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Frequency of *Ectodysplasin* alleles and limited introgression between sympatric threespine stickleback populations

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Abstract The threespine stickleback (*Gasterosteus aculeatus*) is primitively an anadromous or resident marine species but has repeatedly colonized fresh water, where predictable phenotypic divergence usually occurs rapidly. A conspicuous element of this divergence is change of the number and position of lateral armor plates from about 33 that cover the entire flank (complete) to <10 anterior plates (low). This difference is caused primarily by variation at the *Ectodysplasin* (*Eda*) locus. The low *Eda* allele appears to be rarer in two geographically adjacent anadromous populations from Cook Inlet, Alaska than in most marine or anadromous populations reported from elsewhere, and there is no evidence of elevated gene flow for *Eda* between anadromous and resident lake threespine stickleback populations that breed in sympatry. However, the two anadromous populations are divergent for the frequencies of two complete *Eda* alleles. It is not clear how monomorphic low-plated freshwater populations in Cook Inlet have almost invariably acquired ancestral low *Eda* alleles from anadromous ancestors in which this allele appears to be extremely rare.

Keywords Anadromy · Eda · *Gasterosteus aculeatus* · Hybridization · Lateral plate morph · Reproductive isolation · Speciation

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Introduction

The biological species concept has played a central role in evolutionary biology since Mayr (1942) articulated it. It provides the link between evolutionary mechanisms and typological species, but it has been applied to only a small minority of species. Beginning with Hagen's (1967) and McPhail (1969) classic studies, the biological species concept has been applied explicitly to several sympatric, reproductively isolated, pairs of populations within the nominal species *Gasterosteus aculeatus* (McPhail 1994; McKinnon and Rundle 2002; Boughman 2007). *G. aculeatus* is a taxon that comprises numerous geographically localized and phenotypical-

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ly divergent biological species that can be used to study speciation, isolating mechanisms, and gene flow between closely related species.

Gasterosteus aculeatus is a small Holarctic fish. It is primitively anadromous or marine (collectively oceanic) but has repeatedly colonized fresh water, to which it adapts rapidly (Klepaker 1993; Bell 1995, 2001; Bell et al. 2004; Vamosi 2006). Anadromous and resident freshwater populations often breed in sympatry and act as separate biological species (McPhail 1994; McKinnon and Rundle 2002; Boughman 2007) but may also engage in reciprocal introgression (Jones et al. 2006). Most anadromous stickleback populations are monomorphic for the complete lateral plate morph (Fig. 1a), with a modal count of 33 plates per side (Hagen 1967; Klepaker 1996; Aguirre et al. 2008), and resident freshwater stickleback are frequently monomorphic for the low morph (Fig. 1b), which has <10 plates per side, restricted to the anterior third of the body (Bell 1984; Bell and Foster 1994).

About 80% of the difference in plate number between low and complete morphs is caused by the *Ectodysplasin* (*Eda*) gene (Colosimo et al. 2004). Low *Eda* alleles (Eda^L) in all but one of 15 freshwater populations reported by Colosimo et al. (2005) formed a single clade that originated from a complete allele (Eda^C) in a single mutational event and spread throughout the Holarctic (see also Cano et al. 2006). This gene tree implies that ancestral Eda^L allels have been carried from drainage to drainage by anadromous stickleback as they colonized fresh water, and they have been reported at low frequencies in several monomorphic completely plated oceanic stickleback populations (Table 1). Analyses by Colosimo et al. (2005) using the *Stn 380* marker sequence within intron 2 of *Eda* revealed existence of two Eda^L alleles that produce 172 and 189 base pair (bp) fragments. Similarly, PCR amplification of *Stn 381* from intron 6 of *Eda* yielded 165 and 175-bp products for Eda^C alleles. While differences of the phenotypic and fitness effects of the Eda^C and Eda^L alleles have been studied extensively, variation within each of these dimorphic allelic classes has been ignored.

Eda^C is usually largely dominant over Eda^L in F1 crosses between low-morph resident and complete-morph anadromous stickleback (Bańbura and Bakker 1995; Cresko et al. 2004; but see Hagen 1967), and Eda^L alleles in oceanic populations are usually heterozygous Eda^L/Eda^C genotypes that express the complete morph (Colosimo et al. 2005; but see Barrett et al. 2008). Although resident freshwater and anadromous populations often act as separate biological species in sympatry, hybridization may occur (Hagen 1967; Jones et al. 2006). Thus, reciprocal introgression of Eda^L and Eda^C alleles is possible and may be the source Eda^L alleles that form the basis for evolution of low plate morphs when new freshwater populations are founded by oceanic ancestors (Colosimo et al. 2005).

Apparently monomorphic low-plated resident freshwater stickleback and monomorphically completely plated anadromous stickleback breed sympatrically within Mud Lake, in Cook Inlet, Alaska (Karve et al. 2007), creating the potential for introgressive hybridization. Since the Eda^C allele is dominant (i.e., complete plate morph; Colosimo et al. 2004; Cresko et al. 2004) or codominant (i.e., partial plate morph with intermediate numbers of plates;

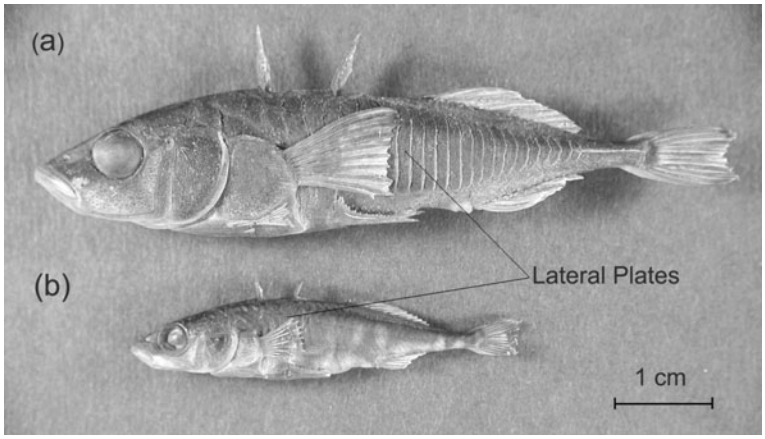

Fig. 1 Completely plated anadromous (**a**) and low-plated resident (**b**) sticklebacks from Mud Lake. From Karve et al. (2007)

Table 1 Frequencies of low *Ectodysplasin* (Eda^L) alleles from marine and anadromous populations of *Gasterosteus aculeatus*. Allelic frequencies are percentages, n is the number of alleles scored, and provinces in Canada and states in the USA are indicated by acronyms (BC, British Columbia; CA, California; WA, Washington). The frequency of Eda^L in the Oyster Lagoon sample is a minimal and approximate estimate because only partial morphs were genotyped, and some complete morphs could have been Eda^L/Eda^C heterozygotes

Eda^L	N	Location	Population type	Publication
19.2	26	Quilcene, WA	anadromous	Kitano et al. (2008)
15.8	38	Clam Bay, WA	marine	Kitano et al. (2008)
11.9	84	Seabeck, WA	anadromous	Kitano et al. (2008)
3.7	218	Navarro R, CA	anadromous	Colosimo et al. (2005)
0.26	~70,000	Oyster Lagoon, BC	marine	Barrett et al. (2008)
0.2	604	Little Campbell R, BC	anadromous	Colosimo et al. (2005)
0.064	1554	Rabbit Slough, AK	anadromous	this study
0.0	48	Duwamish, WA	anadromous	Kitano et al. (2008)
0.000	1216	Mud L., AK	anadromous	this study

Cano et al. 2006; Barrett et al. 2008), its presence should be recognizable phenotypically in heterozygotes in the generally low-plated resident freshwater population. However, Karve et al. (2007) failed to observe any complete morphs among resident freshwater stickleback in Mud Lake (the sample size was not specified). In this study, we attempt to estimate the frequency of Eda^L alleles in a relatively large sample of anadromous stickleback and of Eda^C alleles in a relatively large sample of freshwater stickleback from Mud Lake. We also estimate the frequency of Eda^L alleles in a nearby anadromous population from Rabbit Slough (also in Cook Inlet), where resident stickleback are rare, to determine whether introgression of *Eda* alleles is promoted by sympatry. Based on previous studies (Table 1), we expected to observe low frequencies (~1%) of Eda^L alleles in both anadromous populations, but higher frequencies in the anadromous Mud Lake population. At low frequencies the vast majority of Eda^L alleles in anadromous populations are likely to be in Eda^L/Eda^C heterozygotes and will probably not be expressed. However, they can be detected easily using the *Stn 381* genetic marker, which differs between Eda^L and Eda^C alleles world-wide by an indel (i.e., insertion or deletion) 20–30 bp long (Colosimo et al. 2005). Rare Eda^C alleles in the generally low-plated resident Mud Lake population should nearly all be Eda^L/Eda^C heterozygotes, which would be recognizable as complete or partial morphs. Although they should have reduced fitness in freshwater (Reimchen 2000; Marchinko and Schluter 2007; Barrett et al. 2009a; Marchinko 2009), they might still be present among adult and larger juvenile fish, in which they can be reliably scored (Bell 1981).

Materials and methods

Threespine sticklebacks were sampled from two sites in the Matanuska-Susitna Borough, in Cook Inlet, Alaska, USA. Resident freshwater and anadromous specimens were sampled in June 2007 along about 50 m of shoreline on the north shore of Mud Lake (61.056 N, 148.949 W). Rabbit Slough (61.534 N, 149.268 W) is a small drainage that discharges into Knik Arm of Cook Inlet and is located about 16 km west of Mud Lake. Numerous anadromous stickleback were collected there during May and June 2006 for a large series of genetic crosses; resident stickleback were very rare (~0.1%) in Rabbit Slough.

Specimens were captured using unbaited minnow traps (chamber 44.45 cm long, 22.86 cm diameter; openings 2 cm; mesh 0.32 or 0.64 cm) set overnight (about 20 h). In Mud Lake, they were set 10 m to 20 m offshore in <30 cm depth on a shallow sloping, muddy bottom. Traps were set to span the bottom of the culvert through which Rabbit Slough flows under Glenn Highway to capture anadromous stickleback as they migrated upstream to breed. Trapped fish from both sites were sacrificed with an overdose of MS-222 anesthetic.

Resident *G. aculeatus* were distinguished from anadromous specimens in both sites by size, body shape, the shape, and relative size, and position of the pectoral fin, and the size of the fin spines and pelvic girdle (Fig. 1). Compared to anadromous specimens, resident stickleback are usually smaller (<55 mm SL [standard length]; distance from the tip of the premaxilla to the end of the vertebral column) and less deep bodied, and have smaller, more rounded pectoral fins that are closer to the head, and have shorter fin spines and a pelvic girdle (McPhail 1994; Karve et al. 2007; Aguirre et al. 2008; authors' unpubl. obs.). Although some aspects of body shape map to the region of the *Eda* locus, this factor explains only a small percentage of total shape difference between freshwater and anadromous stickleback (Albert et al. 2008). Complete morph stickleback in resident freshwater populations that are polymorphic for lateral plate phenotypes, including Loberg (Bell et al. 2004), Tommelson, and High Ridge lakes, nearby (Aguirre and Bell, unpubl. data) differ substantially from anadromous stickleback for the traits mentioned above and can readily be distinguished on this basis (Aguirre 2007). Thus, body form appears to be free to vary independently of plate morph. Resident stickleback from Mud Lake were fixed in a 10% buffered formalin solution for a month, soaked in water overnight, and transferred to 50% isopropyl alcohol. They were subsequently stained in an alkaline aqueous solution (KOH, <1% wt/wt) of Alizarin Red S to color the bones red, destained in KOH, rinsed repeatedly for 2 days in water to remove the KOH, and returned to 50% isopropyl alcohol. Lateral plate morph phenotypes were scored by visual inspection of the stained fish to determine the frequency of lateral plate morphs in the resident freshwater population in Mud Lake.

Caudal fins were removed from sacrificed anadromous specimens and preserved in 95% ethanol for genotyping. DNA was isolated from the fin clips using the phenol-chloroform method. The fins were digested overnight in 600 μl solution of 10 mM Tris pH 8.0, 100 mM NaCl, 10 mM EDTA, 0.5% SDS and 10 μl proteinase K (20 $mg \cdot ml^{-1}$). An equal volume of 1:1 phenol-chloroform solution was added and DNA was separated by centrifugation at 12 100 rpm, washed with ethanol, and suspended in 100 μl of TE. Our working stock was 1:25 dilution in H_2O. PCR amplification of the *Stn 381* marker was used to identify Eda^C and Eda^L alleles (Colosimo et al. 2005). This short sequence contains an indel (insertion or deletion) that produced 165 and 175-bp fragments for two Eda^C alleles and a 193-bp fragment for an Eda^L allele in the study by Colosimo et al. (2005). Our methods (see below) produced slightly smaller fragments (i.e., 163, 172, 190–193 bp), but they fell into three discrete classes and are consistent with plate phenotypes of the specimens that produced the fragments. Since all anadromous stickleback that we examined had a complete series of lateral plates and Eda^L is recessive to Eda^C, Eda^L alleles in anadromous stickleback must occur with an Eda^C allele in a heterozygous state, requiring use of molecular criteria to determine the presence of Eda^L alleles.

PCR reactions were carried out in 10 μl volumes consisting of 1× PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 2 mM $MgCl_2$, 0.25 mM dNTP, 0.3–0.4 μM primers, and 0.25–0.5 units of Taq DNA polymerase (Invitrogen). PCR conditions consisted of one cycle at 95°C for 1 min 45 s, 56°C for 45 s, 72°C for 45 s; followed by four cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 45 s; then 30 cycles of 92°C for 30 s, 56°C for 45 s, and 72°C for 45 s; and a final extension of 72°C for 7 min. PCR products were run on a three-rowed 2.5% agarose gel with 72 wells at 90 volts for about 90 min. Negative controls were run in every second gel, and an 1:1 mixture of the PCR products from homozygous Eda^{C165}/Eda^{C165} (anadromous) and homozygous Eda^{L193}/Eda^{L193} (freshwater) individuals was run in each gel as a positive control (Fig. 3a). The Eda^L amplicon is easily distinguishable from the two Eda^C fragments by gel electrophoresis because of their 20 bp or 30 bp differences (Figs. 2, and 3a).

PCR can preferentially amplify one allele in heterozygotes, but we have used this method to genotype Eda in resident freshwater stickleback from Loberg Lake, which is polymorphic for lateral plate morphs (Bell et al. 2004). Complete morphs in these populations are rare, and thus, virtually all of them should be Eda^L/Eda^C heterozygotes. We PCR amplified the *Stn 381* marker sequence from two partial and one complete morph collected in 2003 from Loberg Lake. They should be Eda^C/Eda^L heterozygotes because the frequency of low morphs (i.e., Eda^L/Eda^L homozygotes) was high (82.4%). We also used three complete-morph Eda^C/Eda^C homozygotes from Rabbit Slough, and a sample with a 1:1 mixture of amplified DNA from a Rabbit Slough Eda^C/Eda^C

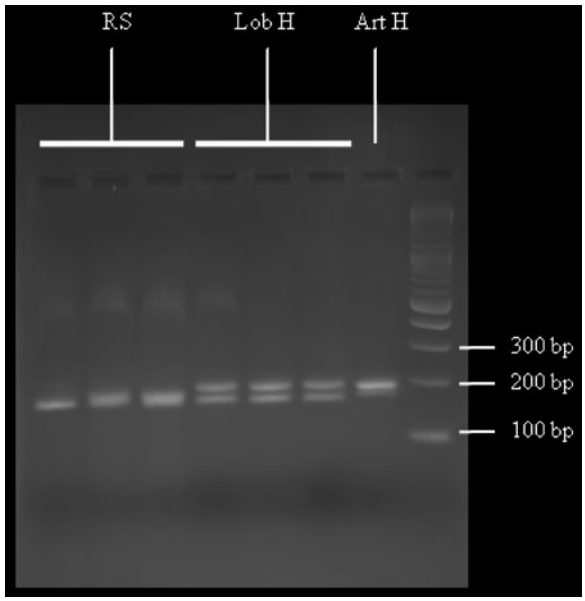

Fig. 2 Gel visualization of fragments from *Stn 381* marker for *Eda* alleles from (left to right) three Rabbit Slough Eda^C/Eda^C homozygotes (RS), three partial and complete morphs from Loberg Lake, which are presumptive Eda^L/Eda^C heterozygotes (Lob H), and one 1: 1 mixture of amplicons (artificial heterozygote) from Eda^L/Eda^L (Loberg Lake low morph) and Eda^C/Eda^C homozygotes (Rabbit Slough complete morph)

homozygote and a Eda^L/Eda^L homozygote from Loberg Lake. These samples were run on a 2.5% agarose gel, and all three Loberg Lake specimens are clearly identifiable as heterozygotes using this method (Fig. 2). Thus, the methods we used to genotype anadromous stickleback from Mud Lake and Rabbit Slough reliably detect Eda^C/Eda^L heterozygotes.

We also reamplified the *Stn* 381 marker in a random subset of 57 anadromous stickleback from the Rabbit Slough and Mud Lake samples using a fluorescent forward (Hex) primer and had them analyzed them on an Applied Biosystems 3730 DNA Analyzer at the University of Arizona Fragment Analysis Facility. These results confirmed the results from agarose gel that they are Eda^C/Eda^C homozygotes. Suspected Eda^L/Eda^C heterozygotes with a clear separation between bands in an agarose gel were also reanalyzed using fluorescent primers and a sequence analyzer. Thus, results using a sequence analyzer confirm our control study using Loberg Lake Eda^L/Eda^C heterozygotes and indicate that the *Eda* allele frequencies we report are accurate.

Although 175 and 165-bp Eda^C alleles can sometimes be distinguished on an agarose gel (Fig. 3b), scoring these alleles was often unreliable, and genotype frequencies from gel analysis were pooled into a single Eda^C class. Small random subsamples of anadromous specimens from Mud Lake (n=34) and Rabbit Slough (n=23) were reamplified using the fluorescent primer and analyzed on a sequencer, as described above, to estimate the frequencies of the 175 and 165 bp Eda^C alleles. An RXC test (Sokal and Rohlf 1995) was used to compare the relative frequencies of the 175 and 165 bp Eda^C fragments in the Mud Lake and Rabbit Slough subsamples.

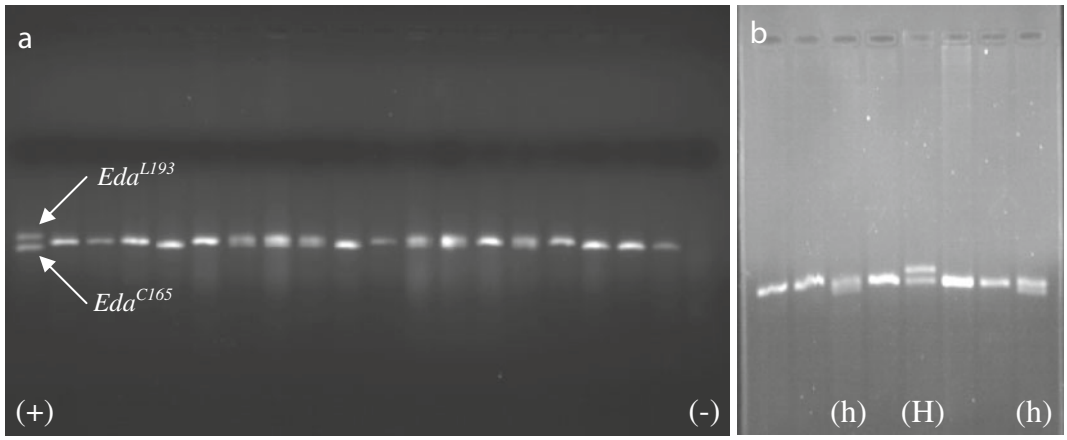

Fig 3 Gel visualization of fragments from *Stn 381* marker for *Eda* alleles from Rabbit Slough *Gasterosteus aculeatus*. **a** Positive control (+) "heterozygote" (i.e., 1:1 mixture of amplicons from complete Eda^{C165}/Eda^{C165} (165 bp) and low Eda^{L193}/Eda^{L193} (193 bp genotypes) and empty negative control (-) lane. **b** Eda^C/Eda^L heterozygote (H) and Eda^{C165}/Eda^{C175} heterozygotes (h) from Rabbit Slough

Results

None of the 608 completely plated anadromous stickleback (1216 haplotypes) from Mud Lake were Eda^C/Eda^L heterozygotes; every specimen was a Eda^C/Eda^C homozygote. Thus, the observed frequency of Eda^L alleles in the anadromous Mud Lake population appears to be below 0.082% (<1/1216). However, this estimate does not take sampling error into account. For example, with a frequency of one Eda^L allele in 1216, the probability of obtaining all Eda^C alleles, as we did, is high (using the Binomial distribution, $p=[1215/1216]^{1216}=0.368$). The probability of obtaining a sample consisting exclusively of Eda^C alleles falls below 5% if the frequency of Eda^L alleles is 3 in 1216 ($[1213/1216]^{1216} = 0.0496$). Thus if present, the frequency of Eda^L alleles in the anadromous Mud Lake population is likely (with >95% confidence) to be <0.247% ([3/1216]*100).

All but one of 2952 putative resident Mud Lake stickleback examined visually for plate morphology were low morphs. One specimen was a complete morph that measured 52.25 mm SL, above the mean for resident Mud Lake stickleback and at the lower end of the size range for anadromous fish (Karve et al. 2007). However, upon close inspection, it resembled anadromous stickleback for body form, the size, shape and position of the pectoral fin, and the relative size of the spines and pelvic girdle. Although its morphology suggests that it is a small anadromous stickleback, we cannot establish this with certainty. We also cannot score its *Eda* geneotype because it was found in the resident freshwater sample after it had been fixed in formalin, impeding genotyping. If it were a small anadromous Eda^C/Eda^C homozygote, the estimated limits on the Eda^L allele frequencies presented above for anadromous fish would remain virtually unchanged. If this specimen were a freshwater Eda^C/Eda^L heterozygote, the frequency of Eda^C alleles among resident fish would be 0.0169% (1/5904 alleles). The probability of obtaining a sample with one Eda^C allele and 5903 Eda^L alleles falls below 5% if the frequency is 5 Eda^C alleles in 5904 ($5904*[5/5904]^1*[5899/5904]^{5903}=0.034$; with 4 Eda^C alleles $p=0.073$), so, in the unlikely event that this lone specimen is a freshwater resident with an Eda^C/Eda^L genotype, then the frequency of Eda^C alleles in the freshwater Mud Lake population is probably <0.084%.

One out of 777 completely plated fish from Rabbit Slough was an Eda^C/Eda^L heterozygote (Fig. 3b) which gives an estimated frequency of low-morph alleles of 0.064% (1/1554 haplotypes). Using the procedure outlined above, the frequency of Eda^L alleles is probably (with >95% confidence) below 0.322%.

Frequencies of the Eda^C 165 and 175 fragments in the Mud Lake subsample were 0.41 and 0.59 and in Rabbit Slough were 0.22 and 0.78, respectively, which differ significantly from each other (RXC test, $G_{(Williams)}=4.923$, $p=0.0265$).

Discussion

The Eda^L allele is very rare in two populations of anadromous threespine stickleback from Cook Inlet in which all specimens are phenotypic complete morphs, and it may be absent in one of them. These results are not unique (Table 1), but place the frequency of Eda^L in these populations at the lower end of the range of variation among oceanic populations. However, presence of Eda^L in the Rabbit Slough population expands the geographical range of anadromous populations within which the Eda^L allele has been observed and supports the inference of Colosimo et al. (2005; see also Cano et al. 2006) that this allele has been carried as a rare recessive around the northern hemisphere within anadromous populations from a single point of mutational origin to most low-plated freshwater populations.

Karve et al. (2007) showed that reproductively mature lake-resident and anadromous stickleback are present simultaneously and occur at the same sites within Mud Lake. In the absence of other reproductive isolating mechanisms, these overlaps would result in frequent hybridization and an elevated frequency of Eda^L alleles in the anadromous and of Eda^C alleles in the resident lake populations (Hagen 1967; Hay and McPhail 1975; Jones et al. 2006). Our inability to detect Eda^C alleles in freshwater resident stickleback or Eda^L alleles in anadromous threespine stickleback in Mud Lake indicate either that they do not hybridize or that such hybridization has produced no or negligible introgression at the *Eda* locus. Thus, they represent separate biological species in Mud Lake.

Our results are similar in some respects to Hagen's (1967) early study of isolating mechanisms between a

pair of biological species of stream-resident and anadromous stickleback from the Little Campbell River, British Columbia, Canada. Although he observed a hybrid zone between completely plated anadromous stickleback downstream and a low-plated resident population upstream, gene flow across this hybrid zone into either parental population was too low to produce substantial numbers of phenotypic intermediates (including partial plate morphs) in either of the parental populations, and the populations on either side of the hybrid zone were fixed for alternative alleles of a muscle protein (Hagen 1967). Hagen (1967) concluded that reproductive isolation between these biological species results from differences in their breeding season and preference for different breeding microhabitats. Although he did not detect evidence of positive assortative mating, Hay and McPhail (1975) subsequently showed that members of both species prefer to mate with conspecifics. Hagen (1967) also failed to observe post-mating isolation, but he estimated it only from the relative viability of hybrid progeny in the lab, which may overestimate hybrid fitness in the wild (Schluter 1995; Gow et al. 2007).

Jones et al. (2006) performed a detailed genetic analysis of stickleback population structure in River Tyne, Scotland. Anadromous stickleback run into the lower reaches of this river, where they hybridize with resident freshwater *G. aculeatus*. In contrast to Hagen's (1967) results, about one third of the specimens in the lower reaches of the river are of hybrid ancestry, but heterozygote deficiency and cytonuclear disequilibrium suggest selection against hybrids. Similarly, Tommelson Lake, which is <10 km west of Mud Lake, contains a large-bodied resident freshwater population that is polymorphic for low and complete *Eda* alleles and lateral plate morphs. Lateral plate polymorphism in the Tommelson Lake population appears to be due to introgression by anadromous stickleback (Aguirre and Bell, unpubl. data). Thus, there appears to be considerable variation in the consequences of sympatric reproduction by anadromous and resident stickleback in lakes and streams.

Body size is an important cue for positive assortative mating in threespine stickleback (Nagel and Schluter 1998; McKinnon et al. 2004; Boughman et al. 2005), and the occurrence of introgression between sympatric resident freshwater and anadromous populations may depend on the size difference between them. Resident freshwater threespine stickleback in Mud Lake are much smaller than anadromous stickleback (Karve et al. 2007). Regardless of whether they hybridize or not, sympatric anadromous and resident freshwater threespine stickleback usually retain divergent phenotypic properties and represent separate biological species (McPhail 1994; McKinnon and Rundle 2002; Boughman 2007), confirming Hagen's (1967) original conclusion.

The frequency of Eda^L has been estimated in nine marine or anadromous populations and varies between about 20% and 0% (Table 1). Its estimated frequency in the Rabbit Slough sample was 0.064%, and it has only a 5% chance of exceeding 0.322%. Similarly, no Eda^L alleles were detected in the Mud Lake population, and there is only a 5% probability that the Eda^L allele exceeds 0.247%. Thus, Eda^L alleles are very rare in anadromous Cook Inlet populations. Nevertheless, freshwater stickleback populations in Cook Inlet lakes are almost always monomorphic for the low plate morph (Bourgeois et al. 1994). The gene tree for *Eda* reported by Colosimo et al. (2005) showed clearly that Eda^L alleles in freshwater populations of *G. aculeatus* are usually derived from ancestral Eda^L alleles that were present in their anadromous ancestors. Eda^L alleles can be carried at low frequencies in anadromous populations because they are usually recessive in F1 hybrids between low-plated freshwater and completely plated anadromous stickleback (but see Hagen 1967; Cresko et al. 2004; Barrett et al. 2008). Thus, Eda^L alleles that are fixed in lake populations are acquired directly or indirectly from oceanic populations.

Fixation of Eda^L alleles in freshwater isolates implies (1) that the effective size (N_e) of their founding oceanic populations was typically large enough to contain rare Eda^L alleles, (2) that gene flow after founding introduced Eda^L alleles directly from anadromous or indirectly from other freshwater populations, (3) that our samples from late May and early June underestimate the frequency of Eda^L alleles in the anadromous stickleback that founded freshwater isolates, (4) some combination of these factors or (5) the Mud Lake and Rabbit Slough populations are unrepresentative of the populations that founded existing freshwater stickleback populations around Cook Inlet. Surveys of molecular genetic variation in populations from British Columbia (Withler

and McPhail 1985; Taylor and McPhail 1999, 2000) and Alaska (Aguirre 2007) imply that freshwater populations typically have reduced effective population size (N_e) compared to oceanic populations, but that they generally were not severely bottlenecked and must be derived from reasonably large founding populations. Our random samples could underestimate the probability that Eda^L alleles occurred in founding populations if pleiotropy or linkage between *Eda* and loci influencing migratory behavior increased representation of Eda^L alleles among founders. But this possibility seems unlikely (Barrett et al. 2009b). A recently founded lake population in Loberg Lake, only a few kilometers from Rabbit Slough, rapidly evolved low morphs (Bell et al. 2004), suggesting that contemporary anadromous populations contain an adequate number of Eda^L alleles to respond quickly to selection favoring that phenotype. *Eda* is tightly linked to other potentially important loci (Colosimo et al. 2005), and Albert et al. (2008) mapped multiple body shape traits to its locus, indicating either linkage or pleiotropy. Similarly, Barrett et al. (2008, 2009a) observed fitness differences among *Eda* genotypes in experimental populations at early life history stages, before extensive plate development had occurred, and between Eda^L/Eda^C and Eda^C/Eda^C genotypes, even though they did not differ substantially for plate phenotypes. These results suggest that traits linked to *Eda* could influence the probability of colonizing fresh water, and Eda^L/Eda^C heteroygotes could be overrepresented among anadromous founders of freshwater populations. Further research will be needed to determine why Eda^L alleles are so predictably acquired by freshwater-resident populations of *G. aculeatus* from their oceanic ancestors despite their low frequency in those populations.

After freshwater populations are founded, low plate morphs, and presumably the Eda^L alleles that encode them, may rapidly evolve to a high frequency or reach fixation, implying strong selection (Bell 2001). *Eda* genotype frequencies changed strongly but irregularly through two generations in experimental populations (Barrett et al. 2008), but in a lake population founded by oceanic stickleback, the frequency of low morphs (and presumably Eda^L alleles) increased rapidly among each of several consecutive generations, reaching 75% within about ten generations (Bell et al. 2004; see also Francis et al. 1985; Klepaker 1993; Vamosi 2006). Reimchen (2000) presented evidence that low-plated stickleback are more likely than complete morphs to avoid capture by predatory fishes in fresh water, and Marchinko (2009) presented evidence that low morphs experience lower rates of insect predation. Marchinko and Schluter (2007) also found that low morphs grow much faster in fresh water than completes. However, selection must not invariably favor the low morph in freshwater; freshwater stickleback populations from regions with strong seasonal temperature differences tend to be monomorphic for the complete morph (Hagen and Moodie 1982). In western Europe and western North America, where all three plate morphs occur, freshwater populations in the south are monomorphic for the low morph, and all three morphs occur to the north (Münzing 1963; Bell 1984). In northern California, where all three plate morphs occur, selection seems to favor low morphs in lentic habitats and completes in lotic habitats, forming steep plate-morph frequency clines near ecotones between these habitats (Bell 1982; Baumgartner and Bell 1984). The causes for regional and habitat-specific differences in the frequencies of low-plated freshwater stickleback is unclear. Thus the causes for plate morph differences between oceanic and freshwater stickleback may be more complicated than these experimental results suggest.

The significant difference between the frequencies of Eda^C alleles marked by the 165 and 175 bp amplicons in the Mud Lake and Rabbit Slough anadromous populations contrasts with lack of divergence at five microsatellite loci (Aguirre 2007; Aguirre, unpubl. data). Since these two populations are only 16 km apart and microsatellites generally behave as if they are selectively neutral, the frequency difference between the Mud Lake and Rabbit Slough populations for Eda^C alleles appears to represent fitness (and presumably underlying functional) differences between these alleles or alleles at linked loci. It will be important in the future to distinguish the Eda^{C165} and Eda^{C175} alleles to infer the possible functional and fitness differences between alternative Eda^C alleles.

Further field and laboratory research is also needed to determine how anadromous and resident *G. aculeatus* in Mud Lake breed in sympatry without producing detectable introgression at the *Eda* locus. It is possible that strong positive assortative mating has

evolved, that hybrids between these populations are inviable in nature, or that some combination of these and other isolating mechanisms are operating in this anadromous-lake resident species pair. However, our results confirm the conclusion of Karve et al. (2007) that gene flow between these sympatric populations is absent or very low.

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