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Lower jaw modularity in the African clawed frog (*Xenopus laevis*) and fire salamander (*Salamandra salamandra gigliolii*)

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Modularity describes the degree to which the components of complex phenotypes vary semi-autonomously due to developmental, genetic and functional correlations. This is a key feature underlying the potential for evolvability, as it can allow individual components to respond to different selective pressures semi-independently. The vertebrate lower jaw has become a model anatomical system for understanding modularity, but to date most of this work has focused on the mandible of mammals and other amniotes. In contrast, modularity in the mandible of lissamphibians has been less well studied. Here, we used geometric morphometrics to quantify the static (intraspecific) modularity patterns in *Xenopus laevis* and *Salamandra salamandra gigliolii*. We tested developmental and functional hypotheses of modularity and demonstrate that both species exhibit significant modularity. Functional modularity was supported in both *Xenopus* and *Salamandra*. Allometry has a small yet significant impact on lower jaw shape in both taxa and sex has a significant effect on shape in *Xenopus*. The high lower jaw modularity in both species observed here, combined with the well-established modularity of the amphibian cranium, suggests that modularity is a ubiquitous feature of the tetrapod head.

ADDITIONAL KEYWORDS: Amphibia – development – function – integration – mandible – morphology – static modularity.

INTRODUCTION

Phenotypic variation is the raw material for natural selection and evolutionary change. As such, uncovering the origins of evolutionary diversity requires an understanding of how phenotypic variation is structured, both within and across populations. One essential source of variation in organisms is generally explained by the concept of modularity and integration. Modularity can be defined as the compartmentalization of structural variation into subunits that vary semi-independently (Olson & Miller, 1958). In contrast, integration can be defined as traits covarying in coordinated way (Zelditch & Goswami, 2021). As such, patterns of modularity and integration within and among complex biological structures are thought to influence the potential to respond to selection pressures and therefore the evolution of phenotypic diversity (e.g. Felice *et al.*, 2018).

Integration and modularity can be studied either at an evolutionary (interspecific) level (Felice *et al.*, 2019; Watanabe *et al.*, 2019; Fabre *et al.*, 2020; Rhoda *et al.*, 2021) or at the static (intraspecific) level looking at the variation within a species at a single ontogenetic stage (e.g. Albertson *et al.*, 2005; Zelditch *et al.*, 2008; Bon *et al.*, 2020). Static integration and modularity are of particular interest as natural selection occurs at the level of the population and thus studying trait correlations at this scale can help us to understand the patterns observed at the evolutionary level.

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There is a long history of researchers investigating static modularity by concentrating on the lower jaw as it is functionally and developmentally complex, two key features to the evolvability of an anatomical system (Raff, 1996; Raff & Raff, 2000; Klingenberg, 2005; Hallgrımsson *et al.*, 2009). Most of this research has been focused on rodents and has hypothesized that mammalian mandibles are divided into units based on functionality and/or developmental origins (Klingenberg *et al.*, 2003; Monteiro *et al.*, 2005; Zelditch *et al.*, 2008). Under the functional modularity hypothesis, the independent variation between the muscle-bearing (ascending ramus region) and tooth-bearing (alveolar region) sub-units of the jaw are strictly related to the different biomechanical functions of these regions (e.g. Wagner, 1995, 1996, 1998; Raff, 1996). In contrast, others have claimed that the ultimate cause of modularity between these two functional modules is related to developmental rather than biomechanical factors (Cheverud, 1982, 1988, 1995, 1996; Zelditch *et al.*, 2009). Finally, Fish *et al*. (2011) argued that the boundaries between modules within the jaw are not of developmental origins or function but of the expression patterns of key patterning genes. It has even been argued that despite their different morphologies and anatomical structures, these similar modules can be found in the lower jaw of all gnathostome species. However, Fish *et al.* (2011) suggested that function and development might impose different constraints on module variation with development defining the potential for variation and function fostering it.

To date, phenotypic modularity in the lower jaw has been investigated almost exclusively in mammals. The few studies that have addressed this question in non-mammalian vertebrates tend to show the same functional pattern with muscle- and tooth-bearing modules (e.g. Albertson *et al.*, 2005; Fish *et al.*, 2011; Rhoda *et al.*, 2021). For example, the highly mobile jaws of snakes similarly showed significant modularity with modules structured into functional subunits (Rhoda *et al.*, 2021). Another study on two species of cichlid fish demonstrated that the pattern of morphological integration of the lower jaw of cichlids results from a balance between opposing functional demands (Albertson *et al.*, 2005).

Because modularity and integration are thought to be influenced by development, function or a combination of the two, it is critical to investigate these properties in organisms with complex life history strategies that involve functional and developmental changes in anatomy through the life of the organism. In this study, we investigate whether this functional and developmental pattern can be found in populations of two different species of lissamphibians: the African clawed frog (*Xenopus laevis*) and fire salamander (*Salamandra salamandra gigliolii*). Unlike the mammalian mandible, which is composed of just a single element, the lower jaw of lissamphibians (as well as most other vertebrates) is more structurally complex, consisting of multiple bones. *Salamandra* and *Xenopus* have different life history strategies and are larviparous and biphasic, respectively (Table 1). *Salamandra* species give birth to fully aquatic larval stage juveniles before they metamorphose into a terrestrial adult with a drastic change of environment associated with partial bone remodelling (notably the coronoid disappears and teeth arrangement is switched from monostichous to polystichous on the dentary; Parker & Dunn, 1964; Rose, 2003). In contrast, *Xenopus* transitions from a tadpole to a frog, living almost entirely aquatically in adulthood and retaining the same suspension suction feeding methods via buccal pumping mechanisms (Wassersug, 1974; Deban *et al.*, 2001). The lower jaw of adult *Salamandra* includes three ossified elements: the dentary, prearticular and articular (Rose, 2003; Jiang *et al.*, 2018). The mandible of *Xenopus* is composed of just two ossified elements, the dentary (which is edentulous, as in nearly every extant frog) and angulosplenial (Fabrezi & Emerson, 2003). In both taxa, the mentomeckalian is also present and is variably fused to the dentary (Fabrezi & Emerson, 2003; Rose, 2003) and was not quantified separately in the present analysis.

Thus, both species undergo a metamorphosis, even if their life cycle strategies are different (biphasic for *Xenopus* and larviparous for this population of *Salamandra*). Feeding ecology and strategy at

Table 1. Differences in jaw anatomy and life history in *Salamandra* and *Xenopus*

	Salamandra salamandra gigliolii	*Xenopus laevis*
Number of ossified elements in the jaw	3	2
Life history strategy	Larviparous	Biphasic
Jaw remodelling	Partial	Complete
Larval habitat	Aquatic	Aquatic
Adult habitat	Terrestrial	Aquatic

the adult stage also differ between the two species (suction feeding in aquatic milieu for *Xenopus* and terrestrial tongue projection for *Salamandra*), as well as sequence of ossification (the articular ossifies last in *Salamandra* and the dentary and lateral part of the angulosplenial ossify last in *Xenopus*) and bone remodelling during metamorphosis (overall remodelling in *Xenopus* versus partial remodelling in *Salamandra*). Given their major differences in jaw development and function, we predict that each species will exhibit distinct modularity patterns. Furthermore, there are thought to be some sex-related differences in cranio-cervical morphology in both taxa (Sassoon & Kelley, 1986; Alarcón-Ríos *et al.*, 2017). If sexual dimorphism influences either developmental rates or jaw function in these species, we predict that males and females could differ in jaw morphology and integration.

Here, we use geometric morphometrics to explore the patterns of variation and covariation in mandibular morphology within *Xenopus* and *Salamandra* and how these patterns compare to across- and within-species patterns of cranial variation (Bon *et al.*, 2020; Fabre *et al.*, 2020; Bardua *et al.*, 2021). We compare the static modularity patterns of *Xenopus* and *Salamandra*, testing the hypothesis that they share similar modularity patterns despite having different mandibular morphologies, life cycles and ecologies. Because allometry is sometimes thought to be an important factor influencing trait integration (Bright *et al.*, 2016; Hallgrímsson *et al.*, 2019), we also quantified the strength and significance of allometry on mandibular morphology. Finally, we examine the effects of sex on jaw morphology in *Xenopus*.

MATERIALS AND METHODS

The sample consisted of 77 specimens of *Xenopus* and 31 *Salamandra* specimens. All specimens were fluid-preserved and sourced from the Natural History Museum (London). The sex of the *Xenopus* specimens was known (38 female, 34 male) but *Salamandra* specimens were not identified by sex in the museum accession data. *Salamandra* specimens are representatives of a single population collected near Genoa, Italy. *Xenopus* specimens originated from the European Xenopus Resource Centre at the University of Portsmouth and were donated to the Natural History Museum. Accession numbers for all specimens are available as part of the Supporting Information (https://doi.org/10.5061/dryad.3bk3j9kq2.). Each specimen was scanned by comuted tomography (CT) at the Natural History Museum using a Nikon XT 225 microCT scanner. We used Slicer3D to process the raw CT scan data and create a 3D model of each specimen's mandibular morphology (Fedorov *et al.*, 2012). Using CheckPoint (©Stratovan), we digitized landmarks and semi-landmarks on each specimen, following the divisions of each bony element (Figs 1, 2). As the mandible is composed of two bones in *Xenopus* and three bones in *Salamandra*, different landmark schemes were used for each. For *Xenopus* we used 16 landmarks and 230 semi-landmarks, and for *Salamandra* there were 22 landmarks and 470 semi-landmarks (Supporting Information, Tables S1–S4). In both species, the landmarks demarcate the edges of the bony elements with semi-landmarks connecting them, tracing the edges of the bones. Then, each dataset was subjected to a Procrustes superimposition to remove the effects of isometric size, position and rotation (Rohlf & Bookstein, 1990).

We visualized patterns of variation in each species using principal component analysis (PCA) and then tested the effect of allometry in each species using Procrustes ANOVA, a regression method specifically designed for high-dimensional data such as geometric morphometric data (Collyer *et al.*, 2015). We used the same method to test whether male and female specimens of *Xenopus* have significantly different jaw shapes. We further tested whether males and females have significantly different shape variance using the morphol.disparity function in the geomorph R package v.4.0.4 (Adams & Otárola-Castillo, 2013). This approach quantifies per-group morphological variance as the sum of the diagonal elements of the variance/covariance matrix divided by the number of individuals in each group (Adams & Otárola-Castillo, 2013). We quantified modularity using two different approaches: EMMLi uses a likelihood-based approach to compare different hypotheses of modular organization (Goswami & Finarelli, 2016) and CR (covariance ratio) compares the within-module covariance/between-module covariance ratio to a null distribution (Adams, 2016; Adams & Collyer, 2019). The hypotheses we proposed include several developmental hypotheses: (1) embryonic derivation of individual bones (mandibular neural crest, hyoid neural crest and non-neural crest, see Piekarski *et al.*, 2014), (2) osteological units, (3) sequence of ossification and (4) bones remodelled during metamorphosis (Supporting Information, Figs S1, S2). We also propose a functional model where the osteological units are further split, specifically: (5) between the anterior (teeth-bearing) and the one or two posterior part(s) (muscle attachment and articulation sites when possible, as it is often not well ossified is *Xenopus*) of the jaw. In *Salamandra*, the ossification sequence and embryonic derivation hypotheses are equivalent as regions derived from mandibular-stream cranial neural crest cells ossify first and the articular, which is

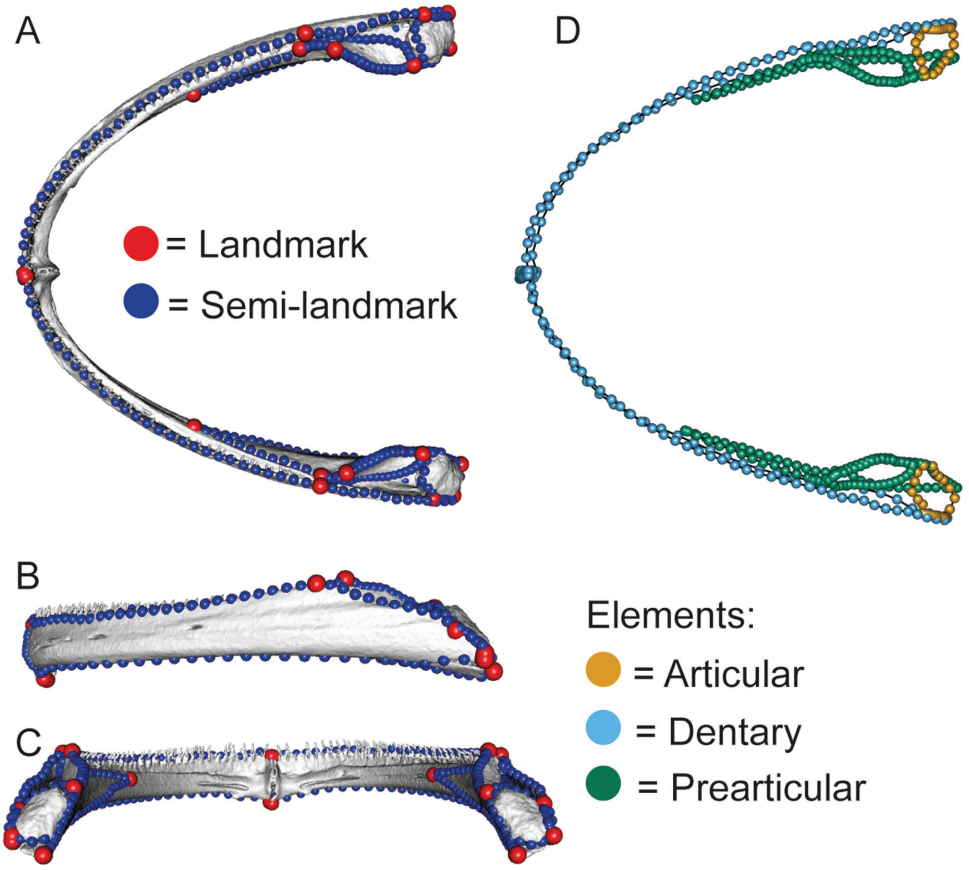

Figure 1. Anatomical landmarks (red) and sliding semi-landmark curves (blue) applied to each *Salamandra salamandra* specimen shown on NHMUK 64C in dorsal (A), left lateral (B) and caudal (C) view. The lower jaw consists of three ossified elements in this taxon (D).

derived from both the mandibular and hypoid neural crest, ossifies second (Rose, 2003; Piekarski, 2014). In *Xenopus*, we did not test the embryonic derivation or remodelling hypotheses as all ossified elements are derived from the mandibular neural crest and all elements are remodelled during metamorphosis, making these hypotheses equivalent to the null model that the entire jaw is a single module. Using the EMMLi method, we tested additional hypotheses by distinguishing between models where each module has the same within-module correlation as each other module ('same within'; Supporting Information, Tables S6, S10) and models where each module has different within-module correlation ('separate within'). We also tested this for between-module correlations ('same within' vs 'same between'). To further interrogate sex-based morphological differences, we used the CR ratio to test whether male and female *Xenopus* exhibit a significantly different strength of modularity. All analyses were carried out in R v.4.2.1 (R Core Team, 2021).

RESULTS

The effect of centroid size on the shape of the *Salamandra* jaws was relatively small yet significant (R^2 = 0.07, P = 0.01, Supporting Information, Table S5) suggesting the impact of jaw size on the morphology of the jaw is low. Similarly, the effect of centroid size on the shape of the *Xenopus* jaws was significant but weak (R^2 = 0.078, P = 0.001, Supporting Information, Table S9).

PCA of the *Salamandra* sample reveals that the first 19 principal component (PC) axes explain 95% of the cumulative variance. The primary element of variation, PC1, represents 22.7% of the total shape variation and described primarily the level of ossification at the articular, narrowness and length of the dentary. Low PC1 scores indicate a shorter and wider jaw with a more ossified articular compared to high PC1 scores (Fig. 3). PC2 (explaining 15.0% of the overall variation) described the anterior–posterior length of the prearticular with specimens with high

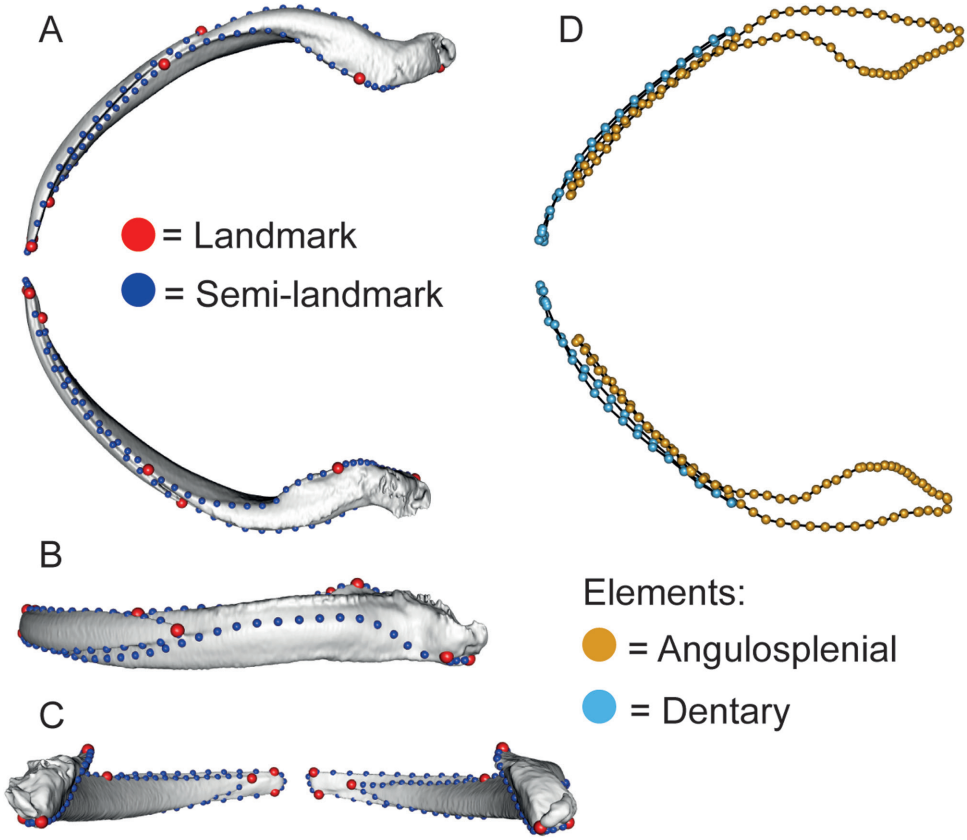

Figure 2. Anatomical landmarks (red) and sliding semi-landmark curves (blue) applied to each *Xenopus laevis* specimen in dorsal (A), left lateral (B) and caudal (C) view. The lower jaw consists of two ossified elements in this taxon (D).

PC2 scores being much shorter than specimens with low PC2 scores (Fig. 3).

In contrast, in *Xenopus* the first 23 PC axes explain 95% of the variation. PC1 represents 23.15% of the shape variation and describes the roundness of the jaw and height of the coronoid processes with specimens with lower PC1 scores being more pointed with shorter, less protruding coronoid processes and those with higher PC1 scores being more rounded with coronoid processes that peak higher (Fig. 4). This component displays the morphological difference between sexes, with female specimens being more varied, exhibiting less rounded jaws with shorter coronoid processes (Fig. 4). This difference in morphospace occupation is supported by a significant difference in Procrustes variance (males: 2.07×10^{-3}, females: 2.77×10^{-3}, $P = 0.041$). However, the effect of sex on the shape of the *Xenopus* jaws was significant yet relatively small ($R^2 = 0.027$, $P = 0.011$, Supporting Information Table S9). The interaction between sex and size does not have a significant effect on jaw shape ($P = 0.377$, Supporting Information, Table S9).

Principal component axis 2, representing 15.46% of the total variation, describes the relative length of the dentary compared to the angulosplenial as well as the medial to lateral concavity of the angulosplenial curve (Fig. 4). Low PC2 scores indicate a shallower concavity of the bone with a lower dentary to angulosplenial ratio whereas higher PC2 scores indicate a more medially curving angulosplenial bone near the posterior end and proportionally longer dentary within the jaw.

Both modularity analyses support the hypothesis that the lower jaw is a modular structure in both taxa. In *Salamandra*, EMMLi recovers the functional modularity hypothesis (four modules) as the highest likelihood hypothesis (Supporting Information, Table S6). This suggests that not only is each bone a separate module, but also that muscle attachment sites of the dentary are decoupled from the tooth-bearing parts of the dentary due to their difference in function. The CR test provides some support for this hypothesis as well. Compared to the alternative hypotheses, functional modularity has the lowest effect size (i.e. strongest signal, Supporting Information, Table S7). However, pairwise comparisons reveal that this hypothesis is not significantly different from the ossification sequence or osteological units hypotheses (Supporting Information, Table S8).

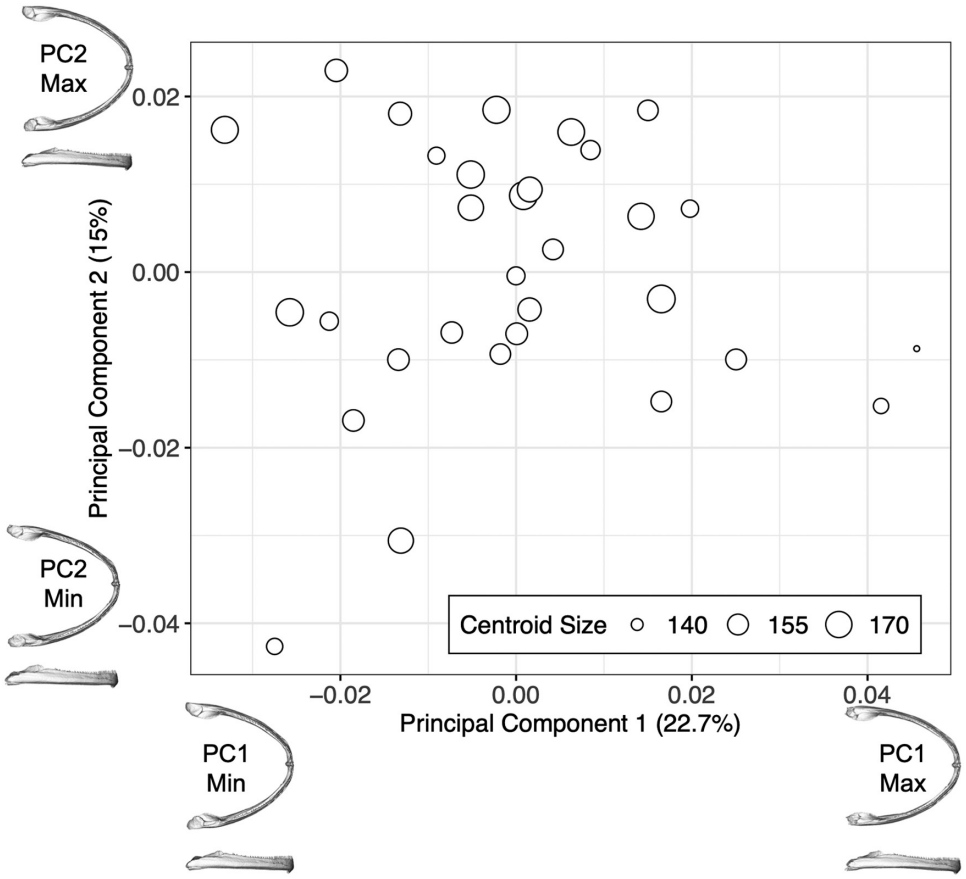

Figure 3. Principal components analysis of the *Salamandra salamandra* lower jaw. Inset lower jaw images illustrate the theoretical shapes described by the minimum and maximum extremes of PC axis 1 and 2 in dorsal and right lateral view.

In *Xenopus*, both CR and EMMLI analyses of modularity support the functional hypothesis. The highest likelihood modularity hypothesis was this three-module model where each bone is an independent module and the angulosplenial is further split into two modules, separating the anterior mouth shape and posterior muscle attachment site (Supporting Information, Table S10). The CR test for the three-module hypothesis produced a CR score of 0.834. This hypothesis has a significantly higher modularity signal than either the osteological units hypothesis or the ossification sequence two-module hypotheses ($P = 0.034$) (Supporting Information, Tables S11, S12). Males and females do not have significantly different strength of modularity (males: CR = 0.8735; females: CR = 0.862; $P = 0.61$).

DISCUSSION

Both *Salamandra* and *Xenopus* exhibit modularity in mandibular morphology and this modularity seems to be partitioned by the function of each jaw sub-region. In *Salamandra*, different approaches to quantifying modularity favoured the functional hypothesis of modularity, but the alternative hypotheses cannot be rejected on the basis of the CR results. These results support the hypothesis that lissamphibians, like mammals (e.g. Cheverud, 1982, 1988, 1995, 1996; Zelditch *et al.*, 2009), exhibit static modularity in the lower jaw. These hypotheses share in common that the part of the jaw that transfers forces to prey items is a distinct module from the load-bearing regions that contain the jaw joint and muscle attachments. Jaw functions are likely to be closely related as partial remodelling of the dentary during metamorphosis corresponds to changes in dentition (Parker & Dunn, 1964) and, presumably, in jaw function with the transition from aquatic to terrestrial life (from suction feeding to lingual feeding).

In *Salamandra*, the CR test fails to reject the ossification sequence and osteological units hypotheses. This is congruent with recent research on modularity in the cranium of this taxon that has demonstrated the skull is highly modular (20 bones partitioned into at least 13 modules; Bon *et al.*, 2020). The pattern of trait

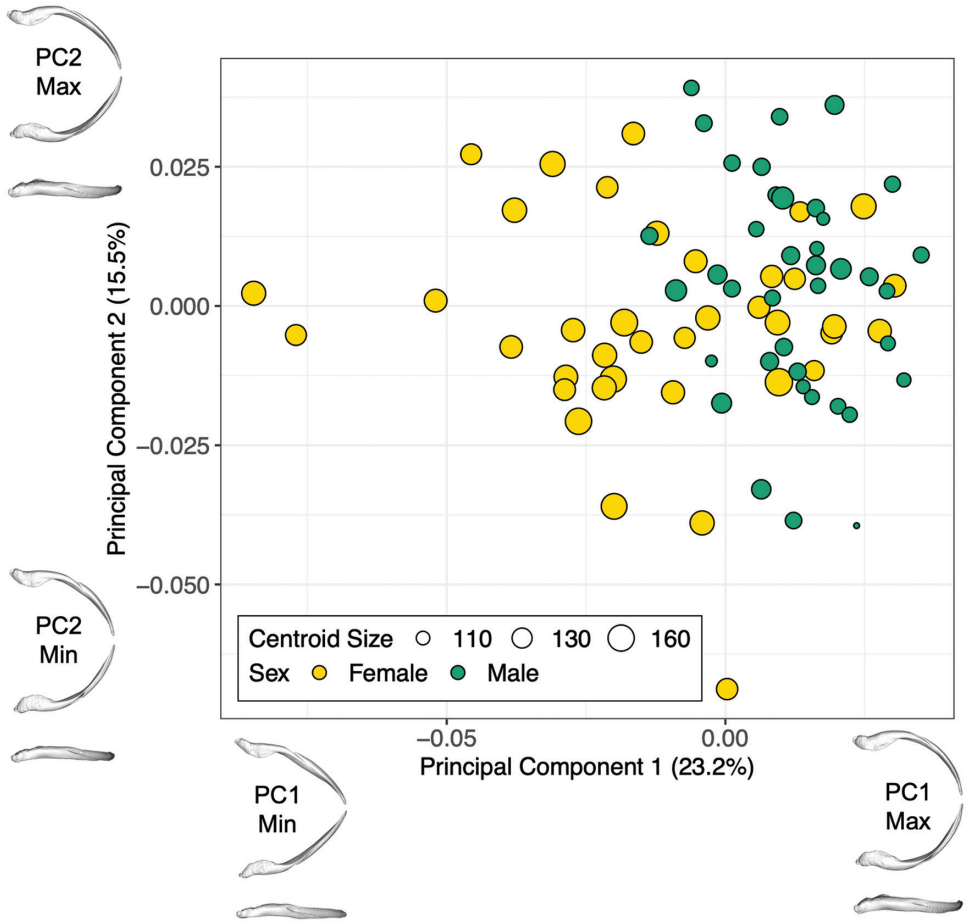

Figure 4. Principal components analysis of the *Xenopus laevis* lower jaw. Inset lower jaw images illustrate the theoretical shapes described by the minimum and maximum extremes of PC axis 1 and 2 in dorsal and right lateral view.

correlation in *Salamandra* includes some modules composed of individual osteological units and others composed of functional complexes of several bones (Bon *et al.*, 2020). The former is indicative of developmental drivers of modularity and the latter is indicative of functional drivers of modularity. Taken together, cranial and mandibular patterns of modularity in *Salamandra* support the hypothesis that the pattern of modularity in the skull is complicated and not attributable to just one set of constraints.

In *Xenopus*, EMMLi and covariance ratio analyses also provided support for a modular pattern of phenotypic variation in the lower jaw. The most likely hypothesis, supported by both analyses, was the three-module functional hypothesis that divided the jaw into the dentary and two functional regions of the angulosplenial. This hypothesis was significantly better supported than the two-module hypothesis that separated the jaw developmentally into bones.

Both taxa in this analysis showed significant support for static modularity, suggesting that individual components of the amphibian lower jaw vary semi-independently and may respond to selective pressures semi-independently. The weaker support for a definitive hypothesis in *Salamandra* suggests that there may be alternative modular organization to the salamander lower jaw that was not tested here. In contrast, in *Xenopus*, the hypothesis of maximal modularity was most supported, dividing the jaw into the dentary, the linguodorsal angulosplenial region and the ventrolabial angulosplenial region. These results suggest the coronoid process of the angulosplenial, the area of attachment for the jaw adductor muscles, can respond to selection pressures independently of the rest of this element.

Our results demonstrate that allometry has a significant but very weak effect on jaw shape in both taxa. Static allometry does not have a significant effect on cranial morphology in *S. salamandra* (Bon *et al.*, 2020). Together, these findings suggest that craniomandibular morphology is not primarily driven by centroid size.

PCA shows that both articular ossification and the length of the jaw are highly variable in *Salamandra*. The variable ossification of the articular can perhaps be explained by the varied age of the specimens as in Urodela the articular tends to ossify late in the metamorphic period, after both the dentary and prearticular (Germain & Laurin, 2009). The second PC axis primarily captures variation in the relative length of the prearticular bone. High levels of variation in the dentary and prearticular could be explained by the role of these bony elements in feeding and how the method of feeding changes due to metamorphosis. Though *Salamandra* initially use suction feeding as larvae due to their aquatic nature, after they metamorphose, their transition to terrestrial feeding causes jaw morphology to instead favour jaw and tongue prehension as the primary feeding mechanism (Deban & Wake, 2000). The muscles required for jaw closing insert onto the prearticular and dentary and thus disparity in the shape of these elements between specimens could be due to age or sex differences. Unfortunately, this remains to be tested. As estimating the age and the sex of these animals is an invasive process, it was not possible for this current study.

In *Xenopus*, males and females occupy overlapping regions of morphospace (Fig. 4), but females occupy a much wider range of shapes and achieve phenotypes from which males are apparently excluded. The female-only phenotype is characterized by a more angular anterior segment of the jaw (Fig. 4). A possible explanation for the morphological constraints on the male specimens is the sexual dimorphism of the *Xenopus* vocalization apparatus. Male specimens of *X. laevis* have a mating call whereas the females do not (Sassoon & Kelley, 1986). We hypothesize that mouth shape could be constrained by its association with hyobranchial muscles and the need to produce recognizable mating calls. This constraint could explain why males occupy a much smaller region of morphospace than females (Fig. 4). If this constraint exists, it is likely to be relatively weak as the effect of sex on shape is significant but weak (Supporting Information, Table S9).

The proximate cause of these sex-related differences is likely to be sex-specific differences in hormones during metamorphosis. The female phenotype superficially resembles an experimentally induced phenotype generated by treatment with exogenous thyroid hormone (Rose & Cahill, 2019). In mammals, sex hormones are known to play a key role in mandibular growth (Marquez Hernandez *et al.*, 2011). As such, a fruitful area for future evo-evo-devo research would be interrogating how hormonal factors influence craniofacial growth during metamorphosis in amphibians.

Across the complete sample of *Xenopus*, the most variable traits were the angle of the anterior jaw and the height of the coronoid process (PC1) and the length of the dentary and concavity of the angulosplenial bone (PC2). These patterns could be related to individual differences in jaw function and performance. For example, functional models of jaw function in Anura demonstrate that frogs eating larger, faster moving prey will have relatively longer and wider jaws as this increases the mouth's opening and closing speeds, as well as the force of the bite (Emerson, 1985). However, *Xenopus* typically favours using its forearms and hindlimbs over jaw prehension during predation (Avila & Frye, 1978), suggesting that jaw strength and prehension speed play a more minor role and are less significant factors influencing variation. As *Xenopus* is almost entirely aquatic and retains the same buccal pump, suction-feeding mechanism from larvae to adulthood, its metamorphic process is less drastic than in other frogs (Cannatella & De Sá, 1993). Nevertheless, remodelling does occur, and the larval musculature of the jaw degenerates before it is replaced (Haas, 2001). The length of the dentary and size of the coronoid processes also increase during metamorphosis, again offering an explanation for the observed morphological disparity in these structures (Trueb & Hanken, 1992).

Taken together, these results demonstrate that both *S. salamandra* and *X. leavis* have complex patterns of mandibular variation and both exhibit modularity in this structure. These taxa have distinct anatomical configurations of the lower jaw, and yet both exhibit similar patterns of modularity with the coronoid process potentially existing as a distinct module. This reflects a similar finding in mammals, which have distinct muscle-bearing and tooth-bearing modules within the lower jaw. Perhaps modularity is a universal trait of the vertebrate lower jaw, regardless of the number of ossified elements.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

DATA AVAILABILITY

All code and landmark data are available on DataDryad at https://doi.org/10.5061/dryad.3bk3j9kq2

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article on the publisher's website.

Table S1. Landmark positions in *Salamandra salamandra*
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Figure S2. Modularity hypotheses for *Xenopus laevis*. For each hypothesis, landmarks and semi-landmarks of the same colour are hypothesized to belong to a single module.