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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 and 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 towards 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.

1. Introduction

Variation is essential for evolution, but *patterns* of available variation can bias the rate and direction of evolutionary change. For example, such biases have been referred to as 'genetic lines of least resistance' whereby adaptive divergence occurs along a trajectory where genetic variation is most available to improve fitness [1]. However, it remains unclear what underlies 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 trade-offs, whereby one anatomical region may respond positively to selection while limiting potentially detrimental effects on another anatomical region [2–5].

While modularity is essential to an understanding of evolutionary potential, biologists currently know little of its genetic basis or its 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 [6–8]. 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 regard to pattern. Distinguishing between these scenarios is important for greater understanding of how modularity may influence phenotypic evolution.

A key step in addressing this 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 [3–5]. 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 trait modularity. 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 [12–15]. 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 for studying skeletogenesis at all stages of development [16]. While the 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 collection of mutations that result in subtle but significant shifts in craniofacial shape (e.g. [17]). As these mutants are identified as adults, they reflect the type of changes that are viable while maintaining functionality [18,19]. In other words, 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 laboratory strains with a wild-caught population of zebrafish. Wild-caught populations probably face very different selection pressures that 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. Indeed, 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 time scales by this conserved but flexible modularity that predisposes lineages to a limited range of trajectories for adaptation.

2. Material and methods

(a) Collection and rearing of fish

Laboratory strains of zebrafish were reared under standardized laboratory conditions and fed flake food and artemia daily until at least 1+ years of age. Wild zebrafish were obtained 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} $n = 50$, *btm*^{t3404} $n = 89$; M.P.H. 2015, ZF models unpublished). All samples were cleared and stained using alizarin red following Potthoff [21]. Photographs of the 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] where landmark coordinates were captured.

(b) Data collection

For investigations of variational modularity, standard practice involves including as much shape information as is reasonably possible [5,23–27]. This increases objectivity by assessing a broad spectrum of possible interactions. Sliding semi-landmarks [28] make possible the description of shapes combining curves and classic homologous landmarks on the same object [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 inter-individual Procrustes distance. 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 analysis 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 eight *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 remodelling) processes (figure 1; electronic supplementary material, table S1). An additional null model representing a lack of modularity was also included in our analyses.

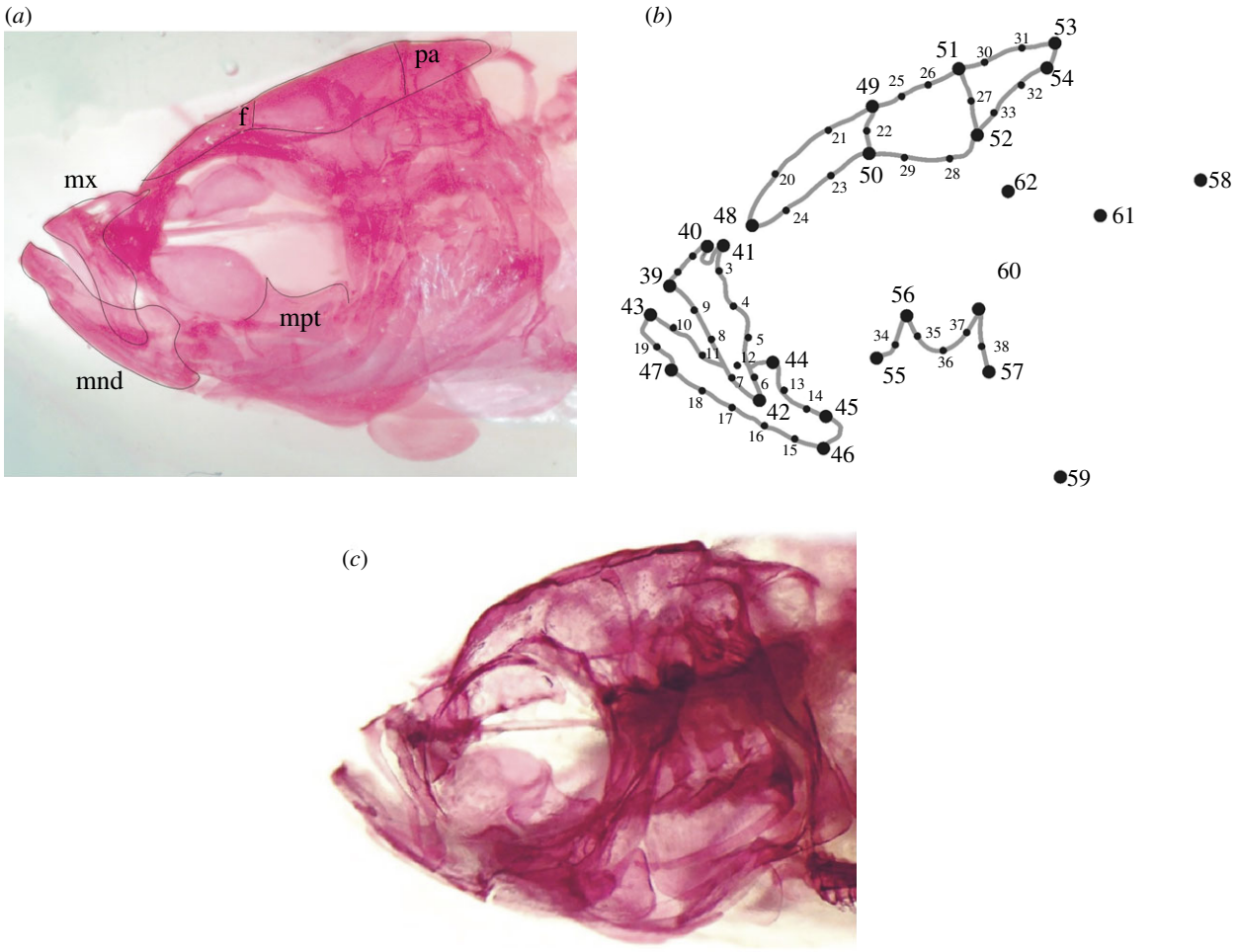

Figure 1. Anatomy of the zebrafish head from a left lateral view. (*a*) The anatomical regions (mnd = mandible, mx = maxillae, f = frontal, pa = parietal, mpt = metapterygoid) and (*b*) landmarks (numbered large black circles) and semi-landmarks (small black circles) used to quantify shape. (*c*) The *btm* mutant, with relatively short oral jaws. (Online version in colour.)

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 powerful options for model selection [5,35]. Model selection approaches are ideal for investigations of modularity because they provide an objective ability to discern models that are best supported from a range of hypotheses. Therefore, model selection provides 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 exist [36]. While this represents an important advancement, the minimum deviance approach explores a much wider range of covariance structures than the likelihood approach (our data suggest 3695 versus 37 models for minimum deviance and likelihood approaches, respectively). This increased range of modularity models is therefore more objective. Furthermore, 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 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 we believe is more evolutionary relevant 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 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 eight hypotheses of modularity were mutually exclusive; therefore, we took advantage of this approach's ability to test all possible non-nested model combinations, providing 3695 tested models. The models tested with this method also allowed for overlapping modules and therefore probably cover a substantial proportion of the developmental and functional processes capable of affecting covariance. 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 used all *a priori* models, and was based on the goodness-of-fit metric γ^*. This metric represented a measure of dissimilarity between observed and expected covariances for each model [35]. Each model comprised a series of partitions

among our landmarks and semilandmarks. Partitions represented a hypothesized module 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 is 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 jack-knife approach in which γ^* values and model ranks were recomputed after removing 1000 randomly chosen subsets comprising 10% of the data.

(e) Testing for similarities in patterns of covariance

If a particular model fitted 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. While 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 centred at a different position, only the direction of these vectors is comparable, which we achieved by examining correlations between γ^* vectors of each group. We did not use all 3695 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. However, for this approach, 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 CoordGen8 [39].

3. 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 minor differences in genetic variation can be present among species [40,41]. Furthermore, our findings demonstrate how the domestication of laboratory strains (an evolutionary process itself) can inadvertently alter patterns of trait covariation, including within so-called wild-type lines that appear outwardly similar (e.g. Tuebingen versus 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 intrinsic to the zebrafish skull

Modularity in the zebrafish skull was pervasive across all laboratory 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 jack-knife analysis. Across strains and mutant lines, the null model of no integration was consistently ranked the lowest among all γ^* values, with jack-knife tests reinforcing this result in all groups. Support for the top-ranked hypothesis for each of AB, Tuebingen, *btm*, *lof* and wild-caught fish was very high, with jack-knife tests corroborating the number one ranking of these hypotheses within 96.1–99.9% of the 1000 runs. For *alf* fish, support for the top-ranked hypothesis was lower with 59.7% of jack-knife runs, but this was due to competition from a very similar 2nd-ranked hypothesis that 35.1% of jack-knife runs supported. The differences between these competing hypotheses were minor making it unlikely that their differences have biological significance (figure 2). Taken together, these results suggest that while modularity is pervasive within zebrafish, patterns vary across lines. Given that modularity is often examined at macroevolutionary scales, this finding provides support for the idea that modularity can evolve at the population level (which lines are akin to) and change rapidly within a given species.

(b) Phenotypically similar wild-type lines express different patterns of modularity

We analysed two highly polymorphic inbred lines, Tuebingen and AB/Oregon, to assess the baseline integration in wild-type zebrafish skulls and the normal variation that is seen across laboratory populations. As our previous analysis of nucleotide diversity across zebrafish strains indicates substantial differences in SNP density and diversity, these strains provide diverse genetic backgrounds [42]. These genetic differences are associated with modest differences in craniofacial shape as measured by Procrustes distance (PD, table 1), and a marked difference in modularity. The top-ranked modularity hypothesis for both strains possessed a lower jaw module (figure 2), and there was a moderate correlation between the top 50 modules for each wild-type line (table 2). However, the second module in the top-ranked hypothesis was distinct between strains. In Tuebingen fish, this module encompassed the upper jaws and opercle region of the skull, while the cranium lacked integration. AB fish, on the other hand, possessed a module that integrates the neurocranium and opercle region of the skull, while the upper jaws lack integration. Thus, the two main wild-type genetic backgrounds used in zebrafish research are associated with distinct modularity patterns. We consider these patterns to be resting states post-domestication upon which we can examine the effects of mutations.

(c) Domestication alters craniofacial shape and variability

Wild-caught zebrafish were used to augment comparisons among laboratory-reared fish. 'Wild-type' strains of zebrafish were derived from pet store stock many decades ago, and have, therefore, been removed from natural conditions for dozens of generations. In this time, they have spread to research laboratories around the globe. Thus, their history is characterized by bottlenecks, inbreeding and altered selection patterns. Domestication can be a potent force of morphological and behavioural change, both intentional

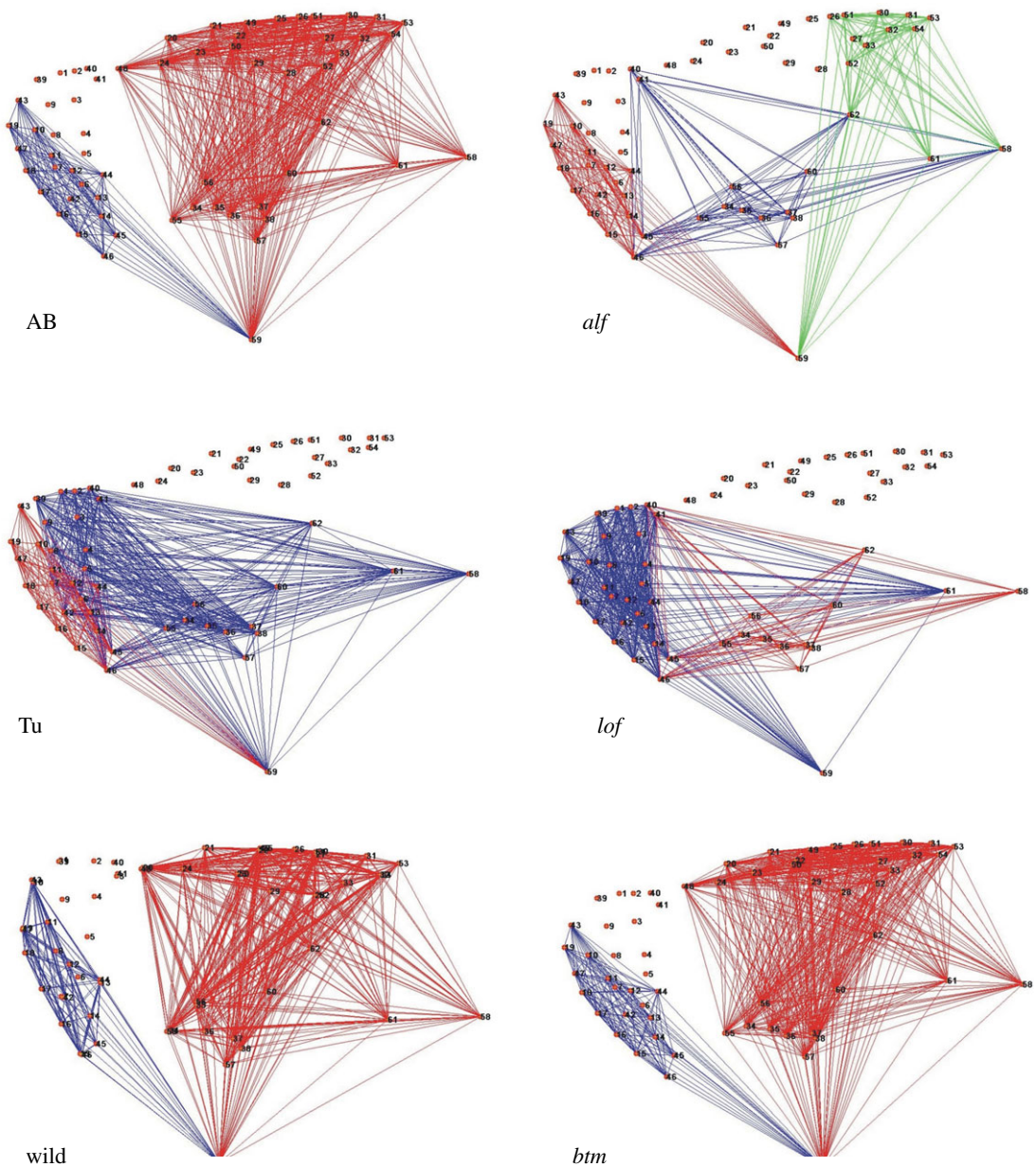

Figure 2. Diagrams depicting the best-supported hypothesis of variational modularity for each of the zebrafish lines. Lines of the same colour within a strain belong to the same module. (Online version in colour.)

Table 1. 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	—		
Tuebingen	0.0348	0.0334	0.0379	0.055	—	
wild	0.1299	0.1341	0.1359	0.1347	0.1340	—

and unintentional (e.g. domestication syndrome) [43–46]. Consistent with this, wild-caught zebrafish exhibit by far the most divergent skull shapes, with pairwise Procrustes distances consistently more than 2× greater than any other non-wild comparison (table 2). Correlations among top-ranked modularity models are also consistently low when wild-caught fish are compared with laboratory strains (table 1). 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 jack-knife analysis, which suggests that core aspects of modularity have been preserved and probably influenced craniofacial shape evolution during domestication.

Table 2. Pairwise correlations (*r*-values) for *alf*, *lof*, *btm*, AB, Tuebingen and wild lines of zebrafish. *R*-values are shown below the diagonal and represent correlations of gamma values (γ^*) for 134 models of 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	—		
Tuebingen	0.52203087	0.54731221	0.34982901	0.58620055	—	
wild	0.15728985	0.24022154	−0.1505487	0.0220932	0.2253471	—

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

Battering ram (*btm*) is a previously undescribed mutant identified in the ZF Models large-scale mutagenesis screen for mutations affecting the adult skeleton (http://www.zf-health.org/zf-models/). The mutant was founded on the Tuebingen background and is characterized by alterations in the coordinated growth of the skull leading to a reduction in the preorbital region (figure 1*c*). 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 wild-type strains (table 1). Notably, however, the *btm* population exhibited a marked shift away from its original Tu background in terms of modularity, and towards the pattern exhibited by AB (figure 2). The best-supported hypothesis was nearly identical between *btm* and AB lines. Moreover, the correlation between the top modularity models for *btm* and AB was the highest of any pair-wise comparison (table 1). The analysis of the *btm* mutant reveals a shift between apparent wild-type resting states such that the *btm* mutation has resulted in the skull to converge on the same pattern of modularity found in AB.

Notably, this also represents a shift to a putative baseline modularity represented by wild-caught fish (see above). Remarkably, this convergence has happened in spite of these strains having distinct genetic backgrounds. This suggests that there may be a limited number of covariance patterns possible in the skull, and that relatively simple genetic changes can result in certain modularity patterns becoming latent (i.e. hidden from selection), while previously dormant patterns become resurrected. This apparent flexibility of modularity has broader implications for phenotypic evolution (see below).

(e) Introgression results in new combinations of variational modules

The mutant *alf* was identified in the Tuebingen 1996 large-scale screen [15] and is due to an alteration in the potassium channel, *Kcnk5b* [47]. *Alf* was founded on the Tuebingen background. To assess how introgression affects patterns of covariance, *alf* specimens analysed in this study were outcrossed to the AB background for two generations. Our hypothesis was that the introgression of AB alleles into *alf* may shift modularity towards a more AB state. Consistent with this hypothesis, the top-ranked model for *alf* does appear to be a composite between AB and Tuebingen states (figure 2). On the one hand, it retains a module that encompasses the orbital and dorsal opercle regions, similar to Tuebingen. Notably, it also possesses a third module that integrates neurocranial and opercle landmarks (figure 2). More generally, *alf* show relatively strong relationships in model space to both AB and Tuebingen (table 1). Thus, introgression can lead to the parsing of variational modules and the 'melding' of covariance present in the parental lineages.

This has important implications for the role of hybridization in promoting phenotypic diversification. While hybridization has long been considered to be a homogenizing force, and a barrier to speciation [48], it has become increasingly obvious in recent years that hybridization can also significantly promote diversification [49]. In particular, transgressive segregation is a process through which hybridization leads to novel phenotypes, which is probably 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 [52]. We show here that *alf(Tu)* × AB skulls are indeed integrated. Moreover, we show that patterns of integration are a novel combination of covariance pattern between AB and Tuebingen strains. In nature, this would translate to hybrid populations that expose a new pattern of variation to selection, which could significantly enhance evolutionary potential.

(f) Overgrowth mutants show convergent modularity

Alf represents a class of fin overgrowth mutants and such increased growth may alter the pattern of modularity in the skull. Although *alf* does not have any obvious craniofacial differences, 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 [53]. *Lof* and *alf* are not allelic and, like *alf*, *lof* does not have a pronounced effect on mean craniofacial shape (table 1). We find that *alf* and *lof* mutants displayed a remarkably high degree of similarity in skull covariance patterns. 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 models for each line are highly similar (table 1). Thus, independent mutations affecting similar physiological processes (i.e. bone overgrowth) appear to converge on a common covariance structure.

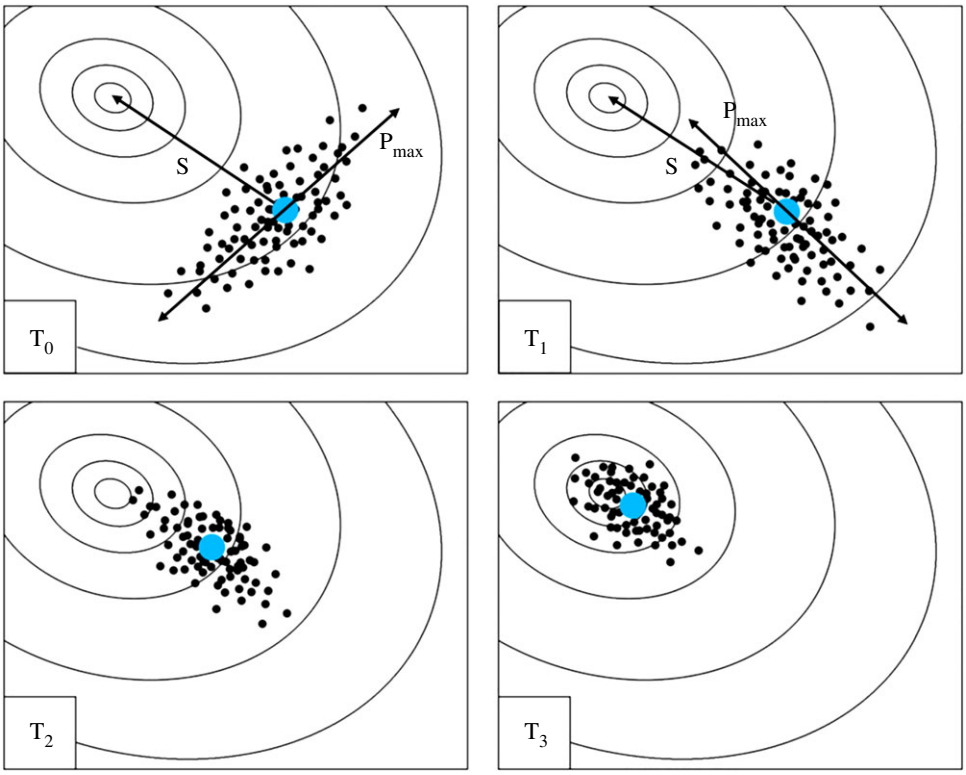

Figure 3. A hypothetical scenario in which a flexible pattern of trait covariance facilitates an evolution response to selection. In each quadrant, the scatterplot represents a two-dimensional (i.e., x,y) morphospace for individuals in a population, superimposed upon an adaptive landscape. At time 0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

(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 five of six lines. The only instance where the module differs is in *longfin* fish where the lower jaw module is augmented with additional upper jaw landmarks (figure 2). Our 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. [52,54,55]). Thus, while further testing is necessary, we suggest that a conserved mandibular module exists 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 cells [56], and forms the foundation upon which subsequent ossification occurs. In addition, four of the six individual bony elements that ultimately constitute the mandible (dentary, retroarticular, quadrate, interopercle) begin ossification at the same stage of development (approx. 5.1 mm NL) [57]. The remaining two structures (anguloarticular and symplectic) ossify soon after (5.5 mm and 6 mm, respectively). Thus, the presence of this variational module is consistent with common developmental origins and timing of differentiation. Moreover, all of these elements are tightly integrated with respect to function (i.e. jaw rotation) which should link them through the iterative process of mechanical stress and subsequent bone remodelling [52,58]. 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 few dimensions relative to other regions of the skull.

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

Evolutionary genetics has long recognized that the genetic basis of traits falls along a continuum from simple to complex [59]. However, there remains a high degree of uncertainty about the genetic underpinnings of higher order properties of development, such as modularity. For example, because modularity is thought to result from biomechanical interactions among traits and through the interaction of multiple genes at localized spatial scales during development, its genetic basis is thought to be complex due to the sheer number of processes involved. Alternatively, recent research on phenotypic integration and modularity has revealed a surprisingly simple genetic basis [5,60,61]. These results are consistent with data presented here, indicating that simple genetic changes can alter what is considered a complex aspect of the phenotype (i.e. modularity). Taken together, such insights suggest two potential outcomes from an evolutionary perspective. First, such a trait underlain by few

loci of major effect would be expected to readily evolve under selection. Second, the phenotypic options for change would be constrained by the limited number of loci involved.

A paradox in craniofacial biology is that variation in this structure has been shown to be underlain by a large number of loci, suggesting a low degree of evolvability, yet it is one of the most disparate characters within and among vertebrate lineages. We suggest that a resolution to this paradox is the genetic decoupling of phenotypic variation and covariation. Early mapping studies in mice showed that the genetic basis of variation and covariation appear to be highly overlapping, which suggests pleiotropy [62]. Likewise, we have also implicated genetic pleiotropy in the covariation of craniofacial traits in African cichlids [63–65]. However, we have also demonstrated that the genetic basis of variation in fish is distinct from that influencing covariation [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 movable 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 trade-offs 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 six-fold greater number of species than mammals [66].

The transient nature of modularity across our zebrafish appears to be mediated by discrete mutations. Therefore, while evidence suggests 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 towards an adaptive optimum (figure 3). Through this process, modularity has the potential to facilitate trait evolution towards an adaptive peak using allelic variants of modest or minor effect. 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.

4. Conclusion

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 variation and bias 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 outwardly obvious the validation of statistical results provides a challenge for the field, especially given the number of approaches available for investigating modularity [35–37,67]. We suggest that such a validation may be possible through connections that can be made between patterns of modularity and genetic variation. Indeed, advances 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 towards experimental approaches whereby the impact of candidate genes can be explored. In this sense, statistical approaches could be considered a first step towards providing hypotheses that can be tested 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 evolvability.

Ethics. Procedures conducted in the USA were approved by IAICUC, while procedures in the UK followed guidelines provided for zebrafish by the Home Office.

Data accessibility. All data used in this manuscript are present in the manuscript and its supporting information. Raw data collected for this study can be found on Dryad Digital Repository [68].

Authors' contributions. K.J.P. conceived the ideas, collected data, analysed the data and led the writing of the manuscript. R.C.A. edited drafts and helped by discussing and helped refine the ideas. Y.H.S., D.T. and A.C. collected data. M.P.H. and S.K. provided samples. All authors contributed critically to the drafts and gave final approval for publication.

Competing interests. We declare we have no competing interests.

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