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Abstract

Characterizing phenotypic divergence using three-dimensional geometric morphometrics in four populations of threespine stickleback, *Gasterosteus aculeatus* (Pisces: Gasterosteidae) in Katmai National Park and Preserve, Alaska

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The threespine stickleback (*Gasterosteus aculeatus* L., 1758) is a vertebrate model for the study of the relationship between phenotype and environment in facilitating rapid evolutionary change. Using four populations from a system of lakes in Katmai National Park and Preserve, Alaska, and microcomputed tomography and three-dimensional geometric morphometrics, we test the hypothesis that stickleback populations, inhabiting freshwater environments display cranial phenotypes that are intermediate between the putative ancestral form and low-plated freshwater populations that demonstrate substantial divergence toward new phenotypic optima. We further test the hypothesis that phenotypic covariance structure is disrupted in the context of such putatively recent adaptive events. We report significant phenotypic differences among all four populations that includes a component of sexual dimorphism. Further, we show evidence of disrupted phenotypic covariance structure among these populations. Taken together, these findings indicate the importance of phenotypic quantification as a key step in elucidating both the ecological processes responsible for rapid adaptive radiations, and the role of developmental mechanisms in biasing evolutionary change.

Key Words

Phenotype – Alaska – braincase – evolution – geometric morphometrics – microcomputed tomography – threespine stickleback – *Gasterosteus aculeatus*– skull

Introduction

Investigating the evolution of population divergence in association with different ecological resources can reveal the consequences of environmental change on populations (Ayala and Fitch 1997; Nosil 2012). Studies of phenotypic divergence in different environments have typically focused on discrete or quantitative variation in behavioural, morphological, and physiological traits related to resource based phenotypic differences, such as body/trait size versus prey/habitat size relationships (e.g. Garland and Losos 1984; Grant 1986; Price 1991; but see Schluter 2000; Rogers et al. 2002; 2013, Di-Poi et al. 2014; Evans et al. 2014). Yet, organisms are integrated to function as a whole, and efforts to assess variance and covariance in different traits may offer evidence of how evolutionary change may be constrained or facilitated by the interaction of phenotypes and their constituent parts (Parsons et al. 2012; Jamniczky et al. 2014; Rogers and Jamniczky 2014). For instance, repeated use of the same underlying genes during parallel and convergent phenotypic evolution may reflect biases and constraints in association with beneficial mutations (Stern and Orgogozo 2009; Gompel and Prud'homme 2009; Conte et al. 2012). Knowledge of these effects might facilitate an understanding of genetic evolution, but steps must be taken to incorporate the phenotypic effects of environmental change towards predicting evolutionary trajectories (Conte et al. 2012).

Covariance between different parts of the phenotype is produced by the action of overlapping developmental processes which are subject to the effects of natural selection (Hendrikse et al. 2007; Hallgrimsson et al. 2009). The relationship between genetic architecture and phenotypic covariance may inform the means by which functionally correlated traits evolve and respond in a correlated fashion to selection (Olson and Miller 1958; Cheverud 1996a; Wagner and Altenberg 1996; Hansen and Houle 2008). Stabilizing selection has been shown to

have a prominent role in maintenance of covariance structure over time (Lande 1980; Cheverud 1984; Stepan 1997^{a,b}; Young and Hallgrímsson 2005; Goswami 2006; Jamniczky and Hallgrímsson 2009), but when the influence of stabilizing selection is reduced (e.g. colonization of new environments or environmental change), covariance structure is easily disrupted and mutations of small effect can have remarkably large phenotypic consequences (e.g. (Hallgrímsson et al. 2006; Richtsmeier et al. 2006; Jamniczky and Hallgrímsson 2009). The role of phenotypic covariance structure in facilitating phenotypic divergence is still unclear, and current work indicates the potential for covariance both to facilitate (Jamniczky and Hallgrímsson 2009; Sanger et al. 2011) and constrain evolutionary change to varying degrees (McGuigan and Blows 2007; Walsh and Blows 2009). A further degree of complexity is introduced through consideration of the largely underappreciated role of phenotypic plasticity in facilitating such change (Wund et al. 2008; Morris and Rogers 2014; Michel et al. 2014; Morris et al. 2014). Integrating estimates of covariance between different parts of the phenotype offers an opportunity to investigate the role of covariance structure following environmental change.

The threespine stickleback (*Gasterosteus aculeatus* L., 1758) provides a unique natural experiment to test hypotheses about phenotypic changes that accompany adaptive radiations. This organism has undergone repeated parallel evolutionary events over the past ~ 10 000 years, invading freshwater habitats from its ancestral marine environment across the Northern Hemisphere (McPhail and Lindsey 1970; Bell and Foster 1994; Peichel 2005). Stickleback in vastly different and geographically distant locations exhibit remarkably similar body and head morphologies correlated with specific habitats (McPhail 1993; Walker 1997; Willacker et al. 2010; Schluter et al. 2010; Aguirre and Bell 2012) which has produced a general consensus describing differences between marine and lacustrine populations (Bell and Foster 1994). In this

conceptualization, anadromous populations are thought to be phenotypically similar to each other and generally representative of the ancestral marine form, and are often used as a proxy for the marine phenotype in morphological investigations (Bell 1976; Bell and Foster 1994; Walker and Bell 2000). Differing lacustrine environments are presumed to have produced another dichotomy between shallow-dwelling benthic foragers (Lavin and McPhail 1985; 1986; 1987; Walker and Bell 2000) and limnetic populations from deeper lakes that retain trophic morphology similar to their marine ancestors (McPhail 1984; Kassen et al. 1995), and which differ along many of the same axes including differences in size, body and head shape, skull shape, armour plating, gill rakers, and colouring (e.g. Hagen and Gilbertson 1972; Larson 1976; Ridgway and McPhail 1984; McPhail 1992; 1993; Robinson 2000; Willacker et al. 2010; McGee and Wainwright 2013). Parallel trends of divergence offer stronger support for the role of natural selection in shaping these patterns, as genetic drift is unlikely to produce predictable changes in independent lineages in correlation with the environment (Schluter and Nagel 1995).

While much of this research is based upon two-dimensional measurement and meristic traits which are likely insufficient to capture the full range of variation present in a given population, other studies have used the tools of geometric morphometrics in two dimensions (Baumgartner et al. 1988; Walker 1997; Caldecutt and Adams 1998; Willacker et al. 2010; McGee and Wainwright 2013), and the extension of this approach to three-dimensional data is a promising avenue of inquiry, as it provides a paradigm within which to better characterize whole-organism phenotypes and better situate the organism within its ecological context. Here we examine the potential role of phenotypic covariance in biasing phenotypic outcomes following adaptive change in a stickleback system in Katmai National Park and Preserve (KNP; Bowles et al. 2016), using the techniques of microcomputed tomography (μ CT) and three-

dimensional geometric morphometrics to quantify phenotypic variation and covariation. We focus in particular on the braincase and skull, given recent work indicating the importance of multivariate characterization of cranial phenotype in relation to stickleback evolution (Jamniczky et al. 2014). We test two hypotheses: first, that stickleback populations, occupying freshwater habitats but experiencing substantial ongoing gene flow (Bowles 2016), display a cranial phenotype that is intermediate between the putative ancestral form and low-plated freshwater populations that demonstrate substantial divergence toward new phenotypic optima; and second, that phenotypic covariance structure is disrupted in the context of such adaptive events. We predict that 1) differences in morphology are linked to divergence of freshwater forms from ancestral phenotypes toward niche-specific ecotypes associated with altered foraging challenges; 2) stickleback fish exhibit transitional morphologies in the skull (and potentially the brain), given the role of the skull in facilitating feeding and the role of the brain in directing behaviour, as they diverge away from the ancestral form; and 3) disrupted phenotypic covariance structure will be identified in freshwater forms that have not yet reached the novel phenotypic optimum toward which they are evolving.

Materials and Methods

Study System

KNP is located approximately 480 km southwest of Anchorage on the northern end of the Alaska Peninsula (Jones et al. 2005). This large (1.7e6 ha) park is divided by the Aleutian Range such that one side drains into Bristol Bay on the Bering Sea, and the other drains into Shelikof Strait in the North Pacific Ocean (Heard et al. 1969; Jones et al. 2005). The Naknek Lake and River system is the main drainage on the west side of the divide produced by the Aleutian Range,

and consists of a series of connected water bodies that drain into the Bering Sea. Naknek Lake is 590.52 km² in area, located at 10.4 m elevation (Jones et al. 2005), and dated at $26\,155 \pm 285$ years (Mann and Peteet 1994; Stilwell and Kaufman 1996). This lake houses a diverse fish community, including known stickleback predatory taxa including *Salvelinus alpinus* (L., 1758), *Salvelinus malma* (Walbaum in Artedi, 1792), *Salvelinus namaycush* (Walbaum in Artedi, 1792), *Lota lota* (L., 1758), *Cottus aleuticus* Gilbert, 1896, *Oncorhynchus kisutch* (Walbaum, 1792), *Oncorhynchus mykiss* (Walbaum, 1792), and *Esox lucius* L., 1758 (Heard et al. 1969). Jo-Jo and Pringle Lakes (informal names) are both small bodies of water within the drainage. Pringle Lake (0.93 km²) drains into the Bering Sea, is at an elevation of 51.05 m, and given that it was formed behind the Newhalen moraine, and thus must be younger than the retreat of that advance, was likely formed approximately 15 000 years ago (+/- error), although complete data is presently unavailable (F. H. Wilson, USGS, written communication 2015). The Pringle Lake fish community is relatively sparse, but is known to contain *E. lucius* (Vanderzwan 2015). Jo-Jo Lake (6.73 km²) is located at an elevation of 11.0 m and was formed 1550 ± 45 years ago (Finney 2006). Jo-Jo Lake also contains a relatively sparse fish assemblage, but that assemblage includes the predatory *E. lucius* and *O. nerka* (Jones et al. 2005). A species inventory does not currently exist for the Naknek River itself (Vanderzwan 2015).

The sticklebacks collected for this study originate from four different locations within KNP – Naknek River at King Salmon (NR; 58.68, -156.67), Lake Camp at Naknek Lake (LC; 58.67, -156.46), Jo-Jo Lake (JO; 58.61, -155.22), and Pringle Lake (PR; 58.56, -155.96). Sticklebacks collected from JO, PR and LC represent lacustrine populations. JO and PR fish are small (least squares mean standard length = 34 mm) and mostly of the low-plated morph (PR: low plated = 76%, JO: low plated = 65%), whereas LC fish, collected at the head of Naknek

River, are larger (least squares mean standard length = 63 mm) and polymorphic for lateral plates (completely plated = 51%, partially plated = 44%, low plated = 5%). NR fish are also larger (least squares mean standard length = 63 mm) and represent an anadromous population (Bowles 2016), mostly of the completely plated morph (83%). In addition, a separate study with 4000 single nucleotide polymorphism (SNP) loci indicates that these populations exhibit significant genetic differentiation that varies from the anadromous NR through LC and JO, with the PR population most genetically divergent ($F_{ST} = 0.037 - 0.13$, $p = 0.001$ for all comparisons; Bowles 2016).

Fish

Fish were collected from each of the four localities described above in the summers of 2011 and 2012. Adult (>30 mm standard length) individuals were sampled from each locality ($n = 125$; NR = 31, LC = 30, JO = 29, PR = 35). Fish were collected using a combination of seine nets (4.5 m long, 1 m high, 5 mm mesh size) and Gee's minnow traps (6 mm mesh size) set along shorelines and left in the water no longer than 24 hours. Fish were euthanized using an overdose of Eugenol (Sigma-Aldrich Co. LLC), fin clips were collected for molecular sex determination and future genetic analysis, and fish were then either placed directly into 95% ethanol, or fixed in 10% formalin for ~24 hours followed by immersion in 70% ethanol for storage. Fish were sampled, handled, and euthanized following a protocol approved by the Canadian Council on Animal Care and the University of Calgary Animal Care Committee (AUP BI09R-41).

Fish were sexed using *Idh* polymerase chain reaction product following a standard protocol (Peichel et al. 2004), where samples showing one band at 302 base pairs were identified as females, and those with bands at 302 and 271 base pairs were identified as males.

Three Dimensional Phenotypic Analyses

The rostral portion of each preserved fish, from the tip of the snout to the caudal extent of the skull, was subjected to μ CT (μ CT 35; Scanco AG, Brütisellen, Switzerland). Specimens were straightened into a standardized position with spines and fins flattened against the body wall and held in place with clear plastic wrap. Fish were scanned at a resolution of 20 μ m (70 kVp, 160 μ A, 500 projections). Raw scans were reconstructed into 3D volumes and imported into Amira 5.4 (FEI Visualization Sciences Group, Burlington MA). Isosurface renderings were produced using standard threshold values of 2500-3000 to account for the differences in bone density. A set of 25 homologous 3D points was then digitized in triplicate, by the same individual in separate sessions, on the left side and midline of each surface rendering with a particular focus on the braincase (Figure 1), and exported for geometric morphometric analysis.

Raw landmarks were first subjected to Procrustes transformation in order to remove the effects of scale, rotation, and translation. Procrustes Analysis of Variance (Procrustes ANOVA) was used to assess the degree to which measurement error (imprecise or inaccurate placement of landmarks) contributed to variance in the dataset (Klingenberg and McIntyre 1998; Klingenberg et al. 2002). Measurement error was not found to have a significant effect, so replicate landmark data were averaged for each individual and these data were used for further analysis.

Procrustes ANOVA was used to assess the contribution of allometric size, as measured by the natural log of centroid size (a measure of landmark configuration size), to variance in the

averaged dataset. Allometric size was found to be significant, with a significant interaction between population and size, but not between sex and size (Table 1). Averaged landmark data were therefore regressed on the natural log of centroid size, pooled by population. Residuals from this regression were then subjected to further analysis.

Procrustes ANOVA was once again used to assess the effect of population and sex on variance in the size-adjusted dataset. Principal Components Analysis (PCA) was applied to the size-adjusted dataset as a whole, as well as to male and female adjusted datasets separately, to characterize the major axes of variation among individuals examined. Male and female datasets were also assessed individually using Procrustes ANOVA to identify differences in population mean shapes for each sex.

Covariance matrix repeatability (Cheverud 1996b), a measure of the degree to which estimated covariance matrices are representative of biological variation rather than sampling error, was calculated following Marroig and Cheverud (2001). Covariance matrix similarity was assessed for the complete size-adjusted dataset using random skewers (Cheverud 1996b; Cheverud and Marroig 2007), a method of calculating and comparing the response of each covariance matrix to 1000 randomly generated selection vectors. We tested the null hypothesis that covariance matrices were unrelated; a significant result indicated similarity between matrices. Global integration within the complete dataset was measured by computing the variance of eigenvalues of each covariance matrix, scaled to total sample variance (Wagner 1984; 1990), and differences among populations were assessed for significance by resampling and re-computing the variance of eigenvalues over 1000 replicates (Manly 1991).

All morphometric and statistical analyses were conducted in R version 3.2.2 for Mac (R Development Core Team 2014). Procrustes ANOVA and PCA were performed as implemented

in the ‘geomorph’ package version 2.1.5 (Adams and Otárola-Castillo 2013). The random skewers matrix comparisons were performed as implemented in the ‘phytools’ package version 0.4-60 (Revell 2011). Code for other analyses was developed by the authors, with the addition of code modified from Claude (2008). All significance testing conducted above employed a non-parametric 1000-round randomized residual permutation procedure (Collyer et al. 2015), corrected for multiple pairwise comparisons using a Bonferroni adjustment where appropriate. Effect sizes are reported as Z-scores, defined as standard deviations of observed sum-of-squares values from sampling distributions of random values obtained through the same randomized residual permutation procedure (Collyer et al. 2015). We used these procedures in order to mitigate the effects of small sample size in the context of high-dimensional data (Collyer et al. 2015).

Results

Allometry and Sexual Dimorphism Significantly Influence Stickleback Phenotype

We observed significant effects of population and size, as well as a population-by-size interaction in the complete averaged dataset (Table 1). We used pooled multivariate regression to adjust for this effect, and then repeated our analyses. We observed significant effects of population and sex in the size-adjusted dataset, but we found no interaction between population and sex (Table 2). We further observed that the Procrustes distances between all four population mean shapes were significant for both the complete dataset (Table 3), and for males and females examined separately (Table 4, Table 5).

Cranial Shape Varies Between Habitats, and Between Males and Females in Low-Plated Populations

The NR individuals in the complete analysis clustered at the positive end of the first principal component of variation (PC1) in the averaged, size-adjusted dataset, while individuals representing the two low-plated freshwater populations clustered at the negative end and the LC individuals occupied the centre of the plot (Figure 2). Individuals at the positive end of the PC1 axis exhibited a deeper skull with a relatively smaller braincase and a relatively dorsoventrally elongate opercle, a smaller orbit, and a rostrocaudally shorter facial region. Individuals at the negative end of the PC1 axis, in contrast, exhibited a longer and shallower skull, with a dorsoventrally compressed opercle and a relatively larger braincase and orbit. This component accounted for 42.7% of the variance in the dataset.

The second principal component (PC2), accounting for a further 9.54% of the variance in the dataset, separated the members of the two freshwater populations from each other, with most JO individuals clustered toward the positive end and PR individuals clustered toward the negative end (Figure 2). Individuals at the positive end of the PC2 axis displayed a relatively deeper facial region in comparison to the braincase, a rostrocaudally shortened braincase, a flattened skull roof and a reduced orbit. Individuals at the negative end of the PC2 axis displayed a more rounded skull roof, with a rostrocaudally elongate braincase and upturned snout, and a larger orbit. This axis also describes changes to the relative width of the skull, with individuals at the positive end exhibiting relatively wider braincases in comparison to their snouts.

The third and following principal components did not reveal any structure within the dataset, and are not discussed further here.

Analysis of males and females separately revealed a broadly similar trend along the first principal component, where NR individuals of both sexes occupied the positive end of the axis and JO and PR individuals occupied the negative end, with LC individuals in between (Figure 3). As in the complete analysis, individuals at the positive end of the PC1 axis in both sexes exhibited a deeper skull with a relatively smaller braincase and a relatively dorsoventrally elongate opercle, a smaller orbit, and a rostrocaudally shorter facial region. Individuals at the negative end of the PC1 axis again exhibited a longer and shallower skull, with a dorsoventrally compressed opercle and a relatively larger braincase and orbit. This component explained 46.6% of the variance in the male dataset, and 39.4% in the female dataset. The second principal component revealed a difference between males and females, where JO and PR males occupied the same region of morphospace (Figure 3A) while JO and PR females occupied opposite ends of the PC2 axis (Figure 3B). Female phenotypes therefore reflected the same trend observed along PC2 in the complete analysis. The second principal component explained 7.63% of the variance in the male dataset, and 13.7% in the female dataset.

Covariance Matrices are Substantially Disrupted

Covariance matrix repeatability was high for all four groups, confirming that variance in the dataset was representative of biological variation and not sampling error. Global integration was found to be variable between populations for the complete, size-adjusted dataset. Eigenvalue variances calculated for NR - JO, LC - PR, and LC - NR comparison did not differ significantly from each other, while significant differences were found for the other comparisons (Figure 4). Responses to random selection vectors identified that the PR and NR covariance matrices were significantly similar to each other, while all other covariance matrices were unrelated (Table 6).

Discussion

Allometric Size Change and Sexual Dimorphism are Key Features of Stickleback Evolution

We show that a major feature of stickleback phenotypic evolution toward freshwater habitats includes a substantial decrease in size, consistent with previous observations (e.g. Bell and Foster 1994) and a recent common environment study documenting a genetic basis for body size in these same populations (Bowles et al. 2016). Interestingly, however, the influence of size on shape in other work has been found to be quite small (Albert et al. 2008). Further research is required to explicitly demonstrate the allometric nature of size variation in the populations studied here, particularly towards exploring the ontogenetic trajectory producing these shape and size differences.

Sexual dimorphism is also present in this dataset, and indicates the possibility that male and female fishes are responding differently to selection in freshwater environments. While the distance between population mean shapes for the JO and PR populations are approximately the same for both sexes (Tables 4, 5), PCA revealed that females of these populations are divergent along the second principal component, which is correlated with a change in width profile of the skull, with JO individuals exhibiting a relatively wider posterior braincase.

Sexual dimorphism is known to play an important role in stickleback brain shape (Park and Bell 2010; Kotrschal et al. 2012), body shape and size (Head et al. 2009; Cooper et al. 2011; Leinonen et al. 2011), armour (Reimchen and Nosil 2004; Aguirre et al. 2008), and feeding mechanism (McGee et al. 2013). Where there are multiple ecological axes under disruptive selection, divergence may be more likely to occur in the absence of sexual dimorphism on at least some of these axes (Bolnick and Doebeli 2003; Cooper et al. 2011). Additionally, habitat

differences may produce plastic changes in morphology (Day and McPhail 1996), and such changes may be important in the context of rapid diversification in stickleback (Park et al. 2012). Alternatively, the presence of sexually dimorphic variation may itself facilitate rapid adaptation, an hypothesis based on the absence of sexual dimorphism in a benthic lake form derived from a strongly dimorphic marine ancestor (Albert et al. 2008). The present study was not designed to identify phenotypic plasticity, nor was it targeted at sexual dimorphism in particular, but future work focused directly on the role of plasticity in the feeding apparatus will be important to address these key questions (Wund et al. 2008; Pfennig and Ehrenreich 2014; Schneider et al. 2014).

Phenotypic Variation in Sticklebacks is Complex

Anadromous stickleback are frequently considered sufficiently conservative in morphology (Bell and Foster 1994) to be used effectively interchangeably with marine forms (Bell 1976), which are also thought to be highly morphologically conserved (Bell et al. 2009). This perspective is largely a product of a sparse fossil record for ancestral marine stickleback (Bell et al. 2009) in association with a paucity of work explicitly examining variation in closely related marine stickleback phenotypes in diverse marine environments (Jamniczky et al. 2015), resulting in the assumption that both temporal and spatial variation in marine and anadromous phenotypes is relatively low (Albert et al. 2008; Rogers et al. 2012; Arnegard et al. 2014). Yet, both marine and freshwater environments are highly variable with respect to biotic (e.g., predator communities) and abiotic factors (e.g., temperature), while recent evidence confirms that adaptive phenotypic variation persists in association with heterogeneous marine environments in Baltic Sea stickleback (DeFaveri et al. 2013; DeFaveri and Merilä 2013). Our results are

consistent with this finding, where we see notable differences in phenotype between NR and polymorphic freshwater-resident LC groups, and suggest that further research into how stickleback use these environments (e.g., competition and ecological opportunity) may help explain the processes driving ecological diversification of these lineages. Specifically, the polymorphic population (located at the head of Naknek River at the entry point to Naknek Lake) is intermediate in shape between the putatively ancestral and freshwater populations (Figure 2), presenting a more elongate, shallow, and gracile skull that includes an enlarged eye and reduced opercle, while remaining distinct from the low-plated freshwater populations that show more exaggerated versions of this cranial shape.

Phenotypic Data Reveal Hints of Selection History

The freshwater phenotypes we examine here are significantly different from each other and do not directly conform to classical descriptions of either limnetic or benthic morphotypes. Both populations are more broadly similar to the limnetic morphotype (Willacker et al. 2010), exhibiting a flattened braincase and relatively large orbit. They also possess an upturned mandible (McPhail 1992, 1993; McGee and Wainwright 2013) and a generally slender, streamlined skull (McGee and Wainwright 2013). The JO population has a relatively square orbit and a horizontally oriented dorsal skull profile, in contrast to the PR population, which displays a rounded orbit and a sloped dorsal skull profile. The opercular apparatus and braincase are relatively deeper but more compact in the PR population, while the same skull elements in the JO population are generally more elongate but shallower and posteriorly broader, at least in females. Recent work has suggested that brain shape varies in association with feeding ecology (Park and Bell 2010), and our results suggest that differences in micro-habitat between the two

freshwater locations studied here may be driving divergence in morphology. However, as we note above, our experimental design does not allow us to differentiate between the presence and strength of selection acting on the trophic apparatus in comparison to other parts of the phenotype, and we also note the presence of potentially greater predation pressure in Jo-Jo lake (two species) as compared to Pringle Lake (one species). Specific hypotheses regarding the nature of the selective forces differentiating these two freshwater populations remain to be tested.

Our results demonstrate that phenotypic covariance structure is substantially disrupted within this assemblage of populations, a finding that is congruent with another recent study examining phenotypic covariance in Alaskan stickleback (Aguirre and Bell 2012). The phenotypic covariance matrices calculated here for the two freshwater populations do not share significant structure (Table 6), and also exhibit differences in global integration (Figure 4). Indeed, only the NR and PR populations show any evidence of shared covariance structure (Table 6), and integration levels are shared between the NR and JO and NR and LC populations independently of the shared integration demonstrated by the LC and PR populations (Figure 4). While covariance structure is largely the product of developmental processes (Atchley et al. 1981; Cheverud 1996a; Zelditch et al. 2006), both theoretical (Lande 1980; Cheverud 1984; Wagner and Altenberg 1996; Jones et al. 2007) and recent morphological work (Steppan 1997a,b; Ackermann and Cheverud 2000; Marroig and Cheverud 2001; Young and Hallgrímsson 2005; Goswami 2006; Jamniczky and Hallgrímsson 2009) indicates that stabilizing selection has a very important role in the maintenance of this structure over evolutionary time. Significant morphological disparity between the two freshwater populations examined in this study (Table 3), associated with the presence of significant differences in covariance structure as measured by differing responses to hypothetical selection vectors (Table 6) and changes in global integration

(Figure 4), may indicate the presence of disruptive selection acting to produce two different phenotypic optima under slightly different environmental conditions.

There are several caveats to our study design. First, small sample sizes (due in part to the time-consuming nature of μ CT data collection) of braincase morphology and variation in preservation technique preclude better estimates of (co)variation. However, we mitigate the effects of small size by using non-parametric approaches to multivariate significance testing (Collyer et al. 2015), allowing us to exploit the unique nature of geometric morphometric data while reaching robust conclusions about phenotypic variation. Second, a lack of genetic data precludes testing certain hypotheses. However, separate analyses have supported significant genetic differentiation between these populations while a common garden experiment has confirmed a genetic basis for size variation between Naknek River, Jo-Jo Lake and Pringle Lake (Bowles et al. 2016). Third, we cannot exclude phenotypic plasticity, and a single population under each condition is limited towards making evolutionary or ecological inferences about the causes of divergence. Along these lines, future research is required to tease apart the roles of selection, constraint and plasticity in structuring phenotypic variation within this system. In particular, testing hypotheses about adaptive radiation requires quantitative characterization of environmental variables for each location, including salinity, food resources, temperature, and predators from multiple populations. Nonetheless, there are at least two possible hypotheses for the patterns of phenotypic variation that we observe. It is possible that these two freshwater morphotypes are part of the range of possible variation in limnetic forms. Alternately, given the other evidence we describe for the action of disruptive selection on freshwater forms in this system, it is possible that these two groups have not yet reached the phenotypic optimum for

their specific freshwater environment and represent transitional forms between polymorphic and truly freshwater, limnetic stickleback (e.g Marchinko et al. 2014).

Further, larger and more thoroughly standardized samples from each population will allow a larger set of landmarks to be collected, facilitating the testing of hypotheses about phenotypic differences, sexual dimorphism, and patterns of covariance in different parts of the skull. Recent work has shown that phenotypic covariance structure in the stickleback trophic apparatus may have an important role in facilitating rapid adaptive radiation (Jamniczky et al. 2014), and the results reported here suggest that exploring the relationship between the morphology of the braincase and trophic apparatus may reveal further insight into the mechanisms of adaptive change. Finally, it is important to note that while we derive hypotheses of trajectory direction from lake age in the present study, other work has shown that phenotypic divergence does not necessarily follow geographical history (Salzburger and Meyer 2004; Seehausen et al. 2008). Future work should include the phylogenetic inference of history in this system. Overall, phenotypic differences in association with predicted evolutionary shape trajectories demonstrate an important role for the integration of quantitative, multivariate phenotypes towards elucidating the mechanisms behind rapid divergence events.

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Tables

Table 1. Procrustes ANOVA of the complete, averaged dataset. Abbreviations: df = degrees of freedom; *F* = *F*-values; *P* = *P*-value; Size = natural log of centroid size; *Z* = Effect size (*Z*-score).

	df	SS	<i>F</i>	<i>Z</i>	<i>P</i>
Size	1	0.17333	102.5267	25.1605	0.001
Population	3	0.04544	8.9595	7.1309	0.001
Size x Population	3	0.01209	2.3832	2.3350	0.001
Residuals	117	0.19780			

Table 2. Procrustes ANOVA of the complete, size-adjusted dataset. Abbreviations: df = degrees of freedom; *F* = *F*-values; *P* = *P*-value; *Z* = Effect size (*Z*-score).

	df	SS	<i>F</i>	<i>Z</i>	<i>P</i>
Population	3	0.20155	41.7738	16.8462	0.001
Sex	1	0.00386	2.4012	2.3130	0.003
Population x Sex	3	0.00577	1.1956	1.2139	0.102
Residuals	117	0.18817			

Table 3. Procrustes distances between populations in the complete, size-adjusted dataset. Distances are reported below the diagonal, with *P*-values for each comparison above the diagonal.

	NR	LC	JO	PR
NR	-	0.001	0.001	0.001
LC	0.0461	-	0.001	0.001
JO	0.0815	0.0511	-	0.001
PR	0.0931	0.0597	0.0427	-

Table 4. Procrustes distances between populations in the male, size-adjusted dataset. Distances are reported below the diagonal, with *P*-values for each comparison above the diagonal.

	NR	LC	JO	PR
NR	-	0.001	0.001	0.001
LC	0.0480	-	0.001	0.001
JO	0.0858	0.0567	-	0.026
PR	0.0957	0.0614	0.0438	-

Table 5. Procrustes distances between populations in the female, size-adjusted dataset. Distances are reported below the diagonal, with *P*-values for each comparison above the diagonal.

	NR	LC	JO	PR
NR	-	0.012	0.001	0.001
LC	0.0461	-	0.001	0.001
JO	0.0817	0.0484	-	0.001
PR	0.0920	0.0609	0.0438	-

Table 6. Covariance matrix repeatability and matrix correlations. Covariance matrix correlation values based on random skewers analysis are given below the diagonal, *P*-values above the diagonal, and repeatability along the diagonal.

	NR	LC	JO	PR
NR	0.977	0.997	0.052	0.000
LC	0.393	0.954	0.963	0.133
JO	0.478	0.412	0.977	0.191
PR	0.500	0.470	0.466	0.947

Figure Legends

Figure 1. 3D landmarks collected for this study. Landmarks are indicated by white points in right lateral view on a 3D reconstruction of a μ CT scan of *Gasterosteus aculeatus* (specimen caught in the Naknek River at King Salmon, AK).

Figure 2. Principal components analysis of the complete, size-adjusted dataset. Wireframe deformation grids represent right lateral views (XY) and right dorsal views (YZ). Abbreviations: PC = principal component.

Figure 3. Principal components analysis of the male and female, size-adjusted dataset. Wireframe deformation grids represent right lateral views (XY) and right dorsal views (YZ). Abbreviations: PC = principal component.

Figure 4. Boxplot of scaled variance of eigenvalues resampling distribution for each population. Letters indicate significantly different comparisons ($P < 0.001$). Observed variance of eigenvalues: Jo-Jo Lake (JO): $2.81\text{e-}6$; Pringle Lake (PR): $1.12\text{e-}6$; Lake Camp (LC): $1.47\text{e-}6$; Naknek River at King Salmon (NR): $2.34\text{e-}6$.

Figure 1. 3D landmarks collected for this study. Landmarks are indicated by white points in right lateral view on a 3D reconstruction of a μ CT scan of *G. aculeatus* (specimen caught in the Naknek River at King Salmon, AK).
115x76mm (300 x 300 DPI)

Figure 2. Principal components analysis of the complete, size-adjusted dataset. Wireframe deformation grids represent right lateral views (XY) and right dorsal views (YZ). Abbreviations: PC = principal component.
131x98mm (300 x 300 DPI)

Figure 3. Principal components analysis of the male and female, size-adjusted dataset. Wireframe deformation grids represent right lateral views (XY) and right dorsal views (YZ). Abbreviations: PC = principal component.
229x293mm (300 x 300 DPI)

Figure 4. Boxplot of scaled variance of eigenvalues resampling distribution for each population. Letters indicate significantly different comparisons ($P < 0.001$). Observed variance of eigenvalues: Jo-Jo Lake (JO): $2.81e-6$; Pringle Lake (PR): $1.12e-6$; Lake Camp (LC): $1.47e-6$; Naknek River at King Salmon (NR): $2.34e-6$.

116x86mm (300 x 300 DPI)