Cranial integration in the fire salamander, *Salamandra salamandra* (Caudata: Salamandridae).

Running title: salamander cranial integration

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ABSTRACT

Phenotypic integration and modularity are concepts that represent the pattern of connectivity of morphological structures within an organism. Integration describes the coordinated variation of traits, and analyses of these relationships among traits often reveal the presence of modules, sets of traits that are highly integrated but relatively independent of other traits. Phenotypic integration and modularity have been studied at both the evolutionary and static level across a variety of clades, although most studies thus far are focused on amniotes, and especially mammals. Using a high-dimensional geometric morphometric approach, we investigated the pattern of cranial integration and modularity of the Italian fire salamander (*Salamandra salamandra giglioli*). We recovered a highly modular pattern, but this pattern did not support either entirely developmental or functional hypotheses for cranial organisation, possibly reflecting complex interactions amongst multiple influencing factors. We found that size had no significant effect on cranial shape, and that morphological variance of individual modules had no significant relationship with respective degree of within-module integration. The pattern of cranial integration in the fire salamander is similar to that previously recovered for caecilians, with a highly integrated suspensorium and occipital region, suggesting possible conservation of patterns across lissamphibians.

KEYWORDS Amphibia- crania- disparity- morphology- phenotypic integration

INTRODUCTION

Identifying the primary factors that shape the evolution of organisms is a long-standing interest in biology. Analysing phenotype is a relevant scale to address this question, allowing consideration of both external (e.g. ecological interactions, climate) and internal (i.e. genetic, development) factors (e.g. Goswami *et al.*,2014; Collar *et al.*, 2010; Moen *et al.*, 2013; Da Silva *et al.*, 2018). However, the types and sources of the data used by studies of external or internal factors are usually different, preventing combined studies of both aspects (Goswami *et al.*, 2014). In 1958, Olson and Miller hypothesized that phenotypic traits are more integrated or correlated (statistically associated) when derived from the same genetic or developmental origin or participating in the same function (Oslon & Miller, 1958). Organisms are made of locally integrated units, also defined by Wagner (1996) as modules, which has also been noted as the reason why characters are often easily identifiable across diverse organisms (Wagner & Altenberg, 1996). Traits within a module are highly connected (i.e. integrated) but less connected to traits of other modules. Integration and modularity therefore refer to the pattern and magnitude of connectivity within an organism. It has been suggested that modularity enables sets of traits to evolve independently of other sets of traits, reducing or removing the constraints of high integration, where changes in one trait may negatively impact the function of a closely integrated trait (Wagner, 1996). Empirical studies have focused on this aspect of evolutionary integration and modularity and have showed either positive correlations between the magnitude of integration and rate of evolution or disparity (Goswami et al., 2014; Randau & Goswami, 2017), or that high integration is indeed correlated with low evolutionary rates (Felice *et al.*, 2018). More recent studies have also found no simple linear relationship between strength of integration and either evolutionary rate or disparity (Bardua et al. 2019b, Watanabe et al. 2019).

Integration and modularity can be explored at the evolutionary level (i.e., at the interspecific level across a whole clade at one ontogenetic stage; Klingenberg 2014), the static level (i.e., at the intraspecific level, within one species at one ontogenetic stage; Klingenberg 2014) and the ontogenetic level (i.e., within one species across ontogenetic stages; Klingenberg 2014). Study of these three levels reveals insights into the underlying biological processes governing evolution. The static level can be used to infer functional, developmental, and genetic integration (Klingenberg, 2014), all of which mutually influence each other, forming a complex network of interactions (See Fig. 1 from Klingenberg 2008). Functional and genetic modularity are thought to evolve to match, through the creation of a modular 'genotype-phenotype map' (Wagner & Altenberg 1996), and it is hypothesized that developmental pathways evolve so that functional and developmental integration also match (‘matching hypothesis’, Wagner & Altenberg 1996). Thus, identification of the pattern of static integration can serve as a starting point to then determine functional, genetic and developmental sources of covariation within a structure and how these factors influence evolvability (Klingenberg, 2014). Evolutionary integration reveals the large-scale patterns of change during evolution, which can be driven by both functional (e.g., performance selection) and genetic (e.g., evolution by selection and drift) modularity (Klingenberg, 2008). Concordant patterns within and between species suggest that modularity is affected by common biological processes through evolution (e.g. developmental, genetic), as has been found in compound leaves where development highly modulates any other variation (Klingenberg *et al.*, 2012). Conversely, discrepancy in patterns of integration between evolutionary and static levels have been found in lizards, suggesting that the functional integration pattern at the static level has appeared by adaptation through selection (Urošević *et al.* 2019). Finally, selection can act on any ontogenetic stage and studies have demonstrated that patterns of integration shift through ontogeny (Zelditch, 1988; Zelditch & Carmichael 1989a, 1989b; Willmore *et al.*, 2006; Goswami & Polly 2010a; Goswami *et al.*, 2012; Ackermann, 2005). All three levels of integration and modularity can therefore together aid our understanding of evolution.

Within tetrapods (limbed vertebrates), evolutionary, ontogenetic, and static modularity have been investigated across a range of taxa. Evolutionary modularity has been investigated in mammals, where many studies found as many as six-modules in the cranium (e.g., Cheverud 1995; Goswami, 2006; Porto *et al.*, 2009; Goswami & Polly, 2010b), influenced by both development and function. Six-module cranial organisation was also recovered at the static and the ontogenetic levels, for example in Macaques (Goswami & Finarelli, 2016), suggesting that modularity in the mammal cranium is constrained by both functional and developmental influences. Within archosaurs, patterns of evolutionary modularity are generally conserved, with highly modular systems recovered across the crania of birds, non-avian dinosaurs and crocodylomorphs (Felice & Goswami, 2018; Felice *et al.,* 2019). Alternatively, the avian skull has also been found to be highly integrated at the evolutionary level (Klingenberg & Marugán-Lobón, 2013). Across squamates, Watanabe *et al.* (2019) found that the cranium comprised nine modules in snakes and ten modules in lizards, revealing highly modular structures influenced by functional constraints across these clades. In contrast, at the static level, Urošević *et al.* (2012) found that the head of the common lizard (*Podarcis muralis*) is a highly integrated structure. Patterns of cranial integration across lacertids (lizards) have been found to vary across evolutionary and static levels, with patterns at the evolutionary level more influenced by development and patterns at the static level more influenced by function, which suggests that functional modularity is adaptive and appeared through selection (Urošević *et al.* 2019). In *Anolis* lizards, patterns of static modularity have also been found to vary across species; a three-module cranial organisation is supported in some *Anolis* (lizards) species but no support for modular organisation was found in other *Anolis* species. Thus, similar to lacertids (Urošević *et al.*, 2019), patterns of cranial integration in *Anolis* lizards may be evolutionary flexible and influenced by unique functional pressures acting on the diverse cranial shapes (Sanger *et al.*, 2012). Across amniotes, studies of evolutionary and static modularity have therefore recovered a range of modular organisations, from fully integrated to highly modular structures, although differences in results may partially reflect differences in type of data collected (Felice *et al*., 2018; Goswami *et al*., 2019).

Relatively few studies have focused on lissamphibians (Anura, Caudata and Gymnophiona). The large range of ecologies and developmental histories across lissamphibians, along with their impressive cranial diversity, suggest patterns of cranial integration in this clade may be complex. However, studies of European newts at the static level have found that the skull is highly integrated with no distinct modules (Ivanović *et al.*, 2005; Ivanović & Kalezić, 2010), a result mirrored at the evolutionary level across the myobatrachid frog family (Vidal-García & Keogh 2017). Interestingly, static integration of the cranium is lower for paedomorphic forms of two European newts compared with fully metamorphosed forms, which can be explained by ontogenetic integration, where integration increases post-metamorphosis (Ivanović et *al.*, 2005). Mixed support was found for a range of three to five module models across different toad species of the *Rhinella granulosa* complex (Simon & Marroig, 2017), and for caecilians, a two-module model was found as best supported for the skull at both the evolutionary and static level (Sherratt, 2011). However, comparison of patterns of integration across clades is hindered by the range of data types, from linear distances (e.g., Simon & Marroig, 2017) to landmarks (e.g., Sherratt, 2011), and some methods prohibit the exploration of multiple models of modularity. Recent advancements in morphometric methods may facilitate the detection of a finer-scale modular signal. Recent high-dimensional studies across caecilians (Bardua *et al.*, 2019b) and within two caecilian species (Marshall *et al.*, 2019) have both recovered similarly highly modular cranial structures, suggesting possible conservation of modularity across static and evolutionary levels. Thus investigation into finer-scale patterns of static and evolutionary integration for additional lissamphibian groups may reveal whether this pattern is conserved across Lissamphibia.

Caudata (salamanders) comprises 10 ecologically and morphologically diverse families and displays a tremendous range of life cycle strategies (e.g., Bonett, 2018; Bonett & Blair, 2017; Bonett *et al.*, 2013; Ledbetter & Bonett, 2019). Extensive phenotypic variation can also extend intraspecifically in salamanders, as in the fire salamander *Salamandra salamandra* (Linnaeus, 1758) which exhibits a range of morphologies, colour patterns and reproductive strategies depending on the subspecies (Sparreboom, 2014; Beukema *et al.*, 2016). Taxonomy of the fire salamander is repeatedly being revised, but *S. salamandra* currently comprises at least 10 subspecies (Sparreboom, 2014). Across these subspecies, the fire salamander is polymorphic in its mode of reproduction, with most populations ovoviviparous but some viviparous (Dopazo & Alberch, 1994; Alcobendas *et al*., 1996; Buckley *et al*., 2007). The high flexibility in development in this species suggests developmental influences on cranial integration may be variable, and provides an interesting opportunity for investigating developmental and functional hypotheses of static modularity. The skull is suitable for investigating patterns of integration, as it is a highly developmentally complex structure (Hanken & Hall, 1993), given the different embryonic origins (neural crest and paraxial mesoderm) and types of ossification (endochondral and intramembranous) across the cranial regions. In addition, the transition from larval to adult morphology for metamorphic subspecies involves deep osteological remodelling in the skull, including the resorption of bones (e.g., vomer, and palatine portion of the palatopterygoid), and the late development of the maxilla and prefrontal (Rose, 2003). The cranium of *Salamanda salamandra* comprises 15 bones, although three of them fuse in one single complex- the prootic and opisthotic fuse to form the otic capsule, which fuses posteriorly with the exoccipital and form the occipito-otic bone (Rose, 2003). Apart from three endochondral bones (the occipito-otic complex, the orbitosphenoid and the quadrate), all bones form by intramembranous ossification (Rose, 2003). The skull is also functionally complex (Moore, 1981; Hanken & Hall, 1993), playing a major role in foraging and feeding, as well as protecting the brain and housing the organs that perceive the main senses (hearing, sight, smell and taste). The skull is therefore an ideal system for investigating fine-scale patterns of integration.

Here we investigate patterns of morphological integration and modularity in the skull of the Italian subspecies of fire salamander [*Salamandra salamandra giglioli* (Eiselt & Lanza, 1956)], which undergoes metamorphosis (Seidel & Gerhardt, 2016). We directly compare eight different modular structures based on hypothesized functional and developmental relationships between cranial regions. We also determine whether integration constrains or facilitates morphological diversity (disparity) by quantifying the relationship between within-module variance and within-module magnitude of integration. Combined, these analyses allow us to assess static (intraspecific) modularity and integration within a population, for qualitative comparison to previous studies of modularity and integration within lissamphibians [e.g., in caecilians (Bardua *et al*., 2019b; Marshall *et al.*, 2019), frogs and toads (Simon & Marroig, 2017; Vidal-García & Keogh 2017) and salamanders and newts (Ivanović *et al.*, 2005; Ivanović & Kalezić, 2010)] and amniotes. This study thus adds to a rich and increasing pool of understanding the evolution and significance of phenotypic integration and modularity for shaping organismal variation at micro- and macroevolutionary scales.

MATERIAL AND METHODS

STUDY SPECIMENS

The sample analysed in this study includes 40 specimens of the subspecies *Salamandra salamandra gigliolii* (Table S1). None of the cloacal regions of the specimens displayed an obvious swelling that could differentiate males from females (Brizzi & Calloni, 1992), thus sex information was not available for these specimens. All the specimens were preserved in alcohol in the collections of the Natural History Museum (NHM), London. The specimens were imaged using micro CT-scanning (Nikon Metrology X-Tek HMX ST 225) at the NHM and resulting tomographs were further segmented in Avizo Lite v.9.3 (FEI, Hillsboro, OR, USA) to obtain 3D models of the crania. Because this study focuses on cranial morphology, the reconstructed meshes were processed in Geomagic Wrap (3D Systems) to remove vertebral elements and the mandible from each cranium, which could hinder the access to the surface of interest. The right half of each skull was then prepared for surface analyses using Geomagic Wrap, by smoothing noise introduced from scanning and removing holes that could prevent the acquisition of surface details (Bardua *et al.*, 2019a). The quality of the left-hand side of the skull was favored in two specimens, for which the skull was thus medially mirrored with the "Mirror" function in Geomagic Wrap.

MORPHOMETRIC DATA

To characterise the shape of the regions of interest and assess how they correlate, a landmarking procedure was performed using Checkpoint (Stratovan, Davis, CA, USA) by the same user to keep the placement of landmark consistent and to avoid user bias. Eighty-five anatomical landmarks were manually placed on the right-hand side of each skull, defining 20 regions in total (Fig. 1 and Table 1) that were identifiable in all specimens of the dataset. These landmarks were homologous across all specimens. Regions delineate bones, or sub-regions of bones when the bone is anatomically polyvalent (e.g. with ventral and dorsal surfaces), and thus potentially modular due to differing functional pressures. The regions are delimited with sliding semi-landmarks ('curves') in between landmarks (Fig. 1 and Table S2). Eighty-five curves in total were drawn over the skull. These curves were then resampled in R (R Core Development Team, 2019) to 689 curve semilandmarks to ensure that they were equidistant and that they capture shape optimally (Botton-Divet *et al.*, 2016). To capture surface information from the regions, 375 sliding surface points were placed over the crania following a semi-automatic procedure using the R (R Core Development Team, 2019) package *Morpho* v.2.6 (Schlager, 2017). First, one specimen of the dataset was defined as the template and surface points were manually placed onto each region. Then, surface points were semi-automatically projected from this template onto each specimen with the "placePatch" function in *Morpho* v.2.6 (Schlager, 2017). Finally, all curve and surface points were slid to become geometrically homologous, minimizing the bending energy criterion, using the ‘slider3d’ function in *Morpho* v.2.6 (Schlager, 2017). A detailed description of this method is available in Bardua *et al* (2019a).

Following sliding, morphometric data were subjected to Procrustes analysis to remove the non-shape aspects of isometric size, rotation, and translation (Rohlf & Slice, 1990). However, because alignment of only one side of a bilateral structure can have a negative impact on the Procrustes alignment (Cardini, 2016), we first mirrored morphometric data to produce a fully bilateral configuration using the “mirrorfill” function in *paleomorph* v.0.1.4 (Lucas & Goswami, 2017). We then performed Procrustes alignment with the “gpagen” function in *geomorph* 3.0.4 (Adams *et al.*, 2019). Finally, the mirrored side was removed from the resulting Procrustes shape coordinates, leaving the right-hand side coordinates only for further analysis.

ALLOMETRY

Allometry corresponds to the impact of size on shape (Klingenberg, 2016). In our study, cranial size was measured as the centroid size (Klingenberg, 2016), which was calculated with the “gpagen” function in *geomorph* 3.0.4 (Adams *et al.*, 2019) during the Procrustes alignment (see Supplementary Table S3). Allometry was assessed with a regression of the shape data on log-transformed centroid size, using the “procD.allometry” function in *geomorph* v.3.0.4 (Adams *et al.*, 2019).

MODULARITY AND INTEGRATION

The eight hypothetical patterns of modularity tested in this study are outlined below. Most simply, we tested for a fully integrated cranium, constrained by its highly diverse functions

(Fig. 2A and Table 2). The cranium could also be divided into two modules based on the two types of ossification (endochondral and dermal), resulting in two different developmental modules (Fig. 2B and Table 2). We also tested two functional hypotheses that were previously tested for caecilians in the literature (Bardua *et al.*, 2019b ; Marshall *et al.*, 2019), in which the skull is either dorsoventrally divided (Fig. 2C and Table 2) or partitioned into four functional modules (Fig. 2D and Table 2). Since bones have different ossification sequences, which could influence skull variation and evolution, we hypothesised that cranial modules are defined by their time of ossification (Fig. 2E and Table 2) as previously explored in the literature (Ivanović & Kalezić, 2010), resulting in four different modules: early, mid, late or metamorphosis modules. We further partitioned and tested a six-module model (Fig. 2F and Table 2) similar to the model found in mammals (Goswami, 2006). Each bone is an identifiable unit that originates from an independent signal, so we also hypothesised that bones comprise their own modules (Fig. 2G and Table 2). Finally, because some bones form distinct regions that are implied in different functions (e.g. the *pars facialis* and the *pars palatina* of the maxilla) and are thus potentially functionally modular, we further hypothesised that the cranium could be highly partitioned into 20 functional modules (Fig. 2H and Table 2).

Modularity was investigated with two different approaches. Firstly, we conducted EMMLi (‘Evaluating Modularity with Maximum Likelihood’) analysis. For this, congruence coefficients were calculated with the “EMMLi” function from the *EMMLi* v. 0.0.3 R package (Goswami *et al.*, 2017). Robustness of the results was tested with a random subsampling down to 10% of the full data, and EMMLi ran iteratively 100 times, using the “subSampleEMMLi” function from the *EMMLi* v. 0.0.3 R package. Mean results from the 100 subsamples were then compared to the analysis of the full dataset.

Secondly, we conducted Covariance Ratio (CR) analysis (Adams, 2016) using the "modularity.test" function from the *geomorph* v.3.0.4 R package. While EMMLi is a model selection approach that compares different models of modular organisation and outputs the most likely model along with its between and within module correlations, covariance ratio analysis is a hypothesis testing method that calculates covariance ratio between the different regions in one specific model. The ratio is the total between-module covariance over the within-module covariance (Adams, 2016), meaning that a ratio of one reflects a lack of modularity. Therefore, support for the preferred model from EMMLi analysis was assessed by calculating the covariance ratio of that model, to confirm that both methods supported similar patterns of trait integration and modularity. We further conducted both analyses with a landmark-only dataset for comparison.

MORPHOLOGICAL VARIANCE

To discern how one module varies from one skull to another, we computed the shape variance (the Procrustes distance between shapes and the mean shape of a module) of each module with the "morphol.disparity" function from the *geomorph* v.3.0.4 R package. To compare the variances between modules, each variance was corrected by dividing by the total number of landmarks defined in the module concerned.

To investigate the relationship between shape variance and the magnitude of within-module integration, we computed a linear regression of corrected variances on within-module correlation.

RESULTS

ALLOMETRY

The regression of shape on log-transformed centroid size was not significant ($R^2 = 0.037$, $p = 0.125$), suggesting that there is no significant effect of size on shape in this cranial structure. Therefore, we did not apply allometric corrections in further analyses.

MODULARITY AND INTEGRATION

The most supported model by EMMLi for the complete dataset is the maximal partitioning modular pattern (Table 3), comprising 20 modules (Fig. 3). However, since we could not test all possible models of modularity, and because previous analyses have suggested that EMMLi has a tendency to pick the most-parametrized model with semilandmark data, we explored the correlation (rho) values among and within modules to assess possible further groupings of modules into larger modules, following the method previously described (Bardua *et al.*, 2019b; Felice & Goswami, 2018; Marshall *et al.*, 2019). Modules were hence further grouped when the between-module estimated correlation was within 0.1 of the smallest within-module trait correlation of the module pair under question. Therefore, we grouped 11 regions into four larger modules, as follows: the squamosal, the pterygoid, the jaw joint and the quadrate were grouped into one jaw suspensorium module, the ventral and dorsal regions of the premaxilla formed a second module, the ventral and dorsal regions of the maxilla a third module, and the ventral and dorsal part of the occipital were grouped with the occipital condyle (Fig. 3 and Table 3). The average random 10% subsampling of the data resulted in a near-identical pattern of trait integration (Supplementary Table S4) compared with the full data, confirming the robustness of our results**.** Through this approach we recovered a 13-module model as best supported.

EMMLi analysis of the landmark-only dataset supported the 15-module model (which subdivided the cranium according to osteological units). However, further assessment of the within- and between-module rho values revealed that no hypothesized cranial module stood out as distinct, as within- and between-module correlations were similar and low, below 0.3 in most cases (See Supplementary Table S5). Within-module correlations were considerably lower in the landmark-only dataset compared with the complete shape dataset, whereas between-module correlations were similar across both datasets. Landmark-only analysis therefore suggested the cranium of the Italian fire salamander was only weakly modular, with no cranial regions standing out as highly integrated, distinct modules.

COVARIANCE RATIO

Covariance Ratio analysis for the full shape data with the most parametrized model was significant (CR = 0.69, p = 0.01) and revealed similar patterns of modularity (Table 3) to those recovered from EMMLi, with CR values closer to 1 for regions related to the jaw suspensorium and occipital. Therefore, we find that the regions of the jaw suspensorium (pterygoid, squamosal, quadrate and jaw joint) and the regions of the occipital (dorsal and ventral sides and condyle) are relatively more integrated, in concordance with EMMLi analysis, but the dorsal and ventral sides of the premaxilla are relatively less integrated (CR = 0.7).

Covariance ratio analysis of the landmark-only dataset found significant support for the 15-module model (CR = 0.85, p = 0.01). Covariance ratio values within this model were generally higher than CR values from the full shape dataset, indicating weaker evidence of modularity with the landmark-only dataset. Moreover, many CR values approached or exceeded one for several pairs of hypothesized modules, indicating that, despite the significant support for an overall pattern of modularity, CR analysis does not show strong support for most of the hypothesized cranial modules when quantified by landmarks alone (Table S6).

MORPHOLOGICAL DISPARITY

Cranial modules within the jaw suspension (pterygoid, squamosal, lateral surface of the quadrate and jaw joint bones) exhibited the highest disparity after correcting for landmark number, and the orbitosphenoid exhibited the lowest disparity (Table 4).

The linear regression of Procrustes variances of the modules on their respective within-module correlations (Fig. 4 and Table 4) was not significant (multiple $R^2 = 0.008$, adjusted $R^2 = -0.081$, $p = 0.763$). Repeating this regression with the original 20 cranial regions revealed a similar, non-significant relationship (multiple $R^2 = 0.008$, adjusted $R^2 = -0.047$, $p = 0.706$) (See Supplementary Table S7).

DISCUSSION

This study comprehensively sampled the cranial morphology of the Italian fire salamander using a high-dimensional approach, and found a complex pattern of modularity, where the Italian fire salamander cranium comprises 13 modules. This pattern of trait integration was supported from EMMLi analysis with both full data and data subsampled to 10%, as well as with CR analysis. The fire salamander skull therefore comprises multiple, semi-independent regions, with fine-scale, localised variation in functional or developmental influences. The modules we found correspond primarily to individual osteological units, as well as a functional module corresponding to the jaw suspensorium (including the pterygoid, quadrate, jaw joint surface of the quadrate and squamosal regions). The influences of type and timing of ossification do not appear to drive the overall pattern of integration, possibly because the superimposition of multiple sources of covariance can confound each other (Hallgrímsson *et al.*, 2009). However, because genetic, developmental and functional modularity are thought to evolve to match (Wagner & Altenberg 1996), other developmental or genetic influences than those tested here may act as processes driving the pattern of modularity that we identify. Identifying the pattern of static integration can therefore help identify the drivers of this pattern (i.e. functional or developmental processes) by narrowing possible factors to those related to the specific patterns observed. The static pattern can also be compared with patterns of integration at the evolutionary and ontogenetic level for determining the factors driving the morphological evolution of salamanders, and for understanding whether our observed pattern of static integration, affected by both development and functional pressures, is conserved through evolution.

An absence of distinct developmental modules in the fire salamander cranium is consistent with a previous study of the alpine newt skull (Ivanović & Kalezić, 2010). These results may reflect confounding or overlapping developmental and environmental interactions through ontogeny, or cranial partitioning may have been too simple given the complexity of developmental influences acting on the skull. However, Ivanović & Kalezić (2010) found no support for any hypothesized modular structure, in contrast to the highly modular pattern that we recover in the fire salamander skull. This large discordance may suggest patterns of integration may be highly flexible within different salamander species. Integration can be flexible even within species with different morphs, and can vary through ontogeny (Ivanović *et al.*, 2005), suggesting patterns of integration may be flexible according to life history. However, differences may also be largely attributable to differences in data type and analyses. Ivanović & Kalezić (2010) used two-dimensional landmark data and consequently could not capture shape information for the orbitosphenoid and some bones of the jaw suspension (quadrate and squamosal). In addition, testing hypotheses comprising two to four modules prevented the detection of finer-scale patterns of integration and thus hinders direct comparison between our study and that of Ivanović & Kalezić (2010). Comparison of integration patterns within different subspecies of fire salamander varying in developmental strategy may reveal whether integration patterns are flexible and influenced by developmental strategy.

In contrast, comparing our results to studies implementing a similarly high-dimensional approach reveals instead a surprisingly conserved pattern of integration, particularly within Lissamphibia. The 13-module model we recover in the fire salamander cranium is very similar to the 12- and 13- module models identified intraspecifically within caecilians (Marshall *et al.*, 2019), and to the 10-module model found across the caecilian clade (Bardua *et al.*, 2019b). This similarity is notable given the osteological differences between salamanders and caecilians, which hinders direct comparison. Like Marshall *et al.* (2019), we find that the parasphenoid (analogous to the caecilian ventral os basale) and the vomer form two independent modules, probably due to functional decoupling, the parasphenoid being the floor of the braincase and the vomer being part of the palate (Rose, 2003). Independent palatal and braincase floor regions have also been found across bird and squamate crania (Felice & Goswami, 2018; Watanabe *et al.*, 2019), although across the caecilian clade these regions formed one module (Bardua *et al.*, 2019b). We also find the three regions comprising the occipital bone form one distinct module, despite their functional differences (braincase protection and connection to the vertebral column). A distinct occipital module is also found in caecilian (Bardua *et al.*, 2019b; Marshall *et al.*, 2019), bird (Felice & Goswami, 2018), non-avian dinosaur and crocodylomorph (Felice *et al.,* 2019) crania. The modular structure identified across the fire salamander skull is more strongly concordant with the pattern recovered within caecilians (Bardua *et al.*, 2019b; Marshall *et al.*, 2019) than amniotes, suggesting a possible divergence in modularity patterns between amniotes and amphibians. The similarity of integration patterns between salamanders and caecilians suggests conservation of modularity despite the great diversity of developmental histories and ecologies across these two amphibian clades.

We recover a highly integrated jaw suspensorium module (quadrate, pterygoid and squamosal) within the fire salamander skull, likely driven by constraints from feeding mechanics. A highly integrated jaw suspensorium region has also been found across and within caecilians, comprising two strongly correlated modules (quadrate-squamosal and pterygoid) (Bardua *et al.*, 2019b; Marshall *et al.*, 2019). In addition, across birds, the quadrate and pterygoid form a module, although the squamosal belongs to the cranial vault (Felice & Goswami, 2018). This jaw joint region of both caecilians and birds exhibits coordinated movement through kinesis, which may be driving its strong integration (Felice & Goswami, 2018; Bardua *et al.*, 2019b). The highly integrated jaw suspensorium module of the fire salamander may be due to tightly linked, functional constraints in this region related to changing feeding requirements through ontogeny. Fire salamanders experience disparate selective pressures through ontogeny, from larval to adult stages, related to changes in both environment and diet (from water to land), and thus in their feeding mechanism (Shaffer &

Lauder, 1988; Wainwright & Reilly, 1994). The highly integrated jaw joint region shared across fire salamanders, caecilians, and birds suggests feeding mechanics plays a large role in shaping the pattern of integration for these clades.

Data type can have a strong impact on studies of integration. Here, whilst our full landmark and semilandmark dataset recovers a highly modular structure, our landmark-only dataset instead suggest a weakly integrated model, with little to no support for many of the hypothesized cranial modules (despite both methods supporting an overall highly-modular pattern for the cranium). This present study, along with previous comparisons of landmark and semilandmark data to landmark-only data, thus reveals how landmark-only data may exaggerate between-region trait correlations and understate within-region trait correlations (Bardua *et al.*, 2019b; Marshall *et al.*, 2019) resulting in landmark-only datasets recovering weaker support for modular structure. This is because landmark-only datasets suffer from boundary bias (Goswami *et al.,* 2019) and do not capture shape information along curves and across surfaces, meaning the shape data are not fully representative of a structure. Analyses using semilandmarks may have the opposite effect, due to non-independence of semilandmarks, but it has been demonstrated in multiple studies that they better capture the morphology of complex structures (Watanabe, 2018; Bardua *et al.*, 2019b; Goswami *et al.*, 2019). While all methods suffer from biases and artefacts, the improved characterisation of shape achievable with a full landmark and semilandmark dataset likely better reflects the structure of the cranium and the interactions among its regions.

We found no significant relationship between integration and shape variance, and thus no support for the hypothesis that integration facilitates or constrains morphological variance in the fire salamander skull. Our results may thus suggest that integration has limited or variable influence on morphological diversification. No significant relationship between integration and variance was also recovered intraspecifically within caecilians (Marshall *et al.*, 2019). Similarly, the crania of domestic dogs display a conserved pattern of integration despite a high cranial variance across the entire order (Drake & Klingenberg, 2010). Studies at the macroevolutionary scale have thus far found contrasting patterns, suggesting the relationship between integration and variance is highly flexible across clades, or that the relationship cannot be expressed linearly. Whilst no significant linear relationship between integration and variance was found across caecilians (Bardua *et al.,* 2019), high levels of integration are associated with low morphological disparity in the crania of birds and mammals (Felice *et al.*, 2018; Goswami *et al.*, 2014), and conversely, the modular fins of ray-finned fish are highly variable (Larouche *et al.*, 2018). More likely, whether integration promotes or constrains morphological evolution of a module may depend heavily on the alignment of the direction that it facilitates in the morphospace and the direction of selection (Hansen *et al.*, 2011; Goswami *et al.*, 2014; Felice *et al.*, 2018). The heterogeneity of results across studies so far therefore suggests that the relationship between integration and disparity may be complex, with no simple relationship between the two metrics in many, if not most, cases.

With a high dimensional approach, we analysed the cranial organisation of the Italian fire salamander (*Salamandra salamandra gigliolі*). The fire salamander's cranium is highly modular, comprising 13 modules, and this modular organisation is highly similar to that recovered in caecilians in terms of both the number and pattern of modules (Bardua *et al.*, 2019b; Marshall *et al.*, 2019). This result suggests a possible conservation of the pattern of integration across lissamphibian crania. We found no support for purely developmental or functional hypotheses of modular organisation, which suggests that the cranial modular pattern in the fire salamander is complex and originates from a mixture of both functional and developmental constraints. Cranial size had little effect on cranial shape in this species, and morphological disparity had no significant relationship with within-module integration, contrary to hypotheses that integration may facilitate or constrain morphological variation. The study of cranial integration of salamanders at the evolutionary level would complement this present study on static integration, providing a more comprehensive understanding of the intrinsic factors shaping the evolution of the salamander skull.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Figure captions

Figure 1. Landmark and semi-landmark positions on the cranium, in (A) ventral, (B) dorsal and (C) lateral views, shown on a Fire salamander *Salamandra salamandra gigliolii (*NHM 1911.2.22.62). Points are colour-coded as follows: landmarks (red), curve semilandmarks (yellow) and surface semilandmarks (blue).

Figure 2. Hypotheses of modular patterns tested in EMMLi analysis. All of the landmarks and semi landmarks from each region were assigned to hypothesised modules and color-coded accordingly. **A**, no module model (no modular organisation); **B**, Developmental module model (purple: dermal, blue: endochondral); **C**, Dorso-ventral module model (blue: dorsal, yellow: ventral); **D**, 4 functional module model (blue: snout, yellow: braincase, green: jaw suspension, orange: occipital-otic complex); **E**, time of ossification module model (blue: early development, green: mid-development, light pink: late development, yellow: metamorphosis); **F**, 7 functional module model (navy blue: snout, light blue: floor of the braincase, light pink: jaw suspension, yellow: skull roof, magenta: palatine, green: occipital-otic complex); **G**, bone module model (frontal: dark blue, maxilla: blue, nasal: yellow; occipital: peach, occipital condyle: grey, orbitosphenoid: black, otic: green khaki, parasphenoid: green, parietal: violet, prefrontal: red, premaxilla: brown, pterygoid: egg shell, quadrate: cyan, squamosal: gold, vomer: hot pink); **H**, Functionally partitioned module model (frontal: dark blue, jaw joint: grey, maxilla: blue, maxilla ventral: cyan, nasal: yellow; occipital dorsal: egg shell, occipital ventral: black, occipital condyle: peach, orbitosphenoid: light orange, otic dorsal: light blue, otic ventral: black, parasphenoid: green, parietal: dark violet, prefrontal: orange, premaxilla dorsal: dark gold, pterygoid: golden brown, quadrate: light

violet, squamosal: green khaki , vomer: magenta). For further details about the results on modularity, please see Table 3, 4 and S3-S5).

Figure 3. The thirteen-module model identified with EMMLi analysis. Top left, network graph of the 20 cranial regions defined in this study, coloured in the thirteen modules identified from EMMLi analysis. The network displays a right-hand side lateral view of the skull. The thickness of a line and the size of a circle respectively correspond to the strength of integration between and within modules. At the right side of the figure, the resulting thirteen modules are visualised on a specimen (*Salamandra salamandra gigliolii* NHM 1911.2.22.62) in lateral, ventral and dorsal views. The thirteen modules correspond to the following regions: occipital in light blue, dorsal (OccD) and ventral (OccV) part of the occipital bone and condyle (OC); dorsal otic (oticD) in yellow and ventral otic (OticV) in light brown; jaw suspension module with squamosal (Sq), quadrate (Qd), jaw joint (JJ) and pterygoid (Pt) in red; parasphenoid (Psph) in black; vomer (Vo) in magenta; orbitosphenoid (Osph) in orange; prefrontal (Prf) in green; Maxilla in blue with the dorsal (MaxD) and ventral (MaxV) sides; premaxilla in brown with dorsal (PmxD) and ventral (PmxV) sides; nasal (Nas) in pale pink; frontal (Fr) in dark blue; Parietal (Par) in dark purple.

Figure 4. Linear regression of Procrustes variances corrected for landmark number on respective within-module integration. The relationship between the two variables is not significant (*p* = 0.76).