Conserved but flexible modularity in the zebrafish skull: Implications for craniofacial evolvability

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Morphological variation is the outward manifestation of development and provides fodder for adaptive evolution. Because of this contingency, evolution is often thought to be biased by developmental processes and functional interactions among structures, which are statistically detectable through forms of covariance among traits. This can take the form of substructures of integrated traits, termed modules, which together comprise patterns of variational modularity. While modularity is essential to an understanding of evolutionary potential, biologists currently have little understanding of its genetic basis, nor its temporal dynamics over generations. To address these open questions we compared patterns of craniofacial modularity among laboratory strains, defined mutant lines and a wild population of zebrafish (*Danio rerio*). Our findings suggest that relatively simple genetic changes can have profound effects on covariance, without greatly affecting craniofacial shape. Moreover, we show that instead of completely deconstructing the covariance structure among sets of traits, mutations cause shifts among seemingly latent patterns of modularity suggesting that the skull may be predisposed toward a limited number of phenotypes. This new insight may serve to greatly increase the evolvability of a population by providing a range of 'preset' patterns of modularity that can appear readily and allow for rapid evolution.

Introduction

Variation is essential for evolution to proceed, but the *patterns* of available variation can bias the rate and direction of evolutionary change. For example, within the context of adaptive divergence such biases have been referred to as 'genetic lines of least resistance' whereby adaptive phenotypic change occurs along a trajectory where genetic variation is most available to improve fitness [1]. However, it remains unclear what may underlie these biases to limit change in particular directions. A key mechanism proposed to influence such evolutionary biases is modularity, which refers to the organization of traits into subsets that are highly integrated and semi-independent of other such subsets [2]. Modularity is proximately determined by underlying developmental processes (e.g. shared fields of gene expression or cell populations) and through functional (e.g. biomechanical) interactions, but at an anatomical level it can be empirically identified through measures of covariance among traits (i.e., variational modules). Ultimately, modularity is believed to bias adaptive evolution through the relaxation of fitness tradeoffs, whereby one anatomical region may respond positively to selection while limiting potentially detrimental effects on another anatomical region [2,3,4,5].

While modularity is essential to an understanding of evolutionary potential, biologists currently have little understanding of its genetic basis, nor its temporal dynamics over generations. Modularity is often assumed to be a stable property of multicellular organisms, even across widely disparate morphologies, that only change over long geological timescales [6,7,8]. This would indicate that the mechanisms underlying modularity are deeply ancestral and possess little to no allelic variation. However, there is growing evidence that patterns of covariance are distinct between closely related species and strains [3,5,9]. This would suggest that modularity has a relatively simple genetic basis, and may respond rapidly to selection. Alternatively, modularity may be responsive to genetic alterations but with only a limited range of outcomes with regards to pattern. Distinguishing between these scenarios is important

for a more comprehensive understanding of how modularity may influence phenotypic evolution.

A key step in addressing this important question is to understand the genetic basis of modularity [4,5]. The craniofacial skeleton is suitable for such investigations of modularity because it is an inherently complex anatomical structure, with a high number of movable bony elements that together perform a wide variety of adaptively relevant functions [3,4,5]. Also, in many organisms the development of the craniofacial skeleton has been well characterized. This provides the basis for a number of functional and developmentally derived hypotheses to be made about how traits may covary. Quantitative genetic analyses of shape change within the cranium of inbred mouse lines have shown that many loci of minor effect influence the shape of different skeletal regions [10,11]. This trend is also supported by genetic screens and mapping of the causal variation of diverse craniofacial disorders [12,13,14,15]. Therefore, while craniofacial *shape* may have a complex genetic basis, which hints at a lack of an evolutionary line of least resistance, the pattern of modularity itself might have a simpler genetic basis [5]. Indeed, evidence is emerging that covariance structure is highly sensitive to mutations [9]. A deeper appreciation for how mutations impact modularity as an independent trait may determine how a complex array of shape-determining genetic variation is revealed in the phenotype.

Here we assess the genetic basis of craniofacial modularity across both shared and varied genetic backgrounds to determine how discrete mutations may affect variational modules. The zebrafish (*Danio rerio*) is a powerful vertebrate model with which to study skeletogenesis at all stages of development [16]. While the vast majority of zebrafish craniofacial mutants described to date exhibit gross, qualitative (i.e., presence/absence) defects that are not amenable to analyses of modularity, our recent screens on postembryonic development of the zebrafish have identified a large collection of mutations that result in subtle but significant shifts in craniofacial shape (e.g. 17). As these mutants are identified at the adult stage, they reflect the type of changes that are permissible while maintaining functionality and hence viability [18,19]. In other words, the phenotypes of these mutants may be

reflective of variance that is possible in natural evolutionary radiations; although not necessarily by similar genetic changes. With this resource, we predicted that mutations with subtle effects on shape may have pronounced influences on modularity in the skull. To gain insights into the potential natural state of modularity, we contrasted findings from lab strains with a wild-caught population of zebrafish. Wild-caught populations likely face very different selection pressures that should limit the effect of mutations on modularity [9]. Our findings have major implications for explaining how a complex character like the craniofacial skeleton can so readily evolve, with results suggesting that minor genetic perturbations can shift patterns of craniofacial modularity, and that such changes in trait covariance are independent of the magnitude of changes in shape. Notably, we also find that while different genetic backgrounds exhibit distinct patterns of modularity, ancestral patterns may 'reappear' in the face of mutation. These findings suggest new properties for modularity and an increased understanding of its genetic basis. Indications are that a simple genetic change may reveal latent patterns of modularity, and/or effectively 'hide' other types of covariance from selection. We suggest that the evolvability of the skull may be facilitated on both short and long-term timescales by this conserved but flexible covariance structure that predisposes lineages to a limited range of trajectories for adaptive phenotypes.

1. Materials and methods

(a) Collection and rearing of fish

Lab strains of zebrafish were reared under standardized lab conditions and fed flake food and artemia daily until at least 1+ years of age. Wild zebrafish were obtained by dipnets from the Kosi river, India during the autumn of 2015. A total of 369 individuals were investigated (Wild caught n =63 Strains: AB n=50, Tuebingen n=71; mutant lines: *alf*^{dt30mh} n=46 [20], *lof*^{dt2} n=50 (XX), and *btm*^{t3404} n =89 (Harris, MP, ZF models unpublished). All samples were cleared and stained using alizarin red following Potthoff [21]. Photographs of the lateral left side were taken for each individual using a

Zeiss Axiocam MRC digital camera mounted on a Zeiss SV12 dissecting scope. Images were imported into TPSdig2 [22], and landmark coordinates were captured in two-dimensional (x,y) space.

(b) Data collection

For investigations of variational modularity it has become standard practice to include as much shape information as is reasonably possible [5,23,24,25,26,27]. This increases objectivity by assessing a broader spectrum of possible interactions among traits. Sliding semi-landmarks [28] make possible the description of shapes combining curves and classic homologous landmarks on the same object, and the incorporation of these data has become standard in the field of morphometrics [29,30]. Here, a total of 24 regular landmarks and 38 semi-landmarks were sampled across the craniofacial region (Figure 1). Landmarks were superimposed by conventional Procrustes superimposition [31], while semi-landmarks were superimposed by allowing them to slide along curves bounded by landmarks to minimize the Procrustes distance among individuals. Superimposition of semi-landmarks was done in TpsRelw [32] using chord distances. Finally, allometric variation in shape was removed by calculating residuals from a regression of shape on centroid size using Standard6a [33].

(c) Hypothesized patterns of modularity

Our analytical approach is based on the operational definition of modularity whereby by covariance among traits arises over ontogeny through sequential and hierarchical process including developmental and physical interactions between structures (cells, tissues) [34]. To begin testing hypotheses of modularity we selected a total of 8 *a priori* models representing the spatial distribution of effects from a diversity of developmental (e.g., cellular condensation) and functional (e.g., effects of muscle, ligament, and tooth attachment on bone deposition and remodeling) processes (Figure1, Table1). An additional null model representing a lack of any integration or modularity was also included in our

analyses.

Several valid methods of analysis exist for examining variational modularity but we favoured an established approach with the ability to perform model selection. Specifically, we focused on a minimum deviance approach that provides especially powerful options for model selection [5,35]. Model selection approaches are ideal for investigations of modularity because they are objective by providing the ability to discern which models that are best supported from a range of valid hypotheses. Therefore, model selection approaches provide an exploratory approach for determining the most relevant patterns of modularity. However, it should be noted that alternative methods for exploring modularity using model selection procedures based upon maximum likelihood have recently been developed [36]. While this represents an important advancement, the minimum deviance approach explores a much wider range of covariance structures than the likelihood approach (preliminary analysis of our data suggests 3695 vs 37 models for minimum deviance and likelihood approaches respectively). This increased range of modularity models provides a much more objective approach that we describe further below. Further, modules delimited using the widely employed Escoufier's RV can be highly integrated with each other so long as intra-modular covariances are higher [37]. This somewhat contradicts with what makes modularity relevant to evolution whereby modules are quasi-independent and free to be modified without interfering with others. Therefore, this justifies our use of the minimum deviance approach which implies this evolutionary freedom because a model will only fit well if modules are independent [35]. All tests and approaches for variational modularity hypotheses were implemented within the matlab package MINT (available at: http://www-personal.umich.edu/~emarquez/morph/index.html). A heuristic visual guide for the main analytical procedures in MINT can be found in our previous research [5].

(d) Testing modularity

The minimum deviance method fits models to the covariance matrix of landmark coordinates which is assessed using a standardized gamma statistic (γ*) [35]. The null hypothesis predicts that the difference between the observed and expected covariance matrices is no greater than expected by chance; thus, a low p value indicates that the model fits the data poorly [5]. The best fitting model is the one that deviates least from the data taking into account the number of fixed parameters.

For our data it was not biologically realistic to expect that our original 8 hypotheses of modularity were mutually exclusive, therefore we took advantage of this approach's ability to test all possible non-nested combinations of the models, giving a total of 3695 tested models. The models tested with this method also allowed for overlapping modules and therefore likely cover a substantial proportion of the developmental and functional processes capable of affecting covariation patterns in the skull. This approach has been successfully applied to the study of modularity in several organisms [3,5], including zebrafish [37].

To determine the best supported hypothesis of modularity we implemented a Monte Carlo model selection procedure. Comparative testing was conducted using all *a priori* models, and was based on the goodness of fit metric γ*. This metric represented a measure of the dissimilarity between observed and expected covariances for each model [35]. In this approach, each model was comprised of a series of partitions among our landmarks and semilandmarks. Partitions represented a hypothesized module or subspace predicted to be highly integrated relative to other such partitions. The statistical significance of γ* was assessed under the null hypothesis that the fit of observed patterns of morphological covariance to a hypothesized model, are no larger than the fit of observed covariance to a randomly-generated matrix [35,38]. Because this Monte Carlo approach can often reveal multiple statistically supported models we performed an additional analysis to help distinguish the best supported models. This involved two steps. First, models were ranked based on their γ* value, and

second, the support for these rankings was determined by a jackknife approach in which γ* values and model ranks were recomputed after removing 1,000 randomly chosen subsets comprising 10% of the data.

(e) Testing for similarities in wider patterns of covariance

If a particular model fit two of our strains or mutants (AB, Tu, *alf*, *lof*, or *btm*) equally well, it would not necessarily mean that they were close to each other in model space. This is because two objects that are equally distant from a third are not required to occupy the same position, especially in a high-dimensional space. In our case the values calculated across models for each group represented reference points to determine their relative position. At the same time these values can describe the nature of the covariance structure within each group, vectors of γ* values can also be useful for comparing the covariance structure among groups. However, because each group may be centered at a different position, only the direction of these vectors can be compared, which we achieved by examining correlations between γ* vectors of each group. We did not use all 3,695 possible γ* values in these correlations; rather, we used the top 50 ranked models for each group, yielding a total of 134 γ* values. This increased the possibility that we were testing associations between the most biologically relevant models.

Finally, we complemented our tests for patterns of modularity by examining shape variation among lines of zebrafish. This involved using the same standardized set of landmarks used above to examine covariation. Instead, in this case we extracted the consensus configuration of landmarks for each zebrafish line for calculations of pairwise Procrustes distances. These distances among consensus shapes were obtained using the software Coordgen8 [39].

2. Results and discussion

Our data support the idea that patterns of craniofacial modularity are flexible in response to simple genetic mutations. These observations have important implications for explaining patterns of evolution, including adaptive radiations where relatively minor differences in genetic variation can be present amongst species [40,41]. Further, our findings demonstrate how the domestication of lab strains (an evolutionary process itself) can inadvertently alter patterns of trait covariation through a process of selection, including within so called 'wild-type' lines that appear outwardly similar to each other (e.g. Tu vs. AB). Surprisingly, our comparisons reveal that certain patterns of modularity can persist while others perhaps re-emerge in a lineage over evolutionary time. We discuss this newly discovered property of modularity below along with a range of other implications.

(a) Modularity is an intrinsic attribute of the zebrafish skull

Modularity in the zebrafish skull was pervasive across all lab strains and wild fish. However, Monte Carlo tests were unable to distinguish among models. Therefore, our interpretations of modularity are based on the relative rankings of γ^* values (i.e. models) that were strongly supported via jackknife analysis. Across our different strains and mutant lines support for the null model of no integration was consistently the lowest based on γ^* values. Jackknife tests also fully supported this ranking in all groups. Support for the top-ranked hypothesis for each of AB, Tu, *btm*, *lof,* and wild-caught fish was very high, with jackknife tests corroborating the number one ranking of these hypotheses within 96.1 to 99.9% of the 1,000 runs (Table 2). For *alf* fish, support for the top-ranked hypothesis was lower with 59.7% of jacknife runs, but this was due to competition from a very similar 2nd-ranked hypothesis that 35.1% of jacknife runs supported. The differences between these competing hypotheses were minor making it unlikely that their differences have much biological significance (Figure 2). Taken together these results suggest that while modularity is pervasive within zebrafish, patterns are subject to vary

245 across lines. Given that modularity is often examined at macroevolutionary scales this finding provides
246 the important that modularity can evolve at the population level (which lines are akin to) and change
247 rapidly within a given species.
248
249 (b) Phenotypically similar wild-type lines express different patterns of craniofacial modularity
250 We analyzed two highly polymorphic inbred lines, Tubingen and AB/Oregon, to assess the baseline
251 integration in wildtype zebrafish skulls and the normal variation that is seen across lab populations. As
252 our previous analysis of nucleotide diversity within zebrafish strains indicate that lines may differ
253 greatly in SNP density and diversity, these strains test the intra-strain variation among groups having
254 diverse genetic backgrounds [42]. This genetic difference is associated with a modest difference in
255 craniofacial shape as measured by Procrustes distance (PD, Table 2), and a marked difference in
256 modularity. The top-ranked modularity hypothesis for both lines possessed a lower jaw module (Figure
257 2), and there was a moderate correlation between the top 50 modules for each wildtype line (Table 2).
258 However, the second module in the top-ranked hypothesis was distinct between lines. In Tu fish, this
259 module encompassed the upper jaws and opercle region of the skull, while the cranium conspicuously
260 lacked integration. AB fish, on the other hand, possessed a module that integrates the neurocranium and
261 opercle region of the skull, while the upper jaws lack integration. Thus, the two main wild-type genetic
262 backgrounds used in zebrafish research are associated with distinct modularity patterns. We consider
263 these patterns to be resting states post-domestication upon which we can examine the effects of
264 mutations. Further, we can also use these patterns to assess the impact of domestication.
265
266 (c) Domestication alters craniofacial shape and variability
267 Wild-caught zebrafish were used to augment comparisons among laboratory-reared fish. “Wild-type”
268 strains of zebrafish were derived from pet store stock many decades ago, and are therefore likely to be

removed from wild conditions for dozens of generations. In this time they have spread to research labs around the globe. Thus, their demographic history is characterized by bottle-necks, inbreeding, and altered selection patterns. Domestication can be a potent force of morphological and behavioral change, both intentional and unintentional (e.g., domestication syndrome) [43,44,45,46]. Consistent with this, wild-caught zebrafish exhibit by far the most divergent skull shapes, with pairwise Procrustes distances consistently more than 2x greater than any other non-wild comparison (Table 3). Correlations among top-ranked modularity models are also consistently low when wild-caught fish are compared to laboratory strains (Table 2). A notable exception to this trend is the top-ranked model of modularity in wild-caught fish, which is nearly identical to that of AB wild-types. Both top models are robustly supported by our jackknife analysis, which suggests that core aspects of the covariance structure have been preserved and have influenced craniofacial shape evolution during domestication.

(d) Simple mutations cause a shift to latent patterns of modularity

Battering ram (*btm*) is a previously undescribed mutant identified in the ZF Models large-scale mutagenesis screen for mutations affecting changes in the adult skeleton (http://www.zf-health.org/zf-models/). The mutant was founded on the Tu background and is characterized by alterations in the coordinated growth of the skull leading to a notable reduction in the preorbital region (Figure 1). This phenotypic effect however is quite variable, and although extreme forms are common, population level shape analyses show that mean craniofacial shape in *btm* is similar to both wildtype strains (Table 2). Notably, however, the *btm* population exhibited a marked shift away from its original Tu background state in terms of modularity, and toward the covariance pattern exhibited by AB (Figure 2). The best-supported hypothesis was nearly identical between *btm* and AB lines. Moreover, the correlation between the top models of integration for *btm* and AB was the highest of any pair-wise comparison (Table 2). The analysis of the *btm* mutant reveals a shift between wildtype resting states such that the

293 *btm* mutation has resulted in the skull to converge on the same pattern of modularity found in the AB.
294 Notably, this represents a shift to a putative baseline pattern of modularity represented by wild-caught
295 fish (see above). What is remarkable here is that this convergence has happened in spite of these strains
296 having distinct genetic backgrounds. These data suggest that there may be a limited number of
297 covariance patterns possible in the skull, and that relatively simple genetic changes can result in the
298 reorganization of variation such that certain patterns become latent (i.e., hidden from selection), while
299 previously dormant patterns become resurrected. This apparent flexibility of skull covariance patterns
300 has broader implications for phenotypic evolution (see below).
301
302 (e) Introgression results in new combinations of variational modules
303 The mutant *alf* was identified in the Tuebingen 1996 large-scale screen [15] and is due to an alteration
304 in the potassium channel, Kcnk5b [47]. *Alf* was founded on the Tu background. In order to assess how
305 introgression affects patterns of covariance, *alf* specimens analyzed in this study were outcrossed to the
306 AB background for 2 generations. Our hypothesis was that the introgression of AB alleles into *alf* may
307 shift modularity towards a more AB state. Consistent with this hypothesis, the top-ranked model for *alf*
308 does appear to be a composite between AB and Tu states (Figure 2). On the one hand, it retains a
309 module that encompasses the orbital and dorsal opercle regions, similar to Tu. Notably, it also
310 possesses a third module that integrates neurocranial and opercle landmarks (Figure 2). More generally,
311 *alf* show relatively strong relationships in model space to both AB and Tu (Table 2). Thus, introgression
312 can lead to the parsing of variational modules and the 'melding' of covariance structures that are
313 present in the parental lineages.
314 This observation has important implications for the role of hybridization in promoting
315 phenotypic diversification. While hybridization has long been considered to be a homogenizing force
316 with respect to biodiversity, and a barrier to speciation [48], it has become increasingly obvious in

recent years that hybridization can also be a significant positive force in promoting diversification [49]. In particular, transgressive segregation is the process through which hybridization leads to the production of novel phenotypes, which is likely achieved through the recombination of alleles in hybrid progeny [50,51]. Linking the processes of transgressive segregation and adaptation, however, rests on the assumption that hybrids retain a fully integrated phenotype [54]. We show here that *alf(Tu)* x AB skulls are indeed integrated. Moreover, we show that patterns of integration are a novel combination of covariance pattern between AB and Tu strains. In nature, this should translate to hybrid populations that expose a new pattern of variation to selection, which could significantly enhance their evolutionary potential.

(f) Overgrowth mutants show convergent patterns of covariance

Alf represents a class of fin overgrowth mutants and such increased growth may alter the pattern of integration seen in the skull. Although *alf* does not have any outward craniofacial phenotype, to ascertain if growth could be a developmental parameter affecting craniofacial modularity, we compared the covariance in *alf* to that in the *lof* longfin mutant, which exhibits comparable overgrowth properties. *Lof* is a spontaneous dominant mutant identified in the aquarium trade [52]. *Lof* and *alf* are not allelic and like *alf*, *lof* does not have a pronounced effect on mean craniofacial shape (Table 2). We find that *alf* and *lof* mutants displayed a remarkably high degree of similarity in patterns of covariance within the skull. This is partially evident from consideration of the top-ranked models for each line, which have a similar variational module that encompasses the orbital and dorsal opercle regions of the skull (Figure 2). More striking however is the observation that the top 50 ranked models for each line are highly similar, as demonstrated by a high degree of correlation (Table 2). Thus, independent mutations affecting similar physiological processes (i.e., bone overgrowth) converge on a common covariance structure. This convergence in modularity supports the assertion that developmental systems are a

major contributor to determining covariance structure.

(g) Robustness in response to genetic perturbation

Our data indicate that some modules are more robust to genetic change than others. Specifically, the lower jaw and associated structures form a relatively stable module that is unchanged in 5 of 6 lines. The only instance where the module differs is in *longfin* fish where the lower jaw module is augmented with the addition of upper jaw landmarks (Figure 2). Our tested mutant lines represent only a small fraction of those available for zebrafish, however they are consistent with a general trend among mineralized tissue mutants whereby the phenotypic effect on the mandible is generally more stable compared to other regions of the craniofacial skeleton (e.g., 53,54,55). Thus, while further testing is necessary, we suggest that a conserved mandibular module may exist due to its specific developmental and/or functional attributes. For example, the progenitor of the mandible is Meckel's cartilage, which is derived from a specific population of neural crest progenitor cells [56], and forms the foundation upon which subsequent ossification occurs. In addition, four out of the six individual bony elements that ultimately constitute the mandible (dentary, retroarticular, quadrate, interopercle) begin ossification at the same stage of development (~5.1mm NL)[57]. The remaining two structures (anguloarticular and symplectic) ossify soon after (5.5mm, and 6mm respectively). Thus, the presence of this variational module is consistent with a common developmental origin and timing of differentiation. Moreover, all of these elements are tightly integrated with respect to function (i.e., jaw rotation). The functional integration of these structures is predicted to link them through the iterative process of mechanical stress and subsequent remodeling of the bone [54,58]. The reinforcement of early developmental patterning by ongoing functional demands could result in the establishment of a mandibular module that is robust to genetic mutation. If so, a prediction for future research would be that the evolution of the lower jaw in *Danio* would be constrained to one or few dimensions relative to other regions of the skull.

365

366 (h) Simplifying the complex: re-emergent modularity as a means for evolvability

367 Evolutionary genetics has long recognized that the genetic basis of traits falls along a continuum from
368 simple to complex [59]. However, there remains a high degree of uncertainty as to the genetic
369 underpinnings of higher order properties of development, such as integration and modularity. On one
370 hand, since modularity is thought to result from biomechanical interactions among traits and through
371 the interaction of multiple genes at localized spatial scales during development, its genetic basis is
372 thought to be complex due to the shear number of processes involved. Alternatively, recent research on
373 phenotypic integration and modularity has revealed a surprisingly simple genetic basis for these traits
374 [5,60,61]. These results are consistent with data presented here, which indicate that simple genetic
375 changes can alter what is commonly considered a complex aspect of the phenotype (i.e. modularity).
376 Taken together, such insights suggest two potential outcomes from an evolutionary perspective. First,
377 such a trait underlain by few loci of major effect would be expected to readily evolve in the face of
378 selection. Second, the phenotypic options for change would be constrained by the limited number of
379 loci involved.

380 A paradox in craniofacial biology is that variation in this structure has been shown to be
381 underlain by a large number of loci, which suggests a low degree of evolvability, and yet it is one of the
382 most disparate characters within and among vertebrate lineages. We suggest that a possible resolution
383 to this paradox is the genetic decoupling of phenotypic variation and covariation. Early mapping
384 studies in mice showed that the genetic basis of variation and covariation appear to be highly
385 overlapping, which suggests pleiotropy [62]. Likewise, we have have also implicated genetic
386 pleiotropy in the covariation of craniofacial traits in African cichlids [63,64,65]. However, we have also
387 demonstrated that the genetic basis of variation in fish is distinct from that influencing covariation
388 [5,63,65]. In line with this, our zebrafish lines show substantial changes in modularity in response to

discrete mutations, but with little effect on craniofacial variation. It is possible that mammals and fish differ in this regard, as relative to fish, mammals have far fewer independently moveable elements in the craniofacial skeleton, which may predispose them to fewer modules and the coupling of variation with covariation. A greater degree of decoupling may be a property of fish which allows them to avoid the tradeoffs that would occur under pleiotropy and in turn increase their evolvability. Indeed, fish are well-known as the most speciose group of vertebrates with a sixfold greater number of species than mammals [66].

The transient nature of modularity across our zebrafish indicates that a flexible covariance structure is possible for the skull. Evidence suggests that shifts in modularity can be mediated by discrete mutations that can cause a pattern to re-emerge. Therefore, while evidence suggest that modularity can be responsive to population-level processes it remains to be seen what range is possible. Nonetheless, by readily altering patterns of trait modularity through discrete mutations new and different types of variants should be exposed to selection (blue dots, Figure 3). In other words, such mutations that alter *covariation* could have the effect of 'releasing' genetic variation underlying morphological *variation*. Such shifts could represent the emergence of an evolutionary trajectory forming the first "large" step toward an adaptive optimum (Figure 3). Through this process modularity has the potential to facilitate trait evolution toward an adaptive peak utilizing allelic variants of modest or even minor effect on shape. These latent patterns may represent a 'reservoir of evolvability' only to re-emerge in response to a new mutation or environment that facilitates rapid phenotypic evolution in complex characters.

3. Conclusions

Our findings highlight the utility of looking beyond the outward phenotype in order to gain a better understanding of the developmental, genetic, and functional processes that shape phenotypic

variation and bias phenotypic evolution. Indeed, our data demonstrate how a focus on morphological variation alone can be misleading. Populations that look similar can have very different underlying modularity affecting variation of their phenotypes, and thus potentially respond to selection in very different ways. Given that variational modularity is not an outwardly obvious trait the validation of statistical results provides a challenge for the field, especially given the number of approaches available for investigating modularity [35,36,37,67]. We suggest that such a validation may be possible through connections that can be made between patterns of modularity and genetic variation. Indeed, advances in techniques now allow patterns of modularity to be treated as a quantitative trait that can be genetically mapped [5]. Such an expansion of methodology can move the assessment of modularity toward more experimental approaches whereby the impact of candidate genes on patterns of covariance can be explored. In this sense the various statistical approaches could be considered a first step toward providing hypotheses that can be tested experimentally with mechanistic approaches. Coupled with the mutational approach illustrated here, these investigations will provide researchers the inroads needed to dissect the proximate causes of phenotypic modules, which will ultimately lead to a far better understanding of the factors that influence organismal evolvability.

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658 **Table 1.** *A priori* developmental and functional modules of modularity tested in this study.

Model	Description
[4-14,19,43,47,58,61] [1-3,14-18, 20-60,62]	Early vs late ossification- bones in the anterior region of the jaws tend to ossify first along with parts of the opercular region (Cubbage and Mabee 1999)
[1-19,39-41,44-46][20-33,48-54][59,61][34-38,55,56,57,60][58][62]	Breathing seeing feeding- regions are divided based on their function in respiration, eye muscle attachment, feeding (the oral jaws), the anterior of the head also comprises a module here
[59,61,1-47] [48-58,60,62]	Movable vs fixed- regions are defined based on their ability to move, or as a muscle attachment point
[34-38,40,41,45,46,55-58,60,62] [1-34, 39, 42-44, 47-54, 59, 61]	Dermal vs cartilage bone- Regions are defined by how bone develops, either through cartilage to ossified bone, or directly to dermal bone (Cubbage and Mabee 1999)
[1-9,34-42,45-46,55-62][10-33,47-54]	Lateral line bones- Bones that are innervated by the lateral line are delineated as a module
[34-38,55-58,60-62][10-19,43-46,59][1-9,20-33,39-42,47-54]	Epaxial/hypaxial- regions where groups of hypaxial muscles lie ventral to the spine are delineated from expaxial muscles of the head

[1-19,40-47] [48-62]	Preorbital 1- the region anterior to the eye is defined as module (Cooper et al. 2010; Parsons et al. 2011)
[1-19,40-47][48,20-25,28-29,34-38,48-50,55-57,60][26,27,30-33,51-54,58,59,61,62]	Preorbital 2- the region anterior to the eye is defined as a module, as are the eye region itself, and opercular region.

675 **Table 2.** Pairwise correlations (r-values) for *alf, lof, btm*, AB, Tu, and wild lines of zebrafish. R-values
676 are shown below the diagonal and represent correlations of gamma values (γ*) for 134 models off
677 modularity derived from the minimum deviance method.

	Alf	*Lof*	*btm*	*AB*	Tu	Wild
alf	-					
lof	0.76890292	-				
btm	0.09678171	-0.29179039	-			
AB	0.29885544	-0.04242859	0.80249807	-		
Tu	0.52203087	0.54731221	0.34982901	0.58620055	-	
Wild	0.15728985	0.24022154	-0.1505487	0.0220932	0.2253471	-

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Table 3. Pairwise Procrustes distances for craniofacial shape variation among lines of zebrafish.

	Alf	*lof*	*btm*	*AB*	Tu	Wild
alf	-					
lof	0.0342	-				
btm	0.0377	0.0237	-			
AB	0.0465	0.0425	0.0372	-		
Tu	0.0348	0.0334	0.0379	0.055	-	
Wild	0.1299	0.1341	0.1359	0.1347	0.1340	-

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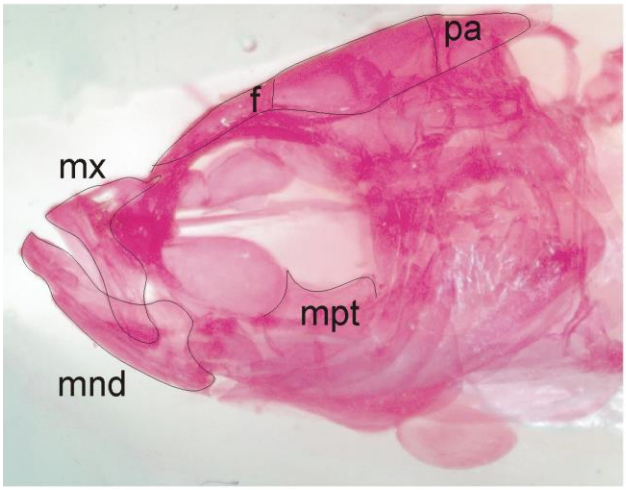
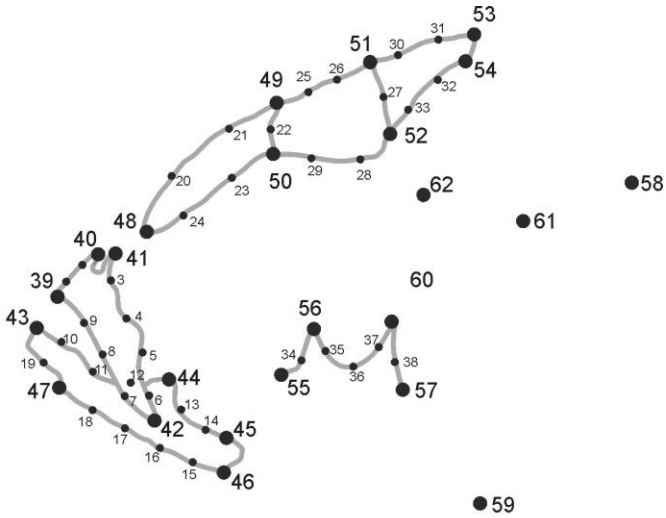
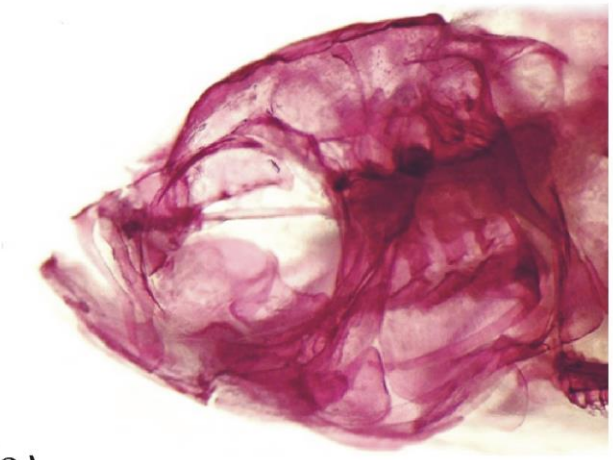


(a)



(b)



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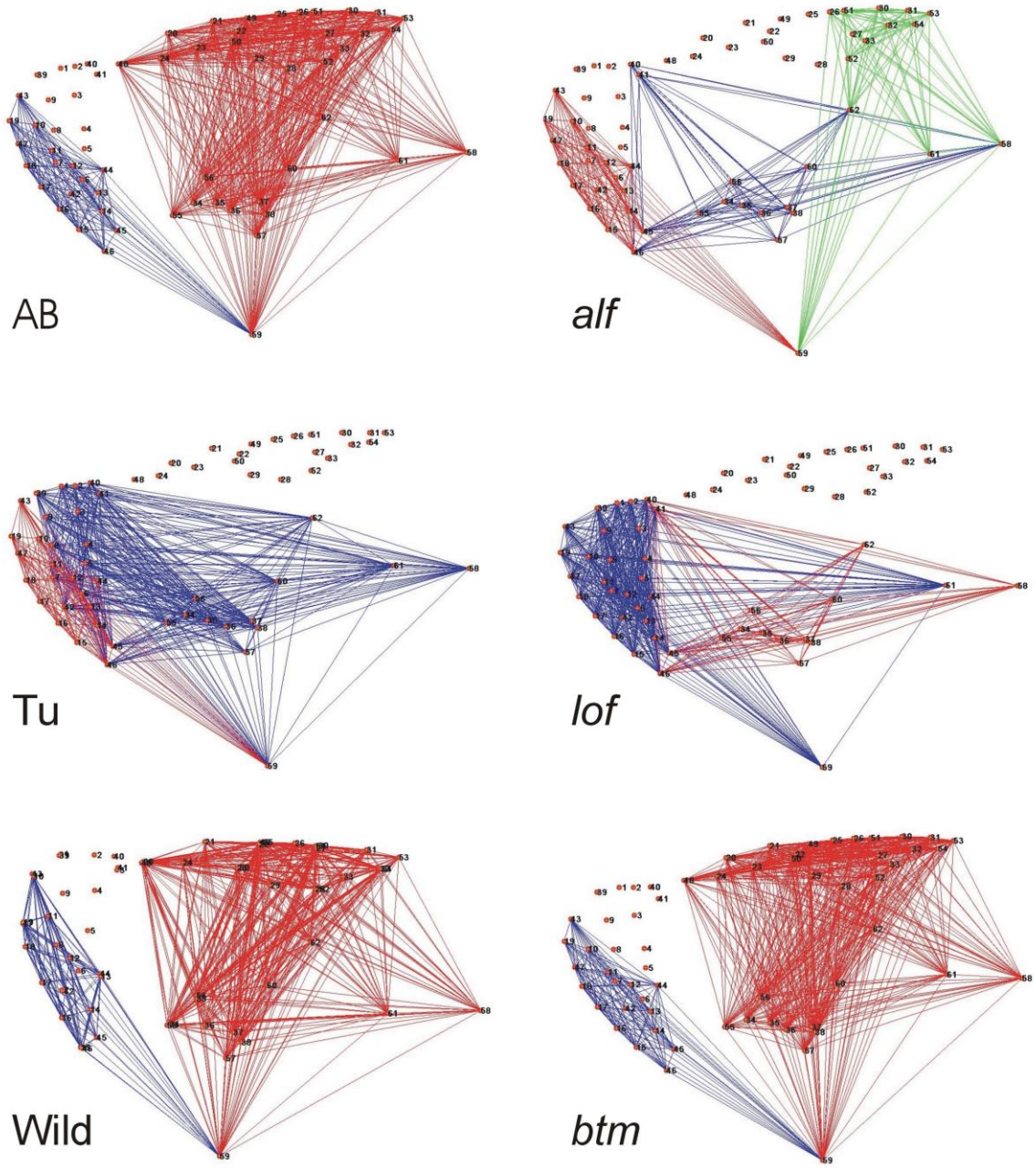

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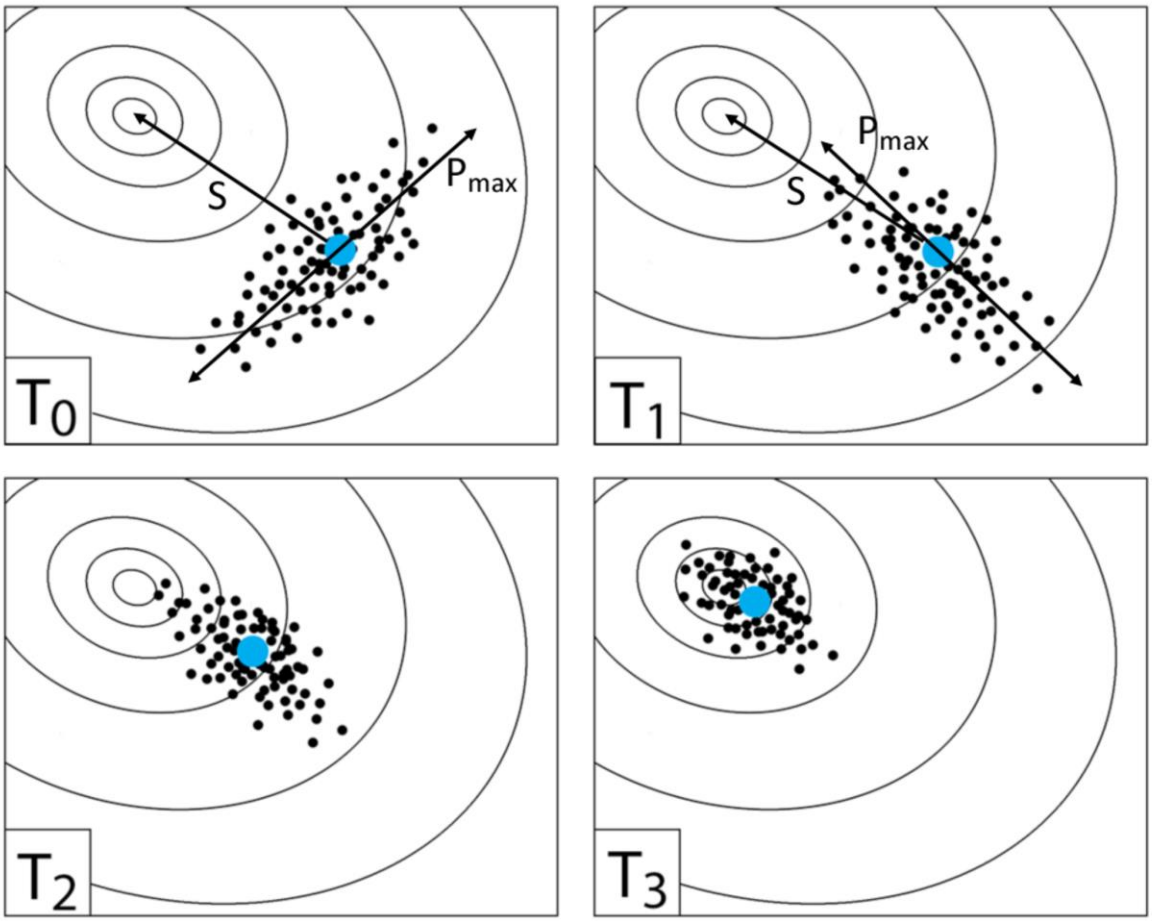
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718 **Figure 1.** Basic anatomy of the zebrafish head from a left lateral view. (a) The anatomical
719 regions of the zebrafish head in a representative sample and the landmarks and semi-
720 landmarks collected for our analysis (mnd = mandible, mx = maxillae, f = frontal, pa = parietal,
721 mpt = metapterygoid). (b) Landmarks (numbered large black circles) and semi-landmarks
722 (small black circles) were used to quantify shape in the zebrafish head. (c) The *btm* mutant,
723 which possesses relatively short oral jaws.

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725 **Figure 2.** Diagrams depicting the best-supported hypothesis of variational modularity for each
726 of the zebrafish lines. Lines of the same colour within a strain belong to the same module.

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729 **Figure 3.** A hypothetical scenario in which a flexible pattern of trait covariance facilitates an
730 evolution response to selection. In each quadrant, the scatterplot represents a two-
731 dimensional (i.e., x,y) morphospace for individuals in a population, superimposed upon an
732 adaptive landscape. At time 0 (T_0), the covariance pattern (i.e., P_{max}) is roughly perpendicular
733 to the axis of selection (S), which is oriented toward an adaptive peak. A dramatic shift in
734 covariance structure due to a relatively simple genetic change could alter patterns of
735 modularity such that P_{max} is more in line with the axis of selection (T_1), without changing the
736 mean shape (blue dot). This would represent a vital first step in an adaptive walk toward a
737 fitness optimum ($T_{2\text{-}3}$), in which subsequent steps are accomplished utilizing loci with modest
738 to small effect sizes. In this way traits with a complicated genetic basis with respect to form
739 could nevertheless show an evolutionary response to selection that is consistent with a
740 geometric model.

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