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Genetic basis for body size variation between an anadromous and two derived lacustrine populations of threespine stickleback *Gasterosteus aculeatus* in southwest Alaska

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Abstract

Body size is a highly variable trait among geographically separated populations. Size-assortative reproductive isolation has been linked to recent adaptive radiations of threespine stickleback (*Gasterosteus aculeatus*) into freshwater, but the genetic basis of the commonly found size difference between anadromous and derived lacustrine sticklebacks has not been tested. We studied the genetic basis of size differences between recently diverging stickleback lineages in southwest Alaska using a common environment experiment. We crossed stickleback within one anadromous (Naknek River) and one lake (Pringle Lake) population and between the anadromous and two lake populations (Pringle and JoJo Lakes), and raised them in a salinity of 4–6 ppt. The F1 anadromous and freshwater forms differed significantly in size, whereas hybrids were intermediate or exhibited dominance toward the anadromous form. Additionally, the size of freshwater F1s differed from their wild counterparts, with within-population F1s from Pringle Lake growing larger than their wild counterparts, while there was no size difference between lab-raised and wild anadromous fish. Sexual dimorphism was always present in anadromous fish, but not in freshwater, and not always in the hybrid crosses. These results, along with parallel changes among anadromous and freshwater forms in other regions, suggest that this heritable trait is both plastic and may be under divergent and/or sexual selection.

Key words: adaptation, body size, common garden, genetic basis, threespine stickleback.

Size at reproductive maturity can impact fecundity, heterospecific mate choice (Conte and Schluter 2013) and overall fitness of individuals (Alm et al. 1959; Peters 1986; Stearns and Koella 1986; Roff 1992; Stearns 1992), and is a trait frequently associated with adaptive radiations (Mayr 1963; Schluter 2000). Body size depends on various environmental (e.g., temperature, light levels, food availability, social organization, population density, migration, predation) (Weatherly 1972) and genetic factors (Snyder and Dingle 1989). In fishes, maturity is typically size-specific and depends on growth rate, which often varies within and among populations (Alm 1959; Iles 1974; McKay et al. 1986; Gjerde and Schaeffer 1989; Siitonen and Gall 1989) and with other phenotypic traits (e.g., disease resistance) (Shine 1988; Shine 1989; Blanckenhorn 2000; Barber et al. 2001; Herczeg et al. 2009). Common garden experiments are important for disentangling environmental from genetic factors contributing to phenotypes.

The threespine stickleback (*Gasterosteus aculeatus*) (Linnaeus 1758) is a small fish that has colonized freshwater lakes and streams

from the sea after the last glacial recession approximately 10–15 000 years ago (McPhail and Lindsey 1970). Several studies have implicated a role for growth (Hatfield and Schluter 1999; Rundle 2002; Barrett et al. 2008; Barrett et al. 2009) and body size (standard length) (McPhail 1977; Snyder and Dingle 1989; Nagel and Schluter 1998; Ishikawa and Mori 2000) in adaptive divergence and as a driver of prezygotic reproductive isolation in threespine stickleback. In addition to demonstrating evolution of larger to smaller body size from marine to stream environments in populations throughout the threespine stickleback range, using experimental size manipulation in a common garden experiment McKinnon et al. (2004) also demonstrated that mate choice can depend on size-selection. These studies have shown that size can be heritable, but others have shown that size is influenced by environmental factors (Conover and Schultz 1995; Conover et al. 2009). Common environment studies can test explicitly the genetic basis of differences in size between newly diverging anadromous and freshwater lake lineages toward informing future approaches that can reveal the genetic architecture and the role of selection on this trait (Bradshaw et al. 1995; Michel et al. 2010; Barrett and Hoekstra 2011; Rogers et al. 2013).

Common environment studies comparing size differences among lacustrine or marine environments exist, but to our knowledge none have been conducted to investigate the genetic basis of differences in size between these diverging lineages. Studies have been done to investigate the potential for diversification of body shape and lateral plate variation in marine threespine stickleback (Leinonen et al. 2011) and the potential for evolution and adaptation of body size in differing salinities using marine threespine stickleback (McGuigan et al. 2011; DeFaveri and Merilä 2014). Heritable body size differences have also been demonstrated between populations inhabiting different lakes (McPhail 1977), and between estuarine and freshwater stickleback from a single river (Snyder and Dingle 1989). Finally, Colosimo et al. (2004) found that differences in the size of lateral plates in very divergent marine and freshwater sticklebacks are heritable, and they found the underlying quantitative trait locus (QTL) associated with this difference. There is therefore a surprising gap in our understanding of the genetic basis of this important phenotypic character between anadromous and lacustrine lineages.

In this study, we tested the hypothesis that differentiation in body size (standard length) between anadromous and freshwater stickleback has a genetic basis. We focused on southwest Alaska, where anadromous stickleback have undergone a recent radiation into freshwater lakes, and are twice the length of those in fresh water. We bred an anadromous population and two lacustrine populations and measured the size of the offspring in a common environment. Based on this size difference and parallel divergence in length between the anadromous and freshwater fish, we predicted that F1s from freshwater lakes would be smaller than those from the anadromous environment, and that hybrids would be intermediate.

Materials and Methods

Sample collection

We collected threespine stickleback from the Naknek River (anadromous site, 58.679°N 156.671°W), JoJo Lake (58.615°N 155.219°W) and Pringle Lake (58.559°N 155.958°W), Alaska (Figure 1), during the summers of 2011 and 2012 using minnow traps and beach seines. We characterized the Naknek fish as anadromous because they demonstrated morphological characters consistent with this life history (e.g., more gill rakers and lateral plates, longer dorsal and pelvic spines) and were captured in a tidally-influenced environment, consistent with previous studies (Hagen 1967; Aguirre et al. 2008). To test for differences in size between wild populations, we euthanized fish from each environment ($n = 24$ from Pringle Lake, $n = 60$ from each of JoJo Lake and Naknek River, Table 1) with an overdose of Eugenol, preserved whole specimens in 10% formalin for 24 hours followed by 70% EtOH for storage, and then took standard length measurements. We also took fin clips and stored them in 95% ethanol for molecular sex determination. At the time of collection, fish in all populations were in reproductive condition: males displayed breeding colors and females easily released eggs. We transported live fish from these sites to the University of Calgary for breeding, and all sampling and transport methods complied with Canadian Council of Animal Care standards (protocol AC13-0040).

Breeding and experimental design

We housed wild stickleback in 113-L aquaria at (mean ± *SD*) 13.38°C ± 0.66°C, 4–6 ppt salinity and pH ~ 8, and fed them daily to satiation with bloodworms, Chironomid (*sp.*) larvae, and *Mysis* shrimp. Our breeding design involved artificially crossing a wild-caught female and male from each population to generate anadromous (Naknek River × Naknek River, NRNR), freshwater-by-anadromous (Pringle Lake × Naknek River, PLNR; JoJo Lake × Naknek River, JLNR), and freshwater crosses (Pringle Lake only, PLPL). This yielded two anadromous, three hybrid, and four freshwater crosses (Table 2).

Crosses were generated in a petri dish. Eggs were stripped from females by applying gentle pressure from the anterior to posterior of the distended abdomen. Sperm was harvested by sacrificing the male to extract the testes, and then macerating the testes to release sperm. Sperm was then added to the eggs by mixing it with distilled water and pipetting it evenly over the egg mass. Eggs were left to incubate at room temperature for approximately 10–15 min. After this, embryos were transferred into a cup with a mesh bottom, and hung in a well-oxygenated tank kept at the same temperature as their wild parents. At the eyed stage, we moved F1s into the tanks where they hatched, which were either 37.9 L or 113.6 L, and kept at room temperature.

Fish were raised in their hatching tanks until they were 2 months of age, when families were split into 113-L aquaria kept at (mean ± *SD*) 15.87 ± 3.66°C, 4–6 ppt salinity, and pH ~ 8, in a single room with 16 h of light per day. Consistent light levels and temperature were maintained year-round. Upon hatching, clutches were fed 1.5 ml of live freshly hatched brine shrimp *Artemia sp.* twice daily for the first 4 weeks, followed by 2 weeks of 3 ml of brine shrimp twice daily, followed by one 100 g cube of frozen *Daphnia sp.* in the morning and 3 ml of brine shrimp in the evening from 6 to 8 weeks. After clutches were split into equal densities at 8 weeks (20 fish per 113-L aquarium, or ~1 fish per 5.7 L of water) they were fed one 100 g cube of *Daphnia sp.* once a day until 12 weeks, followed by one 100 g cube of frozen blood worms once in the morning and once in the evening until they were 9 months of age.

At 9 months, we euthanized fish with an overdose of Eugenol and measured their standard length. At this time, females were releasing eggs and males were building nests (using fiber taken from filter inflows) in all populations, and fish were either equivalent in length or longer than their wild-caught counterparts when they were caught (Figure 3). However, we did not score every female and male for maturity, thus the phenotype that we measured was standard length (size in mm) at 9 months. We raised all fish that hatched, and thus had replicate tanks within families reared in common

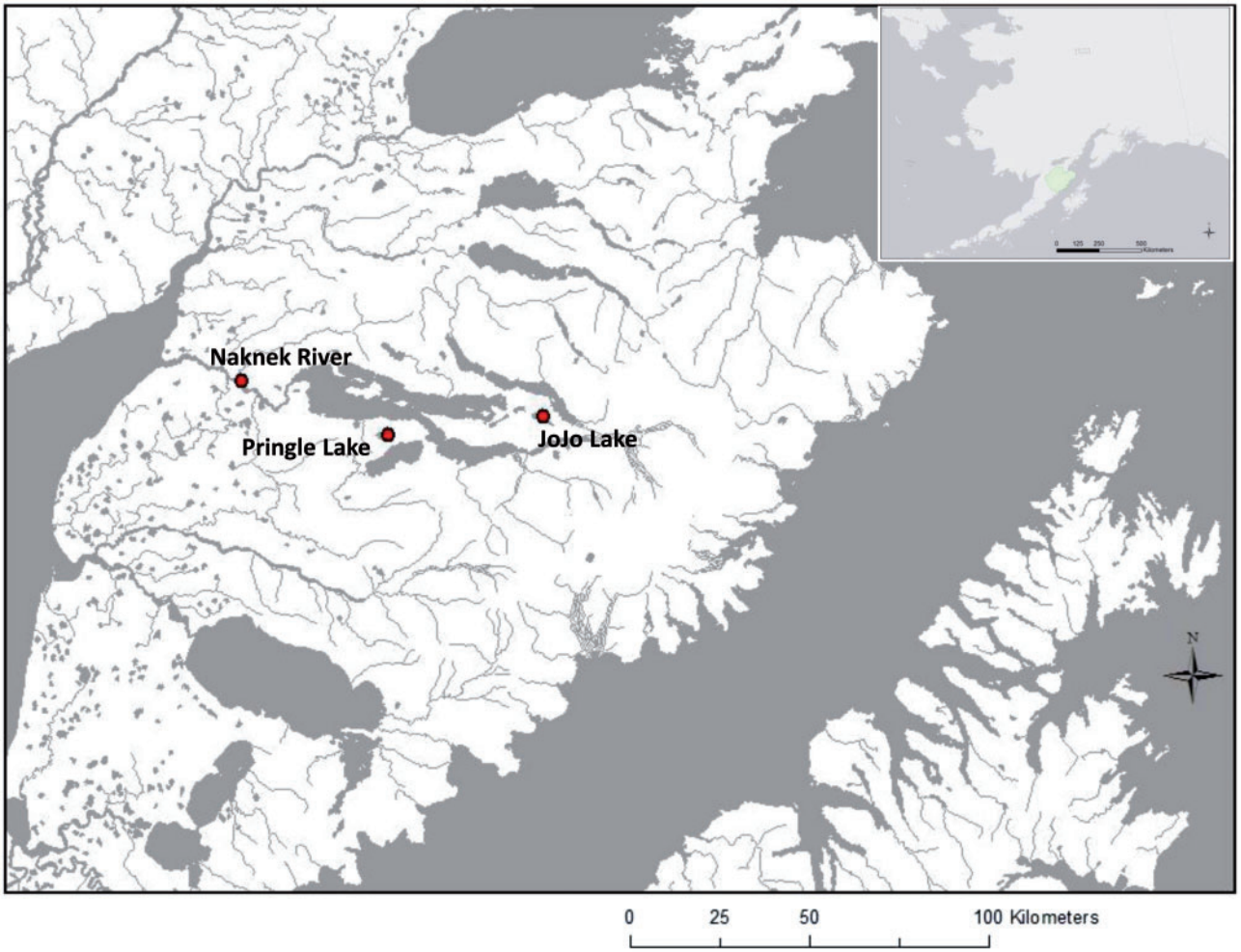

Figure 1. Sampling locations in Katmai National Park and Preserve, Alaska.

Table 1. Numbers of males (M) and females (F) sampled from each wild population

Population	M	F
Naknek River (NR)	35	25
Pringle Lake (PL)	12	12
JoJo Lake (JL)	12	48

environment conditions. Because clutch size varied, the number of tanks per family varied to maintain equal densities (Table 2).

Sex determination

Genomic DNA was isolated from caudal fin clips using either a standard proteinase K phenol chloroform protocol (Sambrook et al. 1989) or a QIAGEN DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA). DNA was amplified at the *Idh* locus (Peichel et al. 2004) by polymerase chain reaction (PCR) with C-1000 and S-1000 thermal cyclers (Bio-Rad, Inc., Hercules, CA) in 10 µl reactions containing 1 µl of unstandardized genomic DNA, 0.5 µM of each primer, 0.25 mM dNTPs (Invitrogen), 0.1 mg/mL bovine serum albumin, 1X ThermoPol buffer, and 0.15 units of Taq polymerase (New England Biolabs). Cycling conditions were as follows: 95°C for 105 s, 56°C for 45 s, 68°C for 45 s, 36 cycles of 94°C for 45 s, 59°C for 45 s, 68°C for 45 s, followed by 72°C for 5 min, and then cooled to 8°C. Sex was determined by viewing PCR product on a 2% w/v agarose gel. Individuals with bands at 302 and 271 base pairs were identified as male, and those with one band at 302 base pairs were identified as female.

Size sampling, measurements, and statistical analysis

We analyzed wild fish and lab offspring using the same methods. At 9 months, we randomly sampled 10 fish from each family, selecting fish based on random numbers generated using R v. 3.0.2 (R Core Team 2013). We took standard length measurements from individually scaled photographs using ImageJ (Abràmoff et al. 2004), to ± 0.5 mm accuracy. All photographs were taken using a Canon PowerShot D10 camera mounted 5.75 cm above the specimen. Data were analyzed using mixed models estimating least-squares means, with cross and sex as fixed effects and family within cross as a random effect for offspring (SAS v. 9.3, SAS Institute, Cary NC). For comparisons between wild fish and F1 crosses, each fish sampled from the wild populations was considered to be from a separate family. Separate mixed models were used for comparisons using the JLNR cross because a lack of JoJo × JoJo crosses precluded a single four-way comparison with PLPL and NRNR crosses. Denominator degrees of freedom for *F*-tests were calculated using the Kenward–Roger technique (Kenward and Roger 1997), and least-squares

Table 2. F1 families and the number of offspring used per family

Cross no.	Wild parent 1 (F)	Wild parent 2 (M)	F1 cross acronym	Number of tanks per F1 family (10 fish sampled from each tank)	Numbers of males (M) and females (F)
1	Naknek	Naknek	NRNR	2	8M, 12F
2	Naknek	Naknek	NRNR	1	8M, 2F
3	Pringle	Naknek	PLNR	3	12M, 18F
4	JoJo	Naknek	JLNR	1	5M, 5F
5	Naknek	JoJo	JLNR	2	10M, 10F
6	Pringle	Pringle	PLPL	1	4M, 6F
7	Pringle	Pringle	PLPL	3	9M, 19F
8	Pringle	Pringle	PLPL	2	8M, 12F
9	Pringle	Pringle	PLPL	1	4M, 5F

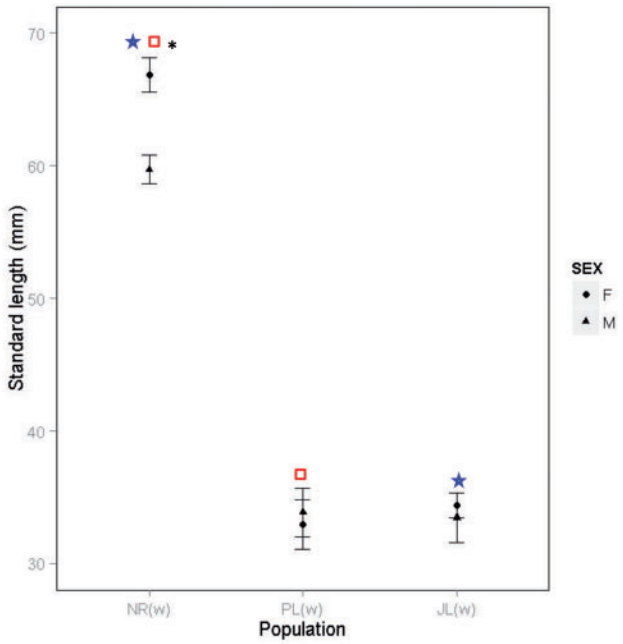

Figure 2. Least-squares means ± *SE* of standard lengths of wild threespine sticklebacks from Naknek River (NR: anadromous), Pringle Lake (PL) and JoJo Lake (JL). Wild fish are denoted with (w). Stars and squares indicate significant difference in standard length between the populations that share that same shape, and asterisks show sexual dimorphism within populations (see Table 3 for analysis results).

means were compared using Tukey's correction for multiple comparisons ($\alpha = 0.05$) for comparisons between the wild populations and NRNR vs PLNR vs PLPL (Kramer 1956).

Results

Wild anadromous stickleback (Naknek River) were approximately twice the length of their freshwater counterparts, and sexual dimorphism was significant in the anadromous population but not in either freshwater population (Figure 2, Table 3). The difference in least-squares means between the three wild populations was highly significant, with the two freshwater populations not differing in average length. Sex was not significant as a main effect but its interaction with population was significant. Males and females differed in length in the anadromous Naknek River population (females larger than males), but not in either freshwater population.

Table 3. Type 3 tests of fixed effects, tests of simple effects and differences between least-squares means for the model comparing standard lengths of the three wild populations: Naknek River (NR), Pringle Lake (PL), and JoJo Lake (JL)

Type 3 tests of fixed effects, main effects

Effect	Num *df*	Den *df*	*F*	*P*
POP	2	138	315.68	<0.0001
SEX	1	138	3.66	0.058
POP*SEX	2	138	4.46	0.013

Differences between least-squares means

Effect	POP	POP	*df*	*t*	*P*
POP	JL	NR	138	−21.87	<0.0001
POP	JL	PL	138	0.32	0.95
POP	NR	PL	138	19.08	<0.0001

Tests of simple effects

Effect	POP	Num *df*	Den *df*	F	P
POP*SEX	JL	1	138	0.2	0.66
POP*SEX	NR	1	138	17.86	<0.0001
POP*SEX	PL	1	138	0.11	0.74

**df* (degrees of freedom); Num (numerator), Den (denominator); POP (population).

In the common environment, offspring from anadromous and freshwater populations also differed in length, with hybrids intermediate between the two pure forms (Figure 3, Table 4). NRNR and PLPL crosses differed significantly in average length, while the hybrid offspring were indistinguishable from either within-population cross-type but more similar to the NRNR fish than to PLPL fish. Sex did not have a significant effect on standard length on its own, but did have a significant interaction with population. Sex-specific size differences persist in the NRNR crosses (females larger than males) and PLNR cross (males larger than females), but not in the freshwater PLPL crosses (Figure 2).

Hybrid JLNR fish did not differ significantly from NRNR crosses (Table 5, Figure 3) or the PLNR cross (Table 6, Figure 3), and sexual dimorphism was significant in the PLNR but not the JLNR crosses. Sex by itself had a significant effect in the JLNR vs

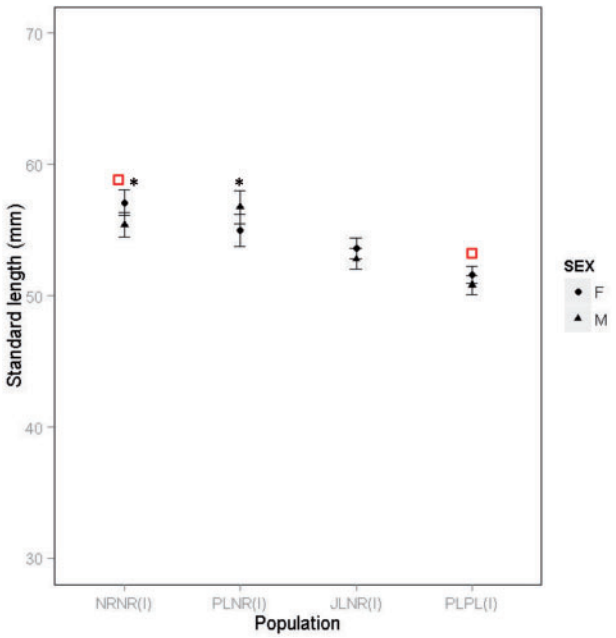

Figure 3. Least-squares means ± *SE* of standard lengths of F1 fish at 9 months of age for Naknek River × Naknek River (NRNR), Pringle Lake × Naknek River (PLNR), JoJo Lake × Naknek River (JLNR) and Pringle Lake × Pringle Lake (PLPL) crosses. Lab-reared F1 fish are denoted with (I). Squares indicate significant difference in standard length between the populations that share that shape, and asterisks show sexual dimorphism within populations (see Tables 4, 5, and 6 for analysis results).

Table 4. Type 3 tests of fixed effects, tests of simple effects and differences between least-squares means for the model comparing standard length differences between Naknek River × Naknek River (NRNR) F1s, Pringle Lake × Pringle Lake (PLPL) F1s and Pringle Lake x Naknek River (PLNR) F1s

Type 3 tests of fixed effects, main effects

Effect	Num *df*	Den *df*	*F*	*P*
POP	2	3.65	13.57	0.02
SEX	1	120	0.37	0.54
POP*SEX	2	119	6.06	0.0031

Differences between least-squares means

Effect	POP	POP	*df*	*t*	*P*
POP	NRNR	PLNR	3.5	0.26	0.96
POP	NRNR	PLPL	3.99	4.66	0.025
POP	PLNR	PLPL	3.4	3.49	0.060

Tests of simple effects

Effect	POP	Num *df*	Den *df*	*F*	*P*
POP*SEX	NRNR	1	121	4.82	0.03
POP*SEX	PLNR	1	117	5.81	0.018
POP*SEX	PLPL	1	117	2.43	0.12

**df* (degrees of freedom); Num (numerator), Den (denominator); POP (population).

NRNR model, albeit with a nonsignificant interaction with population. Parsing this effect, sex was significant in the NRNR crosses, but nonsignificant in the JLNR crosses (Table 5). In the PLNR vs JLNR model, sex by itself was nonsignificant, but sex by population was. Parsing this interaction, sex was significant in the PLNR fish, but not in the JLNR crosses (Table 6).

Table 5. Type 3 tests of fixed effects and tests of simple effects for the model comparing standard lengths of Naknek River × Naknek River (NRNR) F1s to Naknek River × JoJo Lake (JLNR) F1s

Type 3 tests of fixed effects, main effects

Effect	Num *df*	Den *df*	*F*	*P*
POP	1	1.77	9.58	0.11
SEX	1	55.9	5.53	0.022
POP*SEX	1	55.9	0.72	0.40

Tests of simple effects

Effect	POP	Num *df*	Den *df*	*F*	*P*
POP*SEX	JLNR	1	54	1.26	0.27
POP*SEX	NRNR	1	55.5	4.63	0.036

**df* (degrees of freedom); Num (numerator), Den (denominator); POP (population).

Table 6. Type 3 tests of fixed effects and tests of simple effects for the model comparing standard lengths of Naknek River × JoJo Lake (NRJL) F1s, to Naknek River × Pringle Lake (PLNR) F1s

Type 3 tests of fixed effects, main effects

Effect	Num *df*	Den *df*	*F*	*P*
POP	1	1	10.43	0.19
SEX	1	55	0.68	0.41
POP*SEX	1	55	5.52	0.022

Tests of simple effects

Effect	POP	Num *df*	Den *df*	*F*	*P*
POP*SEX	JLNR	1	55	1.18	0.28
POP*SEX	PLNR	1	55	4.94	0.03

**df* (degrees of freedom); Num (numerator), Den (denominator); POP (population).

Wild Pringle Lake fish were shorter than PLPL F1s raised in the lab, whereas wild Naknek River fish were indistinguishable from NRNR F1s (Table 7, Figures 2 and 3). In the model comparing wild-caught Pringle Lake fish to lab-reared F1 Pringle Lake fish, sex did not have a significant main effect or interaction with population. In the model comparing wild-caught anadromous fish and lab-reared anadromous F1s, sex was significant as a main effect, but its interaction with population was not significant. Males and females were significantly different in size in both the wild and lab-reared anadromous fish.

Discussion

This study was motivated by observing that fish from JoJo Lake (JL) and Pringle Lake (PL) were half the length of their anadromous counterparts (Naknek River, NR) (Figure 2). We have shown that body length of threespine stickleback at 9 months is genetically determined, with anadromous stickleback growing larger than their freshwater counterparts and hybrids growing to be intermediate between the two pure cross-types (Figure 3). This is despite reduced interpopulation differences induced by raising these fish in a common lab environment (Figures 2 and 3). Larger anadromous and

Table 7. Type 3 tests of fixed effects and tests of simple effects for the two separate models comparing standard lengths of wild and lab-reared fish. Model one compared wild Pringle Lake (PL) fish to lab-reared Pringle Lake × Pringle Lake (PLPL) F1s (no simple effects tested in this model). Model two compared wild Naknek River (NR) fish to lab-reared Naknek River × Naknek River (NRNR) F1s

Model 1. Pringle Lake wild vs Pringle Lake F1s

Type 3 tests of fixed effects, main effects

Effect	Num *df*	Den *df*	*F*	*P*
POP	1	7.4	216.79	<0.0001
SEX	1	38.5	0.01	0.94
POP*SEX	1	38.5	1.55	0.22

Model 2. Naknek River wild vs Naknek River F1s

Type 3 tests of fixed effects, main effects

Effect	Num *df*	Den *df*	*F*	*P*
POP	1	54.1	0.54	0.46
SEX	1	65.5	13.74	0.0004
POP*SEX	1	65.6	3.87	0.054

Tests of simple effects

Effect	POP	Num *df*	Den *df*	*F*	*P*
POP*SEX	NRNR_F1	1	28	11.85	0.0018
POP*SEX	NR_wild	1	58	8.6	0.0048

**df* (degrees of freedom); Num (numerator), Den (denominator); POP (population).

smaller lacustrine threespine stickleback have been observed repeatedly throughout the circumpolar range of this species (Baker 1994), but the genetic basis of the size difference has not been assessed (but see Snyder and Dingle (1989) for anadromous and stream-types). We speculate that fish from the two lakes are both derived from ancestral fish inhabiting the Naknek River (single watershed, and the NR is the major artery of connection to the Bering Sea), with no significant length difference observed between wild fish from these freshwater populations (Figure 2), although ancestry has not been confirmed.

While the PLNR cross was intermediate between the NRNR and PLPL crosses, the PLNR cross resembled NRNR fish more than they resembled PLPL fish, deviating from our prediction of additivity and suggesting that dominance effects may be important (Ghani et al. 2012; Bell and Aguirre 2013). And, there was no significant size difference between JLNR and PLNR crosses, indicating that lake origin did not influence size. While maternal effects cannot be ruled out using our data, studies have shown that they diminish with age and are limited at maturity (McKay et al. 1986; Nilsson 1990; Shimada et al. 2011). Yet, in the absence of JoJo × JoJo offspring for comparison, and few hybrid families, mechanisms governing the genetic basis of length differences between the pure forms must be interpreted with caution.

The two freshwater populations are probably derived from Naknek River fish, but they are separated by two lakes where fish are much larger at maturity ((least-squares means ± *SE*) Brooks Lake 53.35 ± 0.85 mm, Naknek Lake 47.13 ± 0.49 mm; difference between least-squares means between each of these lakes and Pringle and JoJo Lakes respectively were highly significant in every case) so that their similar length suggests parallel, genetically based phenotypic divergence (Conte et al. 2012). Many agents of selection have been associated with size differences following population divergence. Speculating on but a few, in this system these could include: temperature (Bergmann 1847), migration (Snyder and Dingle 1989), predation (Herczeg et al., 2009; Walsh and Reznick 2009), and prey availability (Schluter 2000). Across taxa, ectotherms in colder temperatures grow to be larger (Bergmann 1847). Correspondingly, year-round temperatures at the estuary at the mouth of the Naknek River are colder than in Lake Brooks, the lake into which PL feeds. Earlier maturation also reduces size at maturity (Shimada et al. 2011), but based on our observation of breeding condition, anadromous and freshwater fish came into reproductive condition at the same time in the lab. This variety of possible selective explanations suggests that future studies should focus on integrating ecological and experimental approaches to disentangling these factors.

Sexual dimorphism has been shown to be significant in stickleback for shape and size (Head et al. 2009; Leinonen et al. 2010; Cooper et al. 2011), including for the populations studied here when a large number of landmarks were used for the cranial region (Pistore A, personal communication). Here, we show that sexual dimorphism for size is significant in the anadromous population in both wild and lab-reared fish, and for 1 hybrid cross, PLNR, but not for PL wild fish, or for hybrid JLNR fish. Given that our results indicate potential dominance effects, with the PLNR cross closer to NRNR fish in size than to PLPL fish, and we know that size is pleiotropic with other traits (Barrett et al. 2009), these results may indicate covariation of multiple phenotypic characters that differ between populations.

Wild PL fish were much smaller than the PLPL lab-reared F1s, whereas wild and lab-reared F1 anadromous fish did not differ in length (Figures 2 and 3). Environmental conditions can significantly affect growth and size in fishes (Alm 1959; Iles 1974), and differences in temperature and growing season opportunity between the laboratory and the field suggest that there is phenotypic plasticity in size (Schluter 1993). The studied lakes are ice-covered from about December to May (Hamon TR, personal communication), potentially limiting food availability for resident fish and inducing smaller size. In all cases, water was warmer and more food was available in the lab than in the wild, potentially allowing lab offspring to grow larger. However, anadromous offspring raised in freshwater grow smaller than if grown in salt water (Marchinko and Schluter 2007; McGuigan et al. 2011), possibly limiting the growth potential of our F1 anadromous fish. Alternatively, the large difference in size seen in the wild between Naknek River fish and the two lake-types may be due in part to differences in age between these fish. It is possible that the life expectancy of anadromous fish is longer than lake-types, especially given the latitude and length of ice-cover on these lakes. Collectively, these data reinforce the utility of common garden approaches to determine extant natural genetic variation in adaptive traits.

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