

Evolutionary diversification of opercle shape in Cook Inlet threespine stickleback

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We investigated the evolution of a large facial bone, the opercle (OP), in lake populations of the threespine stickleback that were founded by anadromous ancestors, in Cook Inlet, Alaska. Recent studies characterized OP variation among marine and lake populations and mapped a quantitative trait locus with a large influence on OP shape. Using populations from diverse environments and independent evolutionary histories, we examined divergence of OP shape from that of the anadromous ancestor. We report preliminary evidence for divergence between benthic and generalist lake ecotypes, necessitating further investigation. Furthermore, rapid divergence of OP shape has occurred in a lake population that was founded by anadromous stickleback in the 1980s, which is consistent with divergence of other phenotypic traits and with OP diversification in other lake populations. By contrast, there has been limited evolution of OP shape in a second lake population that may have experienced a genetic bottleneck early in its history and lacks genetic variation for OP divergence. Taken together, the results obtained from these two populations are consistent with studies of other stickleback phenotypic traits that implicate ancestral variation in postglacial adaptive radiation of threespine stickleback in fresh water. © 2009 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2009, 97, 832–844.

ADDITIONAL KEYWORDS: adaptive radiation – *Gasterosteus aculeatus* – geometric morphometrics – microevolution – parallel evolution – rate of evolution.

INTRODUCTION

Analysis of morphological variation among conspecific populations is a cornerstone of evolutionary research. Morphological diversity within species bears witness to the dynamic nature of species, the power of natural selection, and the consequences of other evolutionary mechanisms. Coupled with information on ecological differences among populations, intraspecific geographical variation can lead to hypotheses for natural selection on variable traits (Endler, 1986). However, there are additional requirements for such analyses. There must be at least two contrasting environment types occupied by phenotypically divergent ecotypes, and there must be several replicates of each ecotype to test for phenotype–habitat associations. In addition, however, there must be a good reason to believe that similar ecotypes have evolved their habitat-specific traits independently of one another to

avoid pseudoreplication (Brooks & McLennan, 1991; Harvey & Pagel, 1991). Once phenotype–habitat associations have been established for populations in which the phenotypes evolved separately, it is possible to test hypotheses for natural selection by several means, including functional morphology of alternative phenotypes (Arnold, 1983) and estimation of fitness components in contrasting natural or experimental environments (Schluter, 1993, 1994, 1995; Gow, Peichel & Taylor, 2007). Finally, it is crucial to estimate the genetic and environmental components of morphological differences to distinguish between phenotypic plasticity and evolved differences.

The threespine stickleback fish, *Gasterosteus aculeatus*, is an excellent subject for this comparative approach. It is primitively a boreal and temperate anadromous (sea-run) or marine (collectively oceanic) fish, but anadromy predisposes it to establish freshwater isolates when conditions are favourable (Bell & Foster, 1994; McPhail, 1994; von Hippel & Weigner,

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2004). Deglaciation of Holarctic coastal lowlands has created an abundant opportunity for colonization of freshwater, and countless freshwater isolates have become established independently by oceanic ancestors. Freshwater threespine stickleback occur in ecologically diverse habitats, and similar habitats occur in widely separated areas. Phylogeographic analyses confirm that morphologically similar freshwater populations have evolved from oceanic ancestors repeatedly (Withler & McPhail, 1985; Orti *et al.*, 1994; Taylor & McPhail, 1999, 2000; Colosimo *et al.*, 2005; but see also Reusch, Wegner & Kalbe, 2001). All the conditions required to use threespine stickleback populations in comparative studies are met.

Hagen & Gilbertson (1972) pioneered interpopulation comparisons to develop hypotheses for selection mechanisms in freshwater threespine stickleback and detected associations of armour and trophic phenotypes with habitat types. Focusing on armour phenotypes, they performed additional studies to test the hypothesis that predatory fishes impose selection on armour phenotypes (Hagen & Gilbertson, 1973a; Moodie, McPhail & Hagen, 1973) and that the phenotypic differences are heritable (Hagen, 1973; Hagen & Gilbertson, 1973b). Subsequent to this pioneering research, numerous interpopulation comparisons of freshwater threespine stickleback have been performed to infer evolutionary mechanisms (Bell & Foster, 1994).

We use interpopulation comparisons to investigate the evolution of opercle (OP) shape, which was described by Kimmel *et al.* (2005, 2008). The OP is a large flat facial bone that supports the gill cover in bony fishes. Although the OP varies substantially among teleosts, little was known about the intraspecific variation of OP shape until the study conducted by Kimmel *et al.* (2005, 2008). These authors demonstrated that it differs between ancestral anadromous and derived freshwater populations, that these differences have a genetic basis, and that a major gene for OP shape is on linkage group 19 of Peichel *et al.* (2001), which includes the sex-determining region (Peichel *et al.*, 2004; Ross & Peichel, 2008). They also showed that there is greater shape variation among freshwater populations than in the anadromous samples available to them, conforming to variation of many other traits in threespine stickleback (Hagen & Gilbertson, 1972; Moodie & Reimchen, 1976; Bell *et al.*, 1993; Walker & Bell, 2000; Spoljaric & Reimchen, 2007). More recently, Kimmel *et al.* (2008) observed that difference in OP shape between anadromous and lake fish can partly be explained by the appearance of a novel allometric trajectory in lake fish.

We used geometric morphometrics to further explore OP shape evolution in freshwater stickleback

from Cook Inlet, Alaska. We confirmed previously obtained results (Kimmel *et al.*, 2005, 2008) indicating major differences between anadromous and most freshwater stickleback for OP shape. The novel aspects of the present study include characterization of shape variation among freshwater populations from multiple habitat types, including deep lakes, shallow lakes, and streams, and preliminary evidence that OP shape variation is associated with these habitat types. We also studied OP evolution in the stickleback population from Loberg Lake, which has evolved from anadromous ancestors since 1983, to gain insight into the rate at which OP shapes typical of resident lake populations evolve. Finally, we provide evidence that the genetic variation responsible for OP shape evolution is contained within the anadromous ancestor and does not depend on new mutations that arise after lake colonization. Rapid divergence and similar outcomes in OP evolution in independently-derived populations follow the evolutionary dynamics of adaptation from pre-existing genetic variation (Barrett & Schluter, 2007). Although the results obtained in the present study suggest strongly that differences in OP shape have functional and adaptive significance, experimental studies will be needed to infer the causes of OP shape diversification.

MATERIAL AND METHODS

SAMPLING AND LOCALITIES

We used field-caught and laboratory-reared *G. aculeatus* from several locations in Cook Inlet, Alaska (Table 1). Most of them came from the Matanuska-Susitna Valley, and a few originate from the Kenai Peninsula, to the south. Many of these populations have been studied previously (von Hippel, 2008). Coordinates for sampling sites were taken from US Geological Survey topographic maps on which the collection localities had been marked in the field. Most localities were selected from approximately 200 that were sampled (Bell & Orti, 1994) to incorporate the range of stickleback habitats that occur in Cook Inlet, including anadromous and resident freshwater populations in small streams, shallow lakes dominated by benthic production ('benthics'), and deeper lakes with both benthic and planktonic production ('generalists'). Specialist planktivore populations, such as those reported from lakes in British Columbia, Canada (Schluter & McPhail, 1992; McPhail, 1994), apparently do not occur in Cook Inlet. Nevertheless, this range of habitat types allowed us to evaluate differentiation between ancestral anadromous and derived freshwater populations and among freshwater populations from contrasting habitats.

Table 1. Samples and sites used in the present study

Site	Latitude (N)	Longitude (W)	Habitat/population type	Sampling year	Sample size
Loberg	61.56	149.258	Lake/introduced	1990	6
				1992	43
				1993	23
				1994	42
				1996	46
				1998	43
				2000	41
				2002	35
				2004	42
Corcoran	61.574	149.688	Lake/benthic	2004	34
Mud	61.563	148.949	Lake/benthic	2005	43
Tern	60.533	149.55	Lake/benthic	2004	24
Beaverhouse	61.574	149.863	Lake/generalist	2004	37
Long	61.578	149.764	Lake/generalist	2005	35
Stormy	60.777	151.047	Lake/generalist	2004	24
Little Meadow Creek			Stream	2005	44
Swanson River	60.745	150.794	Stream	2005	20
Ida	61.75	148.633	Lake	2006	30
Rabbit Slough	61.56	149.049	Anadromous (young-of-the-year)	2006	10
Rabbit Slough	61.56	149.049	Anadromous (adult)	2003	42
Mud	61.563	148.949	Anadromous	2005	18

Young-of-the-year (YOY), field-caught anadromous specimens from Rabbit Slough were included to increase the size range available for our analysis of that population.

We also included samples from two lake populations with contrasting recent histories of phenotypic change to examine evolutionary tempos and the source of genetic variation for OP shape. The Loberg Lake population was sampled annually between 1990 and 2004. It was poisoned by the Alaska Department of Fish and Game in 1982 to exterminate the threespine stickleback and recolonized by anadromous stickleback between 1983 and 1989 (Bell, Aguirre & Buck, 2004; Aguirre, 2007). Contemporary evolution in the direction of typical resident lake populations has been observed in this population for several traits, and the population has become very similar to resident lake populations subsequent to its establishment (Bell *et al.*, 2004; Aguirre, 2007). A second population in Ida Lake was first sampled in 1987 and has not evolved towards phenotypes typical of resident lake populations in the last 20 years (M. A. Bell & W. E. Aguirre, unpubl. data).

Samples were made with six to 15 baited (sharp Cheddar cheese) or unbaited minnow traps (chamber: length 44.5 cm, diameter 22.9 cm diameter; mesh, 0.32 or 0.64 cm) set overnight (for approximately 20 h) on the bottom (< 1 m), within 5 m of shore, usually near rocks, submerged tree branches or aquatic plants (for

a detailed description of the methods, see Bell *et al.*, 1993). Specimens were sacrificed in the field by overdose with MS-222 and fixed in formalin.

Specimens were transferred to 50% isopropyl alcohol and stained in Alizarin Red S to visualize bony structures. Only adult males were used to avoid the potential effects of sexual dimorphism, except for the YOY from Rabbit Slough, which are probably not sexually dimorphic (Kitano, Mori & Peichel, 2007). Sexual dimorphism in the size of the head has been detected in Cook Inlet stickleback (Aguirre *et al.*, 2008), and OP shape maps to the sex chromosome of Peichel *et al.* (2004) and both OP shape and position have been observed to be strongly sexually dimorphic (Albert *et al.*, 2008). Thus, the results obtained using female specimens incorporate an added source of variation. We also excluded some specimens that were distended from infection by a tapeworm (*Schistocephalus solidus*) because OP shape might be affected.

MORPHOMETRICS

We measured OP shape for all specimens using landmark-based geometric morphometrics (Bookstein, 1991). These methods generate shape variables from the x , y coordinates of biologically homologous points after removing the effects of differences in translation, rotation, and scale. Once the shape variables are obtained, standard multivariate techniques

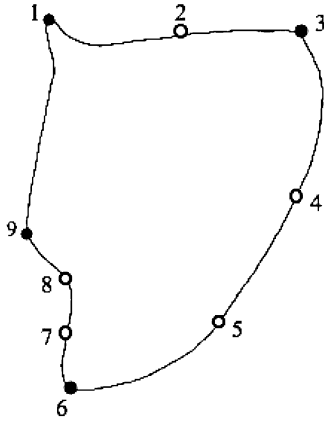


Figure 1. Lateral view of an opercle outline showing the fixed landmarks (●) and sliding semi-landmarks (○) used in the present study.

can be used for exploratory analysis and hypothesis testing. We obtained digital images of the left lateral side of each OP using a Canon PowerShot S45 digital camera mounted on a Leica S6D stereomicroscope. We digitized the outline of each OP with nine landmarks (Fig. 1) using tpsDIG software (Rohlf, 2006). Landmarks 2, 4, 5, 7, and 8 were treated as sliding semi-landmarks (Bookstein, 1997). When possible, sample sizes were at least three to four times the number of landmarks used (i.e. nine) (Bookstein, 1996), but some samples were too small to meet this requirement.

After digitization, a generalized procrustes analysis (GPA; Rohlf & Slice, 1990) was performed on the landmarks, allowing the semi-landmarks to slide along a curve fit to the landmarks. GPA superimposes specimens to a common coordinate system after accounting for differences in position, orientation, and size. 'Sliding' is achieved by a weighted regression, and it improves the fit of a semi-landmark to the line connecting adjacent landmarks by minimizing the difference between corresponding landmarks (for details, see Bookstein, 1997). Initial GPA and 'sliding' were conducted with tpsRELW (Rohlf, 2007). The alignments with the slid semi-landmarks were saved and used to generate 14 shape variables (partial warp scores and uniform component), after a second round of GPA, for the ensuing analysis.

STATISTICAL ANALYSIS

To examine the shape space of the OP and reduce dimensionality of the data, we carried out a relative warp analysis using tpsRELW (Rohlf, 2007), which is essentially a principal component analysis of landmark coordinates after GPA (Bookstein, 1991). We performed the relative warp analysis on the

entire dataset of aligned landmark coordinates. Shape change among populations was visualized using thin-plate spline representations (deformation grids) generated in tpsSPLIN (Rohlf, 2004). These deformation grids represent deviations from a consensus configuration of Rabbit Slough fish, which we treat as the ancestral OP shape. A discriminant function analysis (DFA) was carried out to further evaluate the pattern of differentiation among freshwater ecotypes using the leave-one-out classification option applied to all fourteen shape variables in SPSS, version 11.0. We constructed deformation grids to visualize differences among ecotypes by regressing the first canonical variate on shape using tpsREGR (Rohlf, 2005).

The Ida Lake population differs conspicuously from other resident lake populations and closely resembles anadromous populations for armour structure and body shape, but not in size. We performed a permutation procedure to test whether the Ida OP shape is significantly more similar to anadromous YOY than to anadromous adults. We calculated Euclidean distances between group means of Ida and YOY or adult anadromous samples and compared the observed divergence (D_{obs}) between these two distances with 4999 permutations in which individuals were randomly assigned to samples (D_{rand}). The covariation between samples generated by this procedure provides a test of significance.

We performed a multivariate analysis of covariance (MANCOVA) among groups for shape using the natural log of centroid size (CS) as a covariate to determine whether the allometric component of shape change varies among groups. This test was performed using tpsREGR (Rohlf, 2005), which infers significance using a permutation procedure and is robust to deviations from normality. CS is a measure of scale that is uncorrelated with shape in the absence of allometry (Bookstein, 1991). A previous study (Kimmel *et al.*, 2005) revealed a robust linear relationship between CS and standard length.

We used regression to infer whether OP shape has changed through time in the Loberg Lake population from that of Rabbit Slough (anadromous) subsequent to the lake being colonized by anadromous stickleback between 1983 and 1989 (Bell *et al.*, 2004). First, Euclidean distances between the mean shape of each annual sample from Loberg Lake to that of the 2003 Rabbit Slough sample were regressed on year. Because Euclidean distances do not indicate direction of divergence, we conducted a DFA to assess whether the frequency of anadromous OP morphs decreased over time in the Loberg Lake population. A DFA is robust to moderate violations from normality if the violation is caused by skewness rather than outliers (Tabachnick & Fidell,

1996). We screened for outliers by examining relative warp plots, and cases of obvious outliers were observed only in the two anadromous samples (Rabbit Slough and Mud Lake). The discriminant function was formulated using SPSS, version 11.0, on a training set of shape variables from Rabbit Slough and all lake samples except for the Loberg, Ida, and Rabbit Slough YOY samples. The discriminant function was applied to all Loberg sample years to determine the frequency of anadromous and lake OP morphs during the time series. Only one of 42 anadromous specimens and one of 197 lake fish were misclassified in the training set.

We used the method of Hendry & Kinnison (1999) to estimate haldanes from a time series to compare the rate of evolution in Loberg Lake with other evolutionary rates from natural populations. We used relative warp 1 (RW1) as a multivariate descriptor of OP shape. We regressed $RW1/s_p$ (where s_p is the pooled standard deviation) against the number of generations for seven generations [i.e. 1990–2004; 2 years per generation (Havens *et al.*, 1984)] to calculate the average evolutionary rate in haldanes.

RESULTS

DIVERGENCE OF OPERCULUM SHAPE BETWEEN ANADROMOUS AND LACUSTRINE POPULATIONS

RW1 and 2 explain 45.38% and 19.36% of the OP shape variation, respectively. RW1 largely differentiates lake and anadromous OP shape, and the second relative warp (RW2) distinguishes benthic and generalist lake populations and adult versus YOY anadromous stickleback (Fig. 2). The major difference in OP shape between anadromous and lake populations along RW1 involves: (1) expansion along the antero-posterior (AP) axis and (2) drastic contraction along the dorsoventral axis (between landmarks 1 and 3–6; top and bottom left deformations grids). This divergence in OP shape is consistent with that observed by Kimmel *et al.* (2005).

There also appears to be considerable differentiation between benthic and generalist lake OPs. The DFA carried out to explore this differentiation further separated samples largely along the first axis (Fig. 3), but there is considerable overlap particularly between the trophically contrasting Beaverhouse (generalist) and Mud (benthic) lake

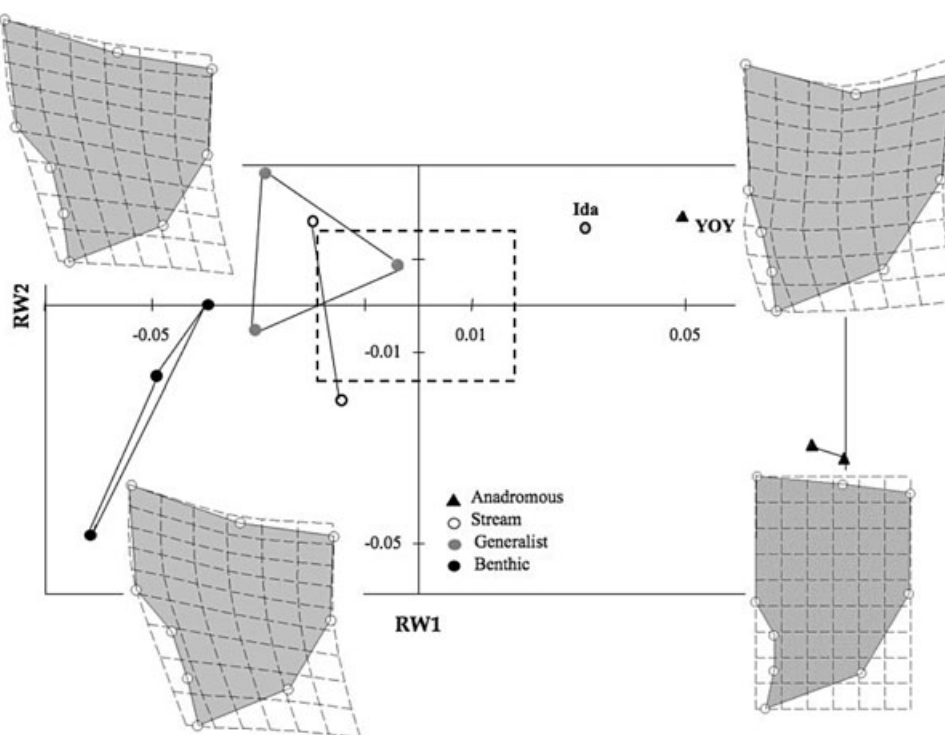


Figure 2. Bivariate shape space for the opercle. The two relative warps (RW) explain approximately 65% of the variation. Deformation grids represent deviations from the consensus of anadromous specimens, which is the putative ancestral shape for derived freshwater fish. Deformations grids are exaggerated two-fold to illustrate shape change. The large rectangle with dashed lines corresponds to the shape space depicted in Fig. 5A for the Loberg Lake time series. YOY, young-of-the-year.

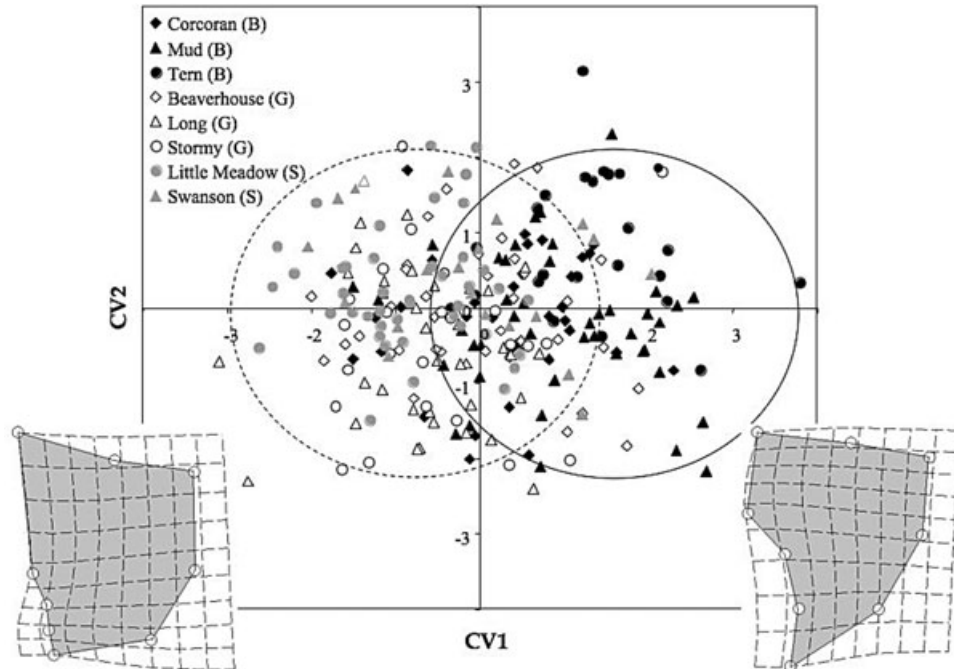


Figure 3. Discriminant functional analysis of shape variables based on the habitat type from which specimens came, including: benthic (B), generalist (G), and stream (S) fish. Canonical variate 1 (CV1) explains 84% of the variation, whereas CV2 accounts for 15%. Only 60% of the original grouped cases were classified correctly with the discriminant functions. The solid circle represents the spread of generalist specimens, whereas the dashed circle represents that of benthic specimens. Deformation grids are exaggerated two-fold to highlight differences between either end of the CV1 axes.

populations. Approximately 60% of grouped (benthic versus generalist) cases were classified correctly using both functions (test of functions 1 and 2: Wilks' $\lambda = 0.643$, $\chi^2 = 110.606$, d.f. = 28, $P < 0.0001$; test of function 2: Wilks' $\lambda = 0.925$, $\chi^2 = 19.536$, d.f. = 13, $P = 0.107$). The exaggerated deformation grids in Figure 3 suggest that the major difference between the OPs from the two lake habitat types is the relative length of the ventral apex (between landmarks 1–3 and landmark 6). Benthics are more like anadromous stickleback in this respect than generalists (Fig. 2, along the RW2 axes), and stream OP shape appears to resemble that of generalist lake forms (Figs 2, 3).

Individual variation appears to be fairly homogeneous within the anadromous populations (Fig. 4), but there are three outliers from the anadromous populations (one in Rabbit Slough, two in Mud Lake) with OP shape very similar to lake shape.

Finally, the results of the MANCOVA (Wilk's $\lambda = 0.19$, $P < 0.0001$) confirm that allometric trajectories of OP growth differ among groups. This pattern was described by Kimmel *et al.* (2008) and is comprehensively investigated in that study.

LIMITED OPERCULUM SHAPE EVOLUTION IN IDA LAKE

The Ida Lake sample resembles anadromous populations for armour structure and body shape (M. A. Bell & W. E. Aguirre, unpubl. data) and clusters closer to anadromous than to freshwater fish along RW1 (Fig. 2). It appears to be closer to anadromous YOY than to anadromous adults (Figs 2, 5). Using the permutation procedure, results confirm that Ida operculum shape is more like that of anadromous YOY than that of adults ($D_{obs} = 0.028$, $P_{rand} = 0.0002$).

DIVERGENT EVOLUTION IN LOBERG LAKE

Figure 6A shows a close-up of the shape space occupied by the Loberg Lake time series (Fig. 2, rectangle box). Annual samples move gradually away from the anadromous OP shape and toward lacustrine OP shape along both RW axes. The vector plots (Fig. 6A) illustrate relative landmark displacements in Loberg samples from ancestral anadromous shape over the years. Regression of the Euclidean distance between Loberg samples and the Rabbit Slough anadromous sample on year exhibits a significant linear trend (Fig. 6B, $r^2 = 0.87$, $P = 0.00024$).

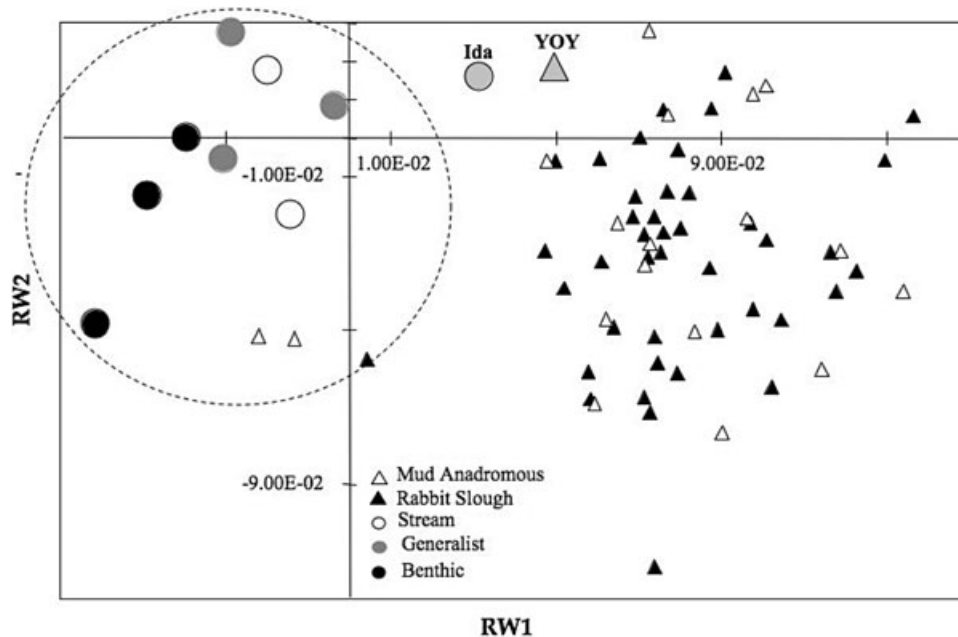


Figure 4. Dispersion of individuals in the two anadromous samples, Rabbit Slough and Mud Lake. The space is essentially the same as shown in Fig. 2. Larger symbols designate centroids of freshwater groups, whereas anadromous specimens are plotted individually. The large dashed circle groups lake populations (with the exception of Ida Lake). Figure 2 is a subspace of the shape space in Fig. 4. RW, relative warp; YOY, young-of-the-year.

Table 2. Classification results from discriminant function analysis of shape variables based on a training set comprising Rabbit Slough (2003) and a subset of lake samples

Year	Percent classified as anadromous operculum morph	Sample size
1990	16.67	6
1992	11.63	43
1993	4.34	23
1994	14.39	42
1996	13.04	46
1998	2.32	43
2000	4.88	41
2002	2.85	35
2004	4.44	42

By the time the first samples were made, the frequency of anadromous OP phenotypes was only approximately 12–16% (Table 2). The DFA indicates that the frequency of anadromous OP shapes decreased from approximately 17% to 4% between 1990 and 2004 (Wilk's $\lambda = 0.263$, $\chi^2 = 307.281$, d.f. = 14, $P < 0.0001$). Finally, the average rate of phenotypic evolution in OP shape was -0.122 haldanes and differed significantly from zero ($P = 0.002$, 95% confidence interval = -0.059 – -0.185 haldanes).

DISCUSSION

The threespine stickleback exhibits striking phenotypic variation among populations in relation to environmental factors (Bell, 1976, 1984; Bell & Foster, 1994; Reimchen, 1994). Several phenotypic traits usually distinguish oceanic and freshwater populations. Although oceanic populations appear to be relatively homogeneous (Bell & Foster, 1994; Walker & Bell, 2000; but see also Klepaker, 1996), freshwater populations vary conspicuously for several traits in relation to predation regime and diet (Bell & Foster, 1994). Recognition of associations between phenotypes and environmental factors has led to successful field and laboratory experiments to test hypotheses for selection mechanisms (Hagen & Gilbertson, 1973a; Schluter, 1993, 1995). The key factor for predation regime is presence or absence of predatory fishes (Hagen & Gilbertson, 1972; Reimchen, 1994; Walker, 1997), and the proportion of benthic prey and plankton in the diet is the major factor for trophic morphology and body shape (Schluter & McPhail, 1992; Schluter, 1993, 1994, 1995; McPhail, 1994; Walker, 1997).

Bell & Foster (1994) argued that lake stickleback populations fall along continua for predation regime and diet, and that these two factors directly or indirectly influence a wide range of behavioural and morphological traits that should covary with environment

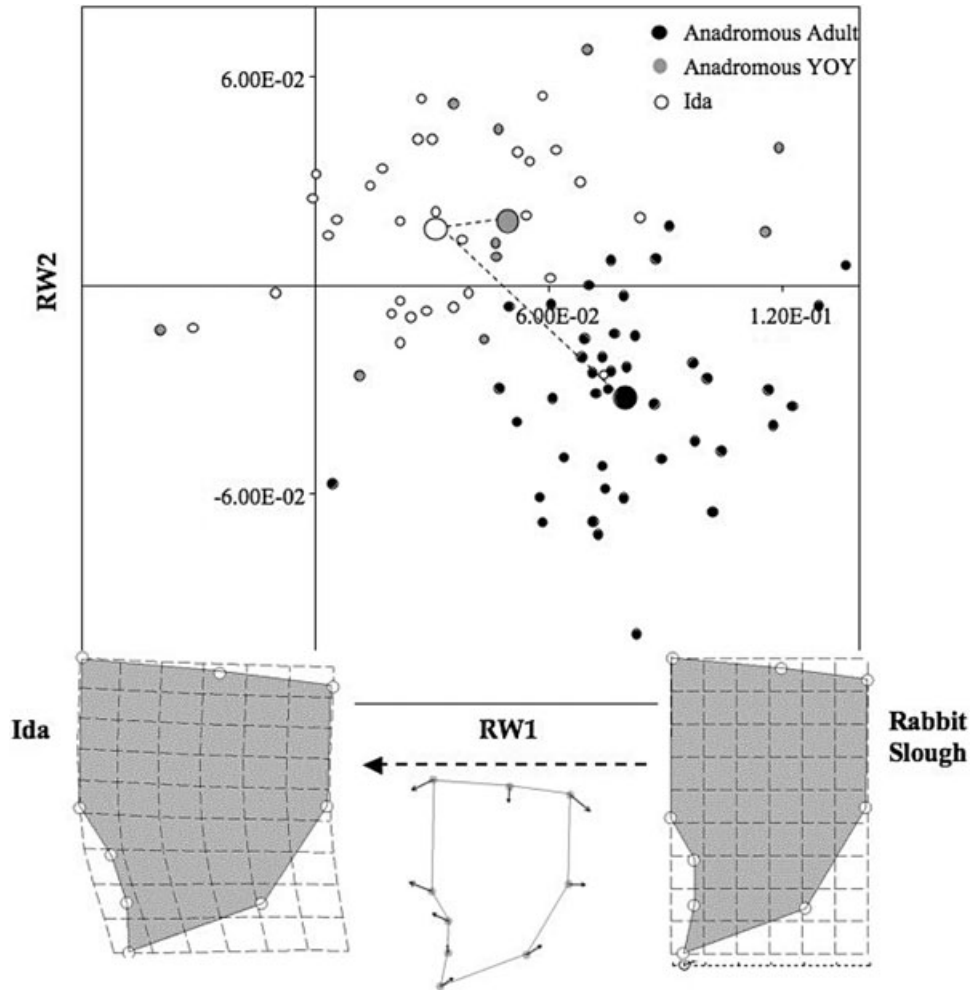


Figure 5. Plot of the Ida Lake and Rabbit Slough adults and young-of-the-year (YOY) samples in shape space. The three larger circles represent group means (centroids). Dashed lines among centroids represent distances between the means used to calculate D_{obs} (for details, see text). The vector plot illustrates landmark displacements in the Ida deformation grid relative to that of the adult anadromous Rabbit Slough stickleback. RW, relative warp.

and one another. They did not include OP shape among the traits that differ between benthic and limnetic lake populations because it was not reported to vary until 2005. Although the functional and fitness consequences of OP shape differences are not understood, Kimmel *et al.* (2005) discovered a characteristic shape difference between oceanic and freshwater populations and variation among freshwater populations. A strong association between OP shape and habitat type indicates a functional difference among OP shape phenotypes and implicates the action of natural selection (Endler, 1986).

Before proceeding, we must justify the treatment of the samples that we compare as phylogenetically and, hence, statistically independent observations (Brooks & McLennan, 1991; Harvey & Pagel, 1991). Although the Corcoran, Beaverhouse, Long, and Little Meadow

Creek populations are all from the Fish Creek drainage, we used divergent freshwater populations from six separate drainages in the Matanuska-Susitna Valley (Loberg, Mud, three Fish Creek drainage populations) or the Kenai Peninsula (Tern, Stormy, Swanson River). Although two populations from relatively deep lakes (Beaverhouse, Long), where plankton from the limnetic zone should be a major component of the diet, are in the same drainage, all other populations from similar habitats are from separate drainages. The Matanuska-Susitna Valley was glaciated or submerged beneath Cook Inlet waters until isostatic rebound approximately 14 000 years ago and the Kenai Peninsula was glaciated until approximately 18 000 to 15 000 years ago (Reger & Pinney, 1996), and so there has been limited time for dispersal among drainages. Aguirre's (2007) phy-

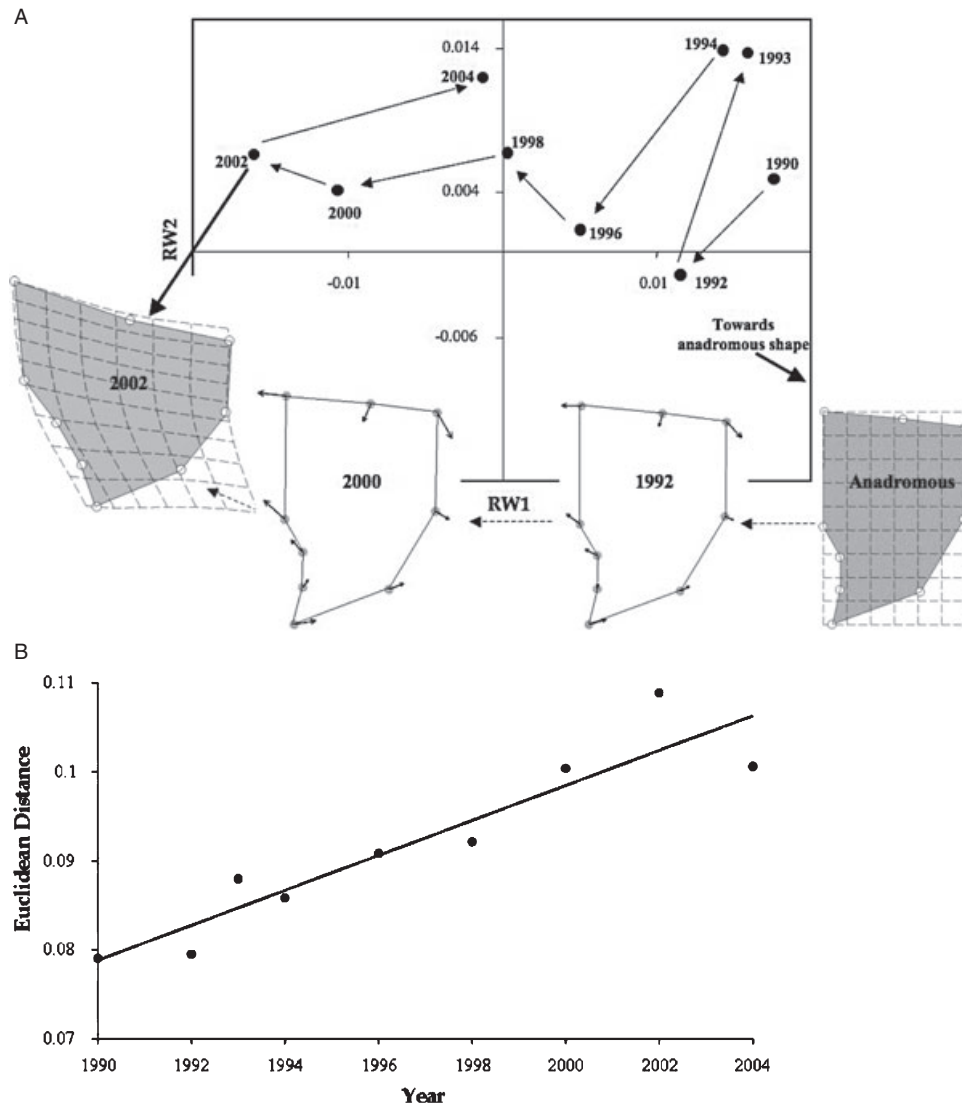


Figure 6. Divergence of opercle shape in the Loberg Lake stickleback. (A), close up of OP shape space occupied by the Loberg Lake time series from the rectangular box in Fig. 2. The deformation grid and vector plots are deviations from Rabbit Slough OP shape. The year represented by each deformation grid or vector plot is indicated in the diagram, and the right-hand plot represents Rabbit Slough average shape. (B), plot of Euclidean distances (between means of shape variables) between annual Loberg Lake samples and the 2003 Rabbit Slough sample. RW, relative warp.

logeographic analysis of lake populations within the Fish Creek drainage, which included samples from Long and Corcoran lakes and Little Meadow Creek, indicated that body form is largely independent of microsatellite (i.e. quasi-neutral genetic) similarity and spatial proximity of populations. If OP shape is as free as body form to evolve independently in adjacent populations, which is suggested by its rapid evolution in the Loberg Lake population, it is reasonable to treat populations from different regions, drainages within the each region, and probably even those within the Fish Creek drainage, as statistically independent cases.

Our comparison of OP shape among populations confirms previously obtained results (Kimmel *et al.*, 2005, 2008) indicating that the oceanic and freshwater populations differ strongly for OP shape (Fig. 2) and provides evidence that variation among freshwater populations is associated with ecological differences (Fig. 3). The OP of freshwater populations is generally expanded along the longitudinal axis (RW1) compared to two anadromous populations. In freshwater, the centroids for OP shape of more benthic populations are separated in the OP shape space from those of stream and generalist populations. Benthic populations exhibit an OP shape with a more pro-

nounced and elongated curvature between the ventral apex (landmark 6) and landmark 9 relative to generalist and stream populations. However, there is a large overlap among some populations and additional populations from these habitat types need to be characterized to determine how significant our findings are.

The Loberg Lake population was founded by anadromous fish between 1983 and 1989, soon after the resident population was eradicated in 1982. The lake was first sampled in 1990 and, subsequently, several traits have diverged (Bell *et al.*, 2004; Aguirre, 2007). OP shape also evolved dramatically in this population within approximately seven generations. The first sample from this population in 1990 was almost monomorphic (96%; Bell *et al.*, 2004) for the ancestral complete lateral plate morph, but only one of six (17%) specimens in that sample had the ancestral OP shape (Table 2). The vast majority of anadromous specimens have the ancestral OP shape (Fig. 4) and, if this was true of the population that founded the Loberg Lake population, OP shape must have evolved much more rapidly than lateral plate morph to reach such a low value by 1990. By 2004, complete morph frequency had declined to 6.5% (Aguirre, 2007), which is comparable to the frequency of anadromous OP morphology (i.e. 4.4%).

Hendry & Kinnison (1999) compiled evolutionary rates in haldanes, and the rate of OP evolution in Loberg Lake (-0.122 haldanes) would be among the highest 22% of the rates that they reported. However, several of their cases that exceeded 0.18 haldanes were from studies based on fewer than three generations. Thus, because evolutionary rates tend to be inversely related to the period over which they were estimated (Gingerich, 1983; Hendry & Kinnison, 1999), and OP shape evolution in Loberg Lake was measured over seven generations during which it was irregular (Fig. 6A), OP evolution rate in Loberg Lake stickleback may actually be more extreme than it appears to be.

Although mean OP shape evolution formed a nearly linear trend ($r^2 = 0.87$; Fig. 6B) between 1990 and 2004, the relative frequencies of specimens assigned by DFA to the anadromous and freshwater OP shape classes was approximately 12% between 1992 and 1996, and declined to less than one-third of that value starting in 1998 (Table 2). Kimmel *et al.* (2005) detected a quantitative trait locus (QTL) on linkage group 19 for a similar measure of operculum shape that accounted for Fig. 6B, 30% of the total variation in their genetic cross, indicating the importance of several other loci, including a weakly significant QTL on linkage group 3. Incongruity of temporal pattern of change for mean OP shape and the proportion specimens with the ancestral OP shape class within the

same set of samples suggests that the smooth divergence of mean OP shape resulted from expression of the major locus discovered by Kimmel *et al.* (2005) plus other minor loci. This is the genetic architecture proposed by Orr (2003) for the evolution of adaptive traits and is similar to that for lateral plate number (Colosimo *et al.*, 2004; Cresko *et al.*, 2004) and extreme pelvic reduction (Shapiro *et al.*, 2004) in threespine stickleback.

By contrast to the dramatic evolution of the Loberg Lake population, the Ida Lake population apparently has not evolved during the same period. This sample was included in the present study because it appeared to resemble anadromous populations for armour and body shape (M. A. Bell & W. E. Aguirre, unpubl. data). Although there is substantial dispersion among the centroids for OP shape in the freshwater samples, the Ida Lake sample is the only one with a positive value along RW1, and it is much closer to the anadromous samples in the OP shape space than any other freshwater sample. It is possible that the Ida Lake population resembles anadromous stickleback for OP shape because selection in this lake favours this and other ancestral phenotypes, or that selection favours the usual array of freshwater phenotypes but the population lacks the genetic variation needed to respond to selection.

Ida Lake and Fish Lake, immediate upstream, differ from other Cook Inlet lakes in that they are inhabited by kokanee, resident freshwater sockeye salmon (*Oncorhynchus nerka*). Selection imposed by kokanee or by the conditions that permit kokanee to inhabit these lakes may also favour the anadromous-like phenotypes of this stickleback population. However, this explanation is unlikely because the primary condition for evolution of kokanee is presence of streams tributary to landlocked lakes, and this should not directly affect selection on stickleback. Predation is the most likely selective effect of kokanee on stickleback evolution, but most lakes in the area, including Loberg Lake, contain other *Oncorhynchus* species (e.g. *Oncorhynchus mykiss*) that prey on threespine stickleback, and these stickleback populations do not exhibit anadromous phenotypes. Although we cannot entirely eliminate the hypothesis that selection has favoured retention of anadromous-like traits in the Ida Lake population, it appears to be implausible.

Limited genetic variation due to genetic drift is a more likely explanation for phenotypic similarity of the Ida Lake population to anadromous stickleback. Ancestral genetic variation has played a major role in divergence of lateral plate (Colosimo *et al.*, 2005) and pigmentation (Miller *et al.*, 2007) phenotypes in freshwater *G. aculeatus*, and loss of ancestral variation has limited divergence in other species (Brad-

shaw, 1991; Blows & Hoffmann, 2005). The Ida Lake population has less genetic diversity at three microsatellite loci than 23 other nearby threespine stickleback populations (Aguirre, 2007; M. A. Bell & W. E. Aguirre, unpubl. data). Low microsatellite diversity and retention of several ancestral phenotypes that typically evolve in freshwater *G. aculeatus* suggest that limited genetic variation for the OP shape has constrained its evolution in the Ida Lake population.

Rapid evolution of OP shape in the Loberg Lake stickleback population, which contains ample microsatellite diversity (Aguirre, 2007), and lack of divergence in the apparently bottlenecked Ida Lake population is consistent with results implicating rare ancestral alleles in the adaptation of anadromous stickleback when they colonize fresh water (*Eda*: Colosimo *et al.*, 2005; *Kit ligand*: Miller *et al.*, 2007). Thus, we expect that 'freshwater' alleles at the OP-shape locus on linkage group 19 (Kimmel *et al.*, 2005) to be present at low frequencies in anadromous populations. The presence of rare outliers with OP shape resembling that of freshwater specimens in our samples from anadromous populations from Mud Lake and Rabbit Slough (Fig. 4) suggests that there is genetic variation for OP shape in anadromous stickleback. Frequent independent evolution of a similar divergent OP shape in freshwater *G. aculeatus* and rapid evolution of OP shape in Loberg Lake suggest that ancestral genetic variation for OP shape in anadromous ancestors is important for this trait as well.

The rate of OP shape evolution in the Loberg Lake population and repeated divergence between anadromous and freshwater populations strongly indicate strong diversifying selection on OP shape in different habitats. However, there is no information concerning the functional effects of OP-shape variation to account for the phenotype–environment associations or rapid evolution we have observed (P. Wainwright, pers. comm.). There are characteristic differences in the size and number of gill rakers and skull and jaw morphology among threespine stickleback from different habitats (Bell & Foster, 1994; McPhail, 1994), which may account for variation in operculum shape. Associations between OP shape and habitat type also suggest a direct effect of oxygen concentration, salinity, and defence against parasites that utilize the gill chamber. However, there is no evidence for such causes. The OP clearly plays a role in propulsion of water through the gill chamber for respiration and suction feeding, and some studies have implicated the OP in abduction of the lower jaw (Anker, 1974, 1978; but see also Westneat, 1990). However, it is not clear how the shape differences between anadromous and freshwater populations or among populations in different freshwater populations could result in strong

enough selection to account for the rapid evolution observed in the Loberg Lake population or consistent divergence among young Cook Inlet populations. This result emphasizes the need for an analysis of OP function.

The ease with which OP shape variation among stickleback populations could be detected demonstrates the feasibility of characterizing this trait using geometric morphometrics (Rohlf & Marcus, 1993; Adams, Rohlf & Slice, 2004). The great ecological breadth of teleost fishes and the ease with which this trait can now be measured create the possibility for comparative studies that exploit teleost ecological diversity to yield clearer insights into the functional significance of OP-shape variation. Once the ecological significance of OP shape is understood in extant species, it may become possible to use it to infer aspects of the ecology of fossil fishes.

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