Evolution and development of facial bone morphology in threespine sticklebacks

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How do developmental mechanisms evolve to control changing skeletal morphology, the shapes and sizes of individual bones? We address this question with studies of the opercle (OP), a large facial bone that has undergone marked morphological evolution in the ray-finned fish. Attributes for developmental analysis motivated us to examine how OP shape and size evolve and develop in threespine sticklebacks, a model system for understanding vertebrate evolution. We find that when Alaskan anadromous fish take up permanent residence in lakes, they evolve smaller and reshaped OPs. The change is a reduction in the amount of bone laid down along one body axis, and it arises at or shortly after the onset of OP development. A quantitative trait locus is present on linkage group 19 that contributes in a major way to this phenotype.

A key feature of evolution in vertebrates is the acquisition of new skeletal morphologies, new bone sizes and/or shapes. Changes might occur in size, the overall dimensions of a bone, or shape alone, such that if one dimension increases, another must decrease. Alternatively, size and shape change might be intercoupled. How the underlying developmental determinants of skeletal morphology change during evolution is unknown, but understanding may be within reach (1, 2). Here we take a simplified approach toward understanding skeletal developmental change during evolution, with focus on a single facial bone, the opercle (OP), which has favorable attributes for developmental genetic study (3). We examine this bone in threespine sticklebacks, fish that have excellent qualities for evolutionary analyses (4–6).

The OP is a prominent, flat bone that is roughly triangular shaped in sticklebacks (7) (Fig. L4). It supports the opercular cover of the gills, its inward–outward movement provides ventilation, and comparative study has shown that the efficiency of opercular pumping depends on its size and shape (8, 9). These morphological features vary markedly among the Actinopterygii or ray-finned fish, of which teleosts represent the largest, most highly derived, and most diverse clade. In basal actinopterygians, or ray-finned fish, morphological features vary markedly among the Actinopterygia (10). OP evolution begins early (12–14), and this feature, along with the bone’s superficial location, where it can be easily visualized, are attributes that facilitate analysis. In zebrafish, mild reduction of Endothelin1, a signaling protein important for dorsal–ventral (DV) patterning (15–17), prominently expands DV growth of the OP (3). The phenotypic changes in endothelin1 mutants mimic features of OP evolutionary changes among actinopterygians; therefore, changes in the Endothelin1 pathway might be responsible for opercular macroevolution (3).

However, essentially nothing is known about OP microevolutionary change-variation within a single species. Does adaptive genetic variation that can be examined developmentally exist in natural populations? An ideal organism to investigate this question is the threespine stickleback Gasterosteus aculeatus (4, 18, 19). Oceanic (anadromous) sticklebacks can take up permanent residence in fresh water, and when they do, their skeletons evolve, including major reduction of body armor (20–22) during as few as 12 years (<10 generations) (23). Hybrids between anadromous and freshwater sticklebacks are fertile, so that genetic loci responsible for evolution can be identified and mapped (24). Indeed, recent analyses have uncovered genetic loci that may globally underlie skeletal armor loss in this species (25–27). In fact, a single developmental regulatory gene, Pitx1, may be largely responsible for pelvic armor loss (25, 28). Remarkably, in Alaskan populations, both this pelvic armor locus and an unlinked locus underlying lateral plate armor reduction segregate as Mendelian genes (27).

Here we examine OP evolution and development in sticklebacks. We discovered that OPs are smaller and less DV elongated in derived, resident Alaskan lake populations than in ancestral anadromous populations. There is no prominent change along the anterior–posterior (AP) axis. Development is reconfigured, possibly at the very earliest stages of bone patterning, and may be caused by changes at only a few genetic loci. These findings will allow us to examine how naturally segregating genetic variants directly affect developmental programs that control adult facial skeletal morphology.

Materials and Methods

Collections and Crosses. Wild-captured Alaskan anadromous fish from Rabbit Slough and lake fish from Boot Lake, Bear Paw Lake, and a fraction of the fish from Whale Lake were the same as we used previously for a study of armor loss, and methods were as described (27). Furthermore, we used the same genetic crosses for complementation and mapping as described. Briefly, complementation crosses were made en masse, and mapping crosses were between single pairs of fish. A collection of Bolo Lake (N 61.526, W 149.0489) stickleback was made at the same time as these others (June 2001). Whale Lake fish from two other collections made in the 1990s were added to our data set (three in all wild-captured sets for this lake). Sets of fish from Mud Lake (N 61.563, W 148.9486) and Long Lake (N 61.578, W 149.7639) were collected in 1998. Fish were reared in a laboratory facility for our developmental studies and the genetic crosses as described (27); see ref. 27 for our methods for genotyping and genetic mapping.

Phenotypic Studies and Data Analysis. Adult wild-captured fish and laboratory-reared juvenile fish >1 month old were anesthetized with tricaine methanesulfonate (MS 222).

We studied wild populations of threespine sticklebacks (4, 8, 16, 27) and laboratory-reared juvenile fish >1 month old.
with 0.0016% Finquel, fixed in neutral 4% formaldehyde, and stained with Alizarin Red S (29). For the population studies described below, and for developmental stages >30 days after fertilization (DPF), the OPs were dissected free from both sides of the face of trypsinized fish. The isolated bones were photographed by using a Nikon E4500 digital camera mounted on a Nikon SMZ1500 stereomicroscope. We observed no consistent left–right OP asymmetries (data not shown) and averaged the measurements for both bones in a single fish (variance discarded). For the genetic crosses, the OPs were photographed in situ on only the right side of the face. For the youngest developmental stages (7–30 DPF), live larvae were vitally stained overnight with Alizarin Red (1% in 1 mM Hepes buffer), anesthetized, and mounted between bridged coverslips. Alizarin fluorescence was imaged with a Zeiss Pascal confocal microscope (10× or 20× objective), and z series scans were made (generally, 2-μm steps in the z axis, ~50 images). Projections were made from these images with Zeiss software (as in Fig. 6, which is published as supporting information on the PNAS web site) and saved. All OP measurements, including the area, were made from digital images with IMAGEJ software (National Institutes of Health). Analyses were made with JMP version 5.1 software (SAS Institute, Cary, NC).

### Results

**OPs Vary in Morphology Between Ancestral and Derived Populations of Alaskan Sticklebacks.** Comparing the morphology of the OPs between anadromous (ancestral) and lake (derived) Alaskan populations of stickleback reveals a prominent change in both size normalized to the standard length (SL) of the fish (see Figs. 1 and 2) and shape. The bones from an anadromous fish (Fig. 1B Upper) are larger and more DV elongated than those from a lake fish (Lower). To quantify the difference, we compared wild-captured fish from two anadromous populations and six lake populations that may have evolved independently from anadromous ancestors (27, 30). We measured and normalized to SL the OP area (A) and the two lengths VP and JP (Fig. 1B), yielding AS, VPS, and JPS. All three measures are significantly higher for the anadromous fish than the lake fish (Table 1). Plotting VPS by JPS yields a morphospace “streak” of points, and the anadromous and lake fish do not overlap along this streak (Fig. 1C; see also Fig. 7, which is published as supporting information on the PNAS web site, for a more visual representation). The difference in VPS accounts for nearly all of the size difference; the difference in JPS between anadromous and lake fish is relatively much smaller (Fig. 1C and Table 1). We conclude that, when anadromous fish evolve into lake fish, their OPs become smaller and less ventrally elongated. The change appears very robust; we also observed the change using other length measurements and multivariate analyses, and, for a subset of the data, landmark-based morphometrics (data not shown).

The OPs in fish from different lakes also differ from one another. We see that the points in Fig. 1C approximately fit a line with a slope of 1.0 (the line shown is a diagonal through the mean of the size-standardized lake distribution (0, 0); see Fig. 6 and Table 2, which is published as supporting information on the PNAS web site). A slope of 1 means that both dimensions of the bones are changing in proportion, and therefore that the difference among lake fish OPs is predominately in size, not in shape. All of these populations show degrees of armor loss as well as reduction of OP size, as compared with the anadromous fish, yet the traits are not completely correlated. For example, Whale Lake fish, with the largest OPs (red points), have extreme armor loss (27, 31). In contrast, Mud Lake fish, with the smallest OPs (light blue points), have spines resembling anadromous fish, although their lateral plates are reduced. OP size also does not seem to be correlated with feeding habits: Mud Lake fish feed near the bottom (a “benthic” population) (32), and Long Lake fish (orange points) feed in open water (“limnetic”). These are different feeding styles, but the OPs in these two populations are similar in size.

### OP Growth Rates Differ Between Ancestral and Derived Sticklebacks

What changes in development produce the derived OP morphologies of the lake populations? We followed development of laboratory-reared fish obtained from within-population single-pair crosses from anadromous and lake parents. For the earliest stages, from the onset of calcification in early larva until ~2 days after hatching, the morphologies show a prominent size difference. Even when measured at this early stage, the lake OPs have already been significantly reduced in size relative to the anadromous OPs (33). This reduction in size continues throughout development, and the derived morphologies are strongly correlated with the final adult size of the bones. For the lake populations that we studied, this correlated change in size is evident from as early as 5 days postfertilization (DPF). At this stage, the OPs from lake fish are already much smaller than those of anadromous fish. The morphologies of the OPs do not develop uniformly along the VP axis (from all of the wild-captured Alaskan fish. VP S and JPS are SL-standardized comparisons. (maximal curvature of the bone. We use the lengths of these lines in shape and size comparisons. (C) A morphospace showing the distribution of OP morphologies from all of the wild-captured Alaskan fish. VP S and JP S are SL-standardized lengths of VP and JP (B), made by taking the residuals of linear regressions of VP length and JP length on standard length (SL) for the lake data set. We excluded the anadromous fish from the regression equation, because the slope of the regression line for the lake fish alone is near 1.0 (Fig. 6). The upper data points are exclusively from anadromous fish (Anchorage River, black; Rabbit Slough, gray). The lower data points are exclusively from lake fish (Boot Lake, dark blue; Mud Lake, light blue; Long Lake, orange; Bear Paw Lake, green; Bolo Lake, purple; Whale Lake, red). The theoretical diagonal line passes through the mean of the lake fish, and indicates variation in OP size without a change in shape.

### Table 1. OP dimensions for the wild-captured fish

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Area</th>
<th>VP</th>
<th>JP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anadromous</td>
<td>28</td>
<td>2.22 ± 0.42</td>
<td>1.53 ± 0.08</td>
<td>0.16 ± 0.04</td>
</tr>
<tr>
<td>Lake</td>
<td>96</td>
<td>0.00 ± 0.17</td>
<td>0.00 ± 0.04</td>
<td>0.00 ± 0.03</td>
</tr>
</tbody>
</table>

$F_{1,122}$ for ANOVA:

<table>
<thead>
<tr>
<th>Group</th>
<th>$t$-value</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anadromous</td>
<td>0.40</td>
<td>0.68</td>
</tr>
<tr>
<td>Lake</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

$F_{1,122}$ for ANOVA, $P < 0.0001$. See Table 2 for a more complete summary. ANOVA statistics, $F$ ratios, and $P$ values are comparisons between Anadromous and Lake fish.
weeks thereafter, we used confocal z scans of living, vitally stained preparations.

We can first see mineralization of the OP in both morphs at 7 DPF, soon after hatching. At this stage, the OP is only a spicule, a line of calcified matrix beginning where it makes its joint with the hynomandibula and extending posteriorly (Fig. 2A). Hence, in both anadromous and lake fish, initial development is along a single axis, the AP body axis, that forms the eventual bone dimension of the adult bone. As the OP elongates along the AP axis, its posterior region then begins to grow out secondarily along the DV axis, forming a triangular shape (and the VP dimension of the bone). DV growth at this stage (9 DPF), and at every stage thereafter, is more prominent in the anadromous fish such that the overall form is more robust in the anadromous fish and more gracile in the lake fish. Both VP and JP continue to lengthen (Fig. 2B) and the adult OP morphologies arise gradually (Fig. 2B and data not shown). We note that early development of neighboring facial bones also starts out as simple lines of calcification, which soon transform into recognizable models of the more complex adult shapes (Fig. 2B).

We quantified these observations by measuring VP and JP during development. We observe, strikingly, that during months of growth of both anadromous and lake fish, VP and JP length increase in proportion to one another, i.e., the points fit straight lines in a VP by JP plot (Fig. 3A; $r^2 = 0.99$). The slope of the line for the anadromous fish, 1.74, is significantly higher than the slope for the lake fish, 1.48 (see legend to Fig. 3). This difference means that the developing anadromous fish maintain a relatively higher rate of VP elongation. The difference in slopes largely predicts the different shapes of the adult bones (data not shown).

By making logarithmic transformations of the same data, another feature of these developmental trajectories becomes clear (Fig. 3B). We can easily fit not one but two straight lines to each data set: "early period," slopes of 0.99; SE 0.012. Test of slopes: $F_1, 1197 = 67.92; P < 0.0001$. (B) An early, distinctive, and highly allometric growth phase shows up for both populations in log–log VP by JP plots of the same data. A larger slope means higher allometry (a slope of 1 means isometric growth). We fit two regression lines to each data set: "early period," $r = 0.99$; SE 0.0109. VP$_{anadromous} = -0.23 + 1.72$ * JP$_{anadromous}$, $r^2 = 0.99$. SE = 0.0109; VP$_{lake} = -0.24 + 1.48$ * JP$_{lake}$, $r^2 = 0.99$. SE = 0.0200. Test of slopes: $F_1, 1197 = 87.92; P < 0.0001$. We note that early development of the OP is only a spicule, a line of calcified matrix beginning where it makes its joint with the hyoid arch shown for the adult in Fig. 1A. Like the OP, the other dermal bones begin development as lines, which then take on their specific shapes.

OP Morphology Is Inherited as a Quantitative Trait. Our developmental study shows that laboratory-reared fish develop OPs of approximately the same morphologies as wild-caught fish from the same populations, indicating that genetic variation for these
traits is partitioned across populations. To begin to understand this variation, we measured the OPs of young laboratory-reared adults arising from between-population crosses. In a cross between fish from two low-armored lakes, Bear Paw and Boot Lake, the F1 OPs were markedly larger than those of the parental populations (Fig. 4B; the two parental means, shown by the colored circles, are nearly identical). However, in this and another lake-by-lake cross (Table 3, which is published as supporting information on the PNAS web site), we do not observe the ventrally elongated shapes of the ancestral anadromous fish. The upper left region of the morphospace character-istic of the anadromous fish (Fig. 4A, gray points) is empty in Fig. 4B. Rather, in the F1 fish, JPs is increased to nearly the same extent as VPs; the cloud of points are near the diagonal in Fig. 4B. Hence, this distribution suggests genetic complementation, with dominant alleles from at least two loci contributing to OP size but not shape (AAbb × aaBB → AaBb). Alternatively, our results may be explained by a single locus with overdominance. In either case, our data suggest that different alleles affect OP size in the two derived populations exhibiting similar OP morphology.

Anadromous by lake crosses suggest that genes other than those contributing only to OP size control its ventral elongation. The anadromous by lake F1 hybrids had OPs distributed roughly in between the parents in the morphospace (Fig. 4C and E). The distributions fall well below that for the anadromous parental population (Rabbit Slough, mean shown by the gray circles). This finding argues forcefully against genes with dominant alleles contributing to shape inheritance, a difference from what we see for size inheritance. For one of the lakes, Bear Paw, the F2 OPs were also approximately intermediate in morphology between the parents (Fig. 4D) and the distribution is broader, as expected because more allelic combinations will be present in F2 progeny than in F1 progeny. As for the F1 OPs, the principal variation is along the VP morphospace axis (Table 3), suggesting a discrete genetic regulation of ventral lengthening (involving change in both size and shape) by a gene set with additive effects.

The F1 hybrid OPs from a second anadromous by lake cross (Rabbit Slough by Boot Lake) deviated from this additive model (Fig. 4F). There is a drift of points along the diagonal of the morphospace. F2 fish from another anadromous by lake cross showed the same deviation, but not so prominently (Table 3). Because neither parental population has the F2 phenotype, the result strongly indicates that new allelic interactions are expanding the size of the bone, either through interactions between alleles within loci (dominance) or interactions of alleles among loci (epistasis).

We calculated the Castle–Wright estimation of the number of effective genetic loci contributing to the morphological changes along the DV axis of the OP when fish evolve from the anadromous to the lake form (33). The method suggests that, for the Rabbit Slough by Bear Paw Lake crosses, the genetic regulation of ventral lengthening (involving change in size and shape) by a gene set with additive effects. Because neither parental population has the F2 phenotype, the result strongly indicates that new allelic interactions are expanding the size of the bone, either through interactions between alleles within loci (dominance) or interactions of alleles among loci (epistasis).

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The F2 data can also be used to genetically map the quantitative trait loci (QTLs) responsible for the phenotypes. With available genetic markers (24), we found one or possibly two of these QTLs for the Rabbit Slough by Bear Paw Lake cross: A highly significant major-effect QTL (logarithm of odds score >6) explaining >30% of the variance in VPs is present on LG19 (Fig. 5). Another weakly significant QTL explaining variance in JPs may be present on locus group (LG) 3 (data not shown).

These map positions suggest that the genetic basis of OP morphological evolution is different from the genetic basis for the evolution of armor loss; in this same cross, lateral plate and pelvic armor genes map to different linkage groups, LG7 and LG18 (27). Other QTLs must contribute to OP variation between anadromous and lake sticklebacks, as indicated by the Castle–Wright estimate, but our F2 family sizes were not large enough to localize these additional QTLs (34).

**Discussion**

OP evolution from ancestral, anadromous to derived, lake fish occurs as a prominent decrease in the amount of elongation of...
We also detected variation in OP size (but not shape) in different lake populations, possibly because of adaptation to different lacustrine environments that we do not presently understand. Genetic drift is also a plausible hypothesis for these findings. Our results might mean only a few loci evolved in our Bear Paw Lake by anadromous crosses and we locate a QTL on LG19 that explains ~30% of the variance along this DV axis. There may be only few evolving loci; using the Castle–Wright equation to determine effective gene number suggests that as few as five loci explain the variance in DV elongation of the OP. Our crosses also yield evidence for a second genetic system that includes dominant and recessive alleles and regulates OP size without effect on its shape. More studies of the lake fish are required to understand more about this “sizing” system and how it might interact with the DV elongation system.

The DV elongation loci underlie the early developmental change we studied in laboratory-reared fish. Genes functioning along the Endothelin1 signaling pathway are excellent candidates for these loci because they specify early pharyngeal skeletal patterning along the DV axis in zebrafish, including early DV elongation and size of the OP (3, 15); this is the phenotypic change we observe in evolving sticklebacks. It is of major interest to learn whether genes and pathways identified in developmental genetic studies are major players in how morphologies evolve in natural populations. OP evolution in sticklebacks lets us address this issue. The DV elongation loci appear to effect morphology quantitatively, because variance is largely additive in our Bear Paw Lake cichlids. Although standard evolutionary theory predicts that mostly variants of small effect will be used during adaptation (48–50), recent theoretical work shows that, during rapid adaptation to radically new environ-

Fig. 5. An OP morphology major-effect locus maps to LG19. LOD score (blue) and percentage of the variance explained (pink) for the size-standardized VP component of OP variation plotted along the entire length of the linkage group are shown. Significant and highly significant marks were determined numerically by using permutation tests of the data across all linkage groups, as was the 95% confidence interval (yellow line) of the main peak by using bootstrap resampling. Data are from the RSxBP F2 progeny shown in Fig. 4D. The presence of two peaks may mean that two QTLs contributing to OP ventral elongation are present on LG19, but also could be due to incorrect assignment of the stn185–187 marker positions. Further analyses will be required.
ments, natural selection may commonly act to fix alleles with large phenotypic and fitness effects (51–54). Our findings add experimental support for this theory.

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