Mixed	lnCS	13.53%	<u><i><.0001</i></u>
		lnTL	11.60%	<u><i><.0001</i></u>
		lnBL	12.99%	<u><i><.0001</i></u>
		Index	5.51%	<i>0.0025</i>
	Female	lnCS	16.52%	<u><i><.0001</i></u>
		lnTL	12.02%	<u><i>0.0004</i></u>
		lnBL	15.31%	<u><i><.0001</i></u>
		Index	5.14%	<i>0.0631</i>
	Male	lnCS	14.64%	<u><i>0.0003</i></u>
		lnTL	16.37%	<u><i>0.0003</i></u>
		lnBL	16.35%	<u><i>0.0002</i></u>
		Index	15.01%	<u><i>0.0003</i></u>
<i>Vipera berus</i>	Mixed	lnCS	4.28%	<i>0.0052</i>
		lnTL	1.64%	<i>0.3457</i>
		lnBL	1.95%	<i>0.222</i>
		Index	1.34%	<i>0.5144</i>
	Female	lnCS	2.88%	<i>0.3633</i>
		lnTL	0.60%	<i>0.9962</i>
		lnBL	0.66%	<i>0.9945</i>
		Index	0.56%	<i>0.9973</i>
	Male	lnCS	7.82%	<i>0.0191</i>
		lnTL	6.21%	<i>0.0709</i>
lnBL		5.36%	<i>0.1296</i>	

Index	6.73%	<i>0.0453</i>
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Table 4 Angles between allometric trajectories between species and sexes, and p -values related to each comparison. P -values are in italics when significant ($p < 0.05$) and underlined when still significant after a Bonferroni correction for multiple tests

Species	Sex	Size Variable	Angle degrees	p -value
<i>Natrix vs Vipera</i>	Mixed	lnCS	79.742	0.25468
		lnTL	68.34	0.07973
		lnBL	61.691	<i>0.03174</i>
		Index	77.798	0.216
	Female	lnCS	83.71	0.34314
		lnTL	73.277	0.13991
		lnBL	54.635	<i>0.00941</i>
		Index	96.687	0.66621
	Male	lnCS	79.485	0.24936
		lnTL	69.337	0.09003
		lnBL	67.173	0.0688
		Index	69.035	0.08681
<i>Natrix helvetica</i>	Female vs Male	lnCS	42.933	<u><i>0.00063</i></u>
		lnTL	49.454	<i>0.0032</i>
		lnBL	47.58	<u><i>0.00208</i></u>
		Index	60.482	<i>0.02625</i>
<i>Vipera berus</i>	Female vs Male	lnCS	35.463	<u><i>0.00006</i></u>
		lnTL	70.447	0.10257
		lnBL	54.636	<i>0.00941</i>
		Index	80.966	0.28069

Table 5 Three-way Procrustes ANOVA to test the impact of body metrics, species and sex (and their interaction) on head shape in the entire sample. Significance is highlighted in italics

Size variable		<i>df</i>	<i>SS</i>	<i>MS</i>	<i>r</i> ²	<i>F</i>	<i>Z</i>	<i>p</i> -value
InCS	InCS	1	0.55356	0.55356	0.26387	209.7498	5.5516	<i>0.001</i>
	species	1	1.17484	1.17484	0.56003	445.1614	7.0572	<i>0.001</i>
	sex	1	0.0036	0.0036	0.00171	1.3632	4.3987	<i>0.001</i>
	InCS:species	1	0.01058	0.01058	0.00504	4.0101	6.4996	<i>0.001</i>
	InCS:sex	1	0.00179	0.00179	0.00085	0.677	3.0362	<i>0.002</i>
	species:sex	1	0.00385	0.00385	0.00183	1.4584	4.6458	<i>0.001</i>
	InCS:species:sex	1	0.00122	0.00122	0.00058	0.4634	2.2133	<i>0.015</i>
	Residuals	132	0.34837	0.00264				
	Total	139	2.09781					
	InBL	InBL	1	0.07241	0.07241	0.03452	26.9924	3.5445
species		1	1.65538	1.65538	0.7891	617.0528	6.6776	<i>0.001</i>
sex		1	0.00414	0.00414	0.00197	1.5438	4.5793	<i>0.001</i>
InBL:species		1	0.00407	0.00407	0.00194	1.5182	4.7	<i>0.001</i>
InBL:sex		1	0.00324	0.00324	0.00155	1.2086	4.1126	<i>0.001</i>
species:sex		1	0.00243	0.00243	0.00116	0.9067	3.6302	<i>0.001</i>
InBL:species:sex		1	0.002	0.002	0.00095	0.7467	3.2027	<i>0.001</i>
Residuals		132	0.35412	0.00268				
Total		139	2.09781					
InTL		InTL	1	1.02448	1.02448	0.48836	380.4459	6.1774
	species	1	0.70058	0.70058	0.33396	260.1632	7.51	<i>0.001</i>
	sex	1	0.00492	0.00492	0.00235	1.8285	5.0125	<i>0.001</i>
	InTL:species	1	0.00457	0.00457	0.00218	1.6964	4.7309	<i>0.001</i>
	InTL:sex	1	0.00139	0.00139	0.00066	0.518	2.4792	<i>0.005</i>
	species:sex	1	0.00361	0.00361	0.00172	1.3417	4.3003	<i>0.001</i>
	InTL:species:sex	1	0.00279	0.00279	0.00133	1.0375	3.8104	<i>0.001</i>
	Residuals	132	0.35546	0.00269				

	Total	139	2.09781					
	Index	1	1.20839	1.20839	0.57603	442.5915	6.361	0.001
	species	1	0.50727	0.50727	0.24181	185.7959	8.1842	0.001
	sex	1	0.00706	0.00706	0.00336	2.5851	5.4843	0.001
	Index:species	1	0.00513	0.00513	0.00245	1.8805	4.9007	0.001
Index	Index:sex	1	0.00218	0.00218	0.00104	0.8003	3.2415	0.001
	species:sex	1	0.00418	0.00418	0.00199	1.5295	4.5872	0.001
	Index:species:sex	1	0.0032	0.0032	0.00152	1.171	3.9581	0.001
	Residuals	132	0.3604	0.00273				
	Total	139	2.09781					

Table 6 Summary of PLS analyses performed on each snake species separately, to test covariation between head shape and relative body metrics. p -values represent significance for the first pair of PLS vector extracted. P -values are in italics when significant ($p < 0.05$) and underlined when still significant after a Bonferroni correction for multiple tests

Species	Sex	<i>RV</i> coefficient	<i>r</i> PLS1 (p -value)	p -value
<i>Natrix helvetica</i>	Mixed	0.3122	0.88 (<.0001)	<u><.0001</u>
	Female	0.3381	0.86 (<.0001)	<u><.0001</u>
	Male	0.3642	0.86 (<.0001)	<u><.0001</u>
<i>Vipera berus</i>	Mixed	0.0682	0.38 (0.3275)	0.1837
	Female	0.0262	0.25 (0.9961)	0.9936
	Male	0.174	0.62 (0.0525)	0.0507