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Reintroduction of threespine stickleback into Cheney and Scout Lakes, Alaska

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ABSTRACT

Background: Marine or anadromous threespine stickleback have colonized many northern Holarctic lakes after glacial recession, and their freshwater descendants have diverged in characteristic ways. Such divergence begins within a few generations, but previous studies have sampled only one generation or initiated sampling several generations after the populations were founded. Rotenone treatment of two Cook Inlet lakes to exterminate invasive northern pike also eliminated native threespine stickleback, creating an opportunity to introduce anadromous stickleback and observe their adaptation to freshwater conditions for multiple generations, beginning immediately after we founded the populations.

Methods: In 2009 and 2011, we released about 3000 reproductively mature anadromous threespine stickleback into each lake. We sampled the source population and made annual samples from the two introduced populations. These samples have been preserved for future analysis. We estimated annual variation in relative abundance, made preliminary morphological observations, and assessed parasite diversity.

Results: Anadromous stickleback released into the lakes produced abundant progeny, many of which survived and became reproductively mature the following spring. Both populations experienced demographic bottlenecks in 2013 and 2014 and began to recover in 2015. Preliminary observations indicate that stickleback in both lakes resemble anadromous stickleback, but by 2015 about 20% of the specimens in one population had a highly heritable, freshwater phenotype. One lake population had roughly twice as many parasite species and a much higher prevalence of a large metazoan parasite than either the anadromous ancestor or the other lake population. Our preliminary observations indicate that substantial

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evolution occurred during the first few generations, our existing samples can be used to study contemporary evolution, and sampling should be continued.

Keywords: adaptive radiation, Cheney Lake, colonizing species, contemporary evolution, experimental evolution, *Gasterosteus aculeatus*, parasite, *Schistocephalus solidus*, Scout Lake, threespine stickleback.

INTRODUCTION

Darwin (1859: 84) asserted repeatedly that evolution cannot be observed in the present: 'We see nothing of these slow [evolutionary] changes in progress, until the hand of time has marked the long lapse of ages . . . we only see that the forms of life are now different from what they formerly were.' Like so much of *The Origin of Species*, this assertion anticipated a criticism of Darwin's proposal of evolution by means of natural selection. Despite subsequent evidence, especially evolution of industrial melanism (Majerus, 1998) and of antibiotic (Baquero and Blázquez, 1997) and pesticide resistance (Gould, 2010), Darwin's assertion discouraged research on 'contemporary evolution' for 140 years. Hendry and Kinnison (1999) stimulated interest in this phenomenon, and subsequent research has confirmed that contemporary evolution is ubiquitous (e.g. Hendry and Kinnison, 2001; Palumbi, 2001; Hendry *et al.*, 2008; Thompson, 2013).

Contemporary evolution of threespine stickleback fish (*Gasterosteus aculeatus*) is not unusually fast, but it is conspicuous, involves diverse traits, and is highly replicated (Bell and Aguirre, 2013). Colonization of fresh water by oceanic (i.e. resident marine or anadromous) stickleback constitutes a major ecological transition. The demands for osmoregulation (Wootton, 1976; Marchinko and Schluter, 2007; Schultz and McCormic, 2013; Divino *et al.*, 2016) and temperature tolerance (Barrett *et al.*, 2011) are greater in fresh water than in the ocean. Large-mouthed predatory fishes are abundant and diverse in the ocean, where there is limited structural refuge, but they are more rare or absent in fresh water, where insect and bird predation occur, and structural refuge may be accessible. Different phenotypes to avoid capture and ingestion should be favoured (Reimchen, 1994, 2000). The stickleback diet contrasts between marine and freshwater habitats (Hart and Gill, 1994). Parasites also differ (Barber, 2007), creating a fitness cost for colonization of fresh water by oceanic threespine stickleback (MacColl and Chapman, 2010). Contrasting habitat structure, predators, and prey usually impose different locomotory demands (Walker, 1997; Spoljaric and Reimchen, 2007). Colonization of fresh water by oceanic stickleback should cause strong directional selection on diverse phenotypes.

Contemporary evolution of oceanic threespine stickleback that have recently colonized fresh water can be interpreted by reference to the vast literature on adaptation in this species (e.g. Hagen and Gilbertson, 1972; Bell and Foster, 1994; Bell and Ortí, 1994; Reimchen, 1994; Walker, 1997; Spoljaric and Reimchen, 2007). Oceanic stickleback have repeatedly colonized fresh water, and the resulting freshwater isolates have diverged from their oceanic ancestors and diversified ecologically and phenotypically (e.g. Lindsey, 1962; McPhail and Lindsey, 1970; Bell, 1976, 1984; Bell and Foster, 1994). Alleles for their adaptation to fresh water have been recycled from freshwater stickleback into anadromous populations by introgressive hybridization, and these recycled alleles provide variation for adaptation each time oceanic stickleback colonize fresh water (Colosimo *et al.*, 2005; Schluter and Conte, 2009; Bell and Aguirre, 2013). The frequency of 'recycled alleles' should increase more rapidly in response to selection than new mutant

alleles because there may be multiple copies of recycled alleles in colonizing populations, and they are not random variants, but are adaptive for fresh water (Barrett and Schluter, 2007). Hundreds of recycled freshwater-adapted alleles have been identified (Jones *et al.*, 2012), and many of them are linked within genomic islands of divergence (Hohenlohe *et al.*, 2012), so they respond to selection as a unit (Feder *et al.*, 2012). Roesti *et al.* (2014) recently identified a genomic signature for such recycled alleles in derived populations and applied it to freshwater stickleback. Phenomena with broad implications for evolutionary theory can be investigated at phenotypic and genomic levels to study adaptation after oceanic stickleback colonize fresh water.

Several studies have been made of contemporary evolution of anadromous threespine stickleback populations that had recently colonized fresh water (Bell and Aguirre, 2013; Lescak *et al.*, 2015). Unfortunately, the earliest samples used in these studies were usually made decades after colonization, missing the earliest evolutionary events. For example, Lescak *et al.* (2015) sampled several resident freshwater populations only 50 years after being founded by anadromous stickleback in uplifted island lakes, and evolution of freshwater phenotypes had already been completed. Similar divergence occurred within two decades after anadromous stickleback colonized another lake and was well under way within 7 years after founding (Bell *et al.*, 2004; Arif *et al.*, 2009; Aguirre and Bell, 2012). Significant changes of heritable phenotypes (Kristjánsson, 2005) and genotypes occurred (Barrett *et al.*, 2008) within one generation after marine stickleback were experimentally transferred into ponds, but these studies were limited to one generation. Adaptation to freshwater conditions appears to begin immediately after anadromous stickleback colonize fresh water, involves a large number of phenotypes and genes, but it apparently happens so fast that natural colonization is unlikely to be discovered in time to observe it.

We exploited an opportunity to introduce anadromous threespine stickleback into Cheney and Scout Lakes in the Cook Inlet basin, Alaska, USA after they were treated with rotenone to exterminate invasive populations of northern pike (*Esox lucius*), which incidentally eliminated the native threespine stickleback populations. The threespine stickleback is native to most low-elevation Cook Inlet lakes (Bell and Ortí, 1994; Bourgeois *et al.*, 1994; Foster *et al.*, 2003; von Hippel, 2008), but the northern pike is exotic (McPhail and Lindsey, 1970; Morrow, 1980; Mecklenburg *et al.*, 2002). It was illegally introduced into the Cook Inlet basin in the late 1950s and has dispersed widely since then (Haught and von Hippel, 2011; Sepulveda *et al.*, 2013; Massengill, 2014). It eats diverse animal prey, including fishes, which may decline or disappear from lakes (Patankar *et al.*, 2006; Haught and von Hippel, 2011; Massengill, 2014; Sepulveda *et al.*, 2015).

The threespine stickleback normally contributes valuable ecosystem functions in lakes (Harmon *et al.*, 2009; Matthews *et al.*, 2016; Rudman and Schluter, 2016). It eats small invertebrates (Wootton, 1976; Hart and Gill, 1994), serves as a host for numerous parasite species (Love and Moser, 1983; Barber, 2007), and is eaten by a diverse array of insects, fishes, and birds (Reimchen, 1994). Thus, the Alaska Department of Fish and Game decided to re-establish threespine stickleback in Cheney and Scout Lakes after they were treated with rotenone. Although anadromous stickleback are poorly adapted to lake conditions and may initially contribute poor ecosystem function, they have excellent potential to adapt to lakes and contribute to ecosystem function in the future (Faith *et al.*, 2010; Hendry, 2016).

We released about 3000 anadromous stickleback from Rabbit Slough, Alaska into Cheney and Scout Lakes in 2009 and 2011, respectively, to restore stickleback ecosystem function and to simulate natural colonization of lakes by anadromous stickleback. Divino *et al.* (2016), Kurz *et al.* (2016), and Wund *et al.* (2016) have already used these introduced

populations to study contemporary evolution. The purpose of this paper is to provide the background for future studies of these populations and establish that they are already adapting to lake conditions.

MATERIALS AND METHODS

Cheney and Scout Lakes and their treatment with rotenone

Cheney Lake is an artificial 3.3 ha impoundment 61 m above sea level with maximum and mean depths of 4.3 and 1.7 m, respectively (Alaska Department of Fish and Game, undated). It formed in 1972 when a gravel pit was flooded (Anchorage Park Foundation, 2016). It is located in a high-density residential setting within the Anchorage Municipality (61.2003°N, 149.7619°W). It has no tributary or discharge streams, although a screen-capped (6.35 mm mesh) subterranean 30 cm diameter pipe discharges from it into Chester Creek, about 1.5 km away. Northern pike were detected in Cheney Lake in 2000, and it was treated with rotenone on 21 and 22 October 2008. Rotenone was still detectable beneath the ice until 20 February 2009. No northern pike or threespine stickleback were captured with gill nets and minnow traps (648 trap-hours of effort) set continuously from 4 to 8 May 2009, but Alaska blackfish (*Dallia pectoralis*) did survive and are still present (www.evolutionary-ecology.com/data/2966Appendix.txt). The lake is stocked with chinook salmon (*Oncorhynchus tshawytscha*), coho salmon (*O. kisutch*), and rainbow trout (*O. mykiss*) (Alaska Department of Fish and Game, undated), which prey on threespine stickleback (Reimchen, 1994). Dense filamentous algae grow near shore.

In contrast, Scout Lake covers 38.5 ha, is 75 m above sea level, and has maximum and mean depths of 6.1 and 4.0 m, respectively (Alaska Department of Fish and Game, undated). It is located in a wooded, sparsely developed suburban area in Sterling, Kenai Peninsula Borough (60.5353°N, 150.8322°W). It is a natural seepage lake with no tributary or outlet streams. Northern pike were discovered in 2005, and the Alaska Department of Fish and Game concluded that they were reproducing in 2006 (Massengill, 2014). Scout Lake was treated with rotenone on 13 and 14 October 2009. No live fish were captured after 14 October 2009, and caged fish were inviable until at least 14 June 2010. Sixty-six trap-hours of effort on 11 May 2010 produced no stickleback (2966Appendix.txt). It is stocked with coho salmon, rainbow trout, and Arctic grayling (*Thymallus arcticus*) (Alaska Department of Fish and Game, undated). Rooted plants are common near shore, but filamentous algae are not.

Capture, processing, and release of anadromous threespine stickleback

We set unbaited, 6.35 mm mesh Gee minnow traps across the outlet of a culvert through which Rabbit Slough flows under the Parks Highway (61.5344°N, 149.2677°W). We removed live anadromous stickleback from the traps daily, transported them to the University of Alaska Anchorage, and held them in a 1500-litre outdoor pool. We released 2964 Rabbit Slough specimens between 29 May and 3 June 2009 into Cheney Lake on the day of capture and 3047 into Scout Lake between 4 June and 4 July 2011 (Table 1). Stickleback were accumulated for a few days before release and released over a longer period of time into Scout Lake because they were less abundant in 2011, and the lake is 2½ hours' drive from the laboratory. Except for the last 744 stickleback released into Scout Lake, we used nail clippers to remove the first dorsal spine from all released stickleback and

Table 1. Numbers of anadromous Rabbit Slough stickleback released live on the dates indicated into Cheney Lake in 2009 and Scout Lake in 2011 or preserved for morphological and DNA analysis

Lake	Date	Released live (*N*)	Preserved (*N*)
Cheney	29 May 2009	608	25
	30 May 2009	732	50
	1 June 2009	961	50
	2 June 2009	663	0
	3 June 2009	0	85
	Total	2964	210
Scout	4 June 2011	862	0
	8 June 2011	297	0
	15 June 2011	1144	0
	4 July 2011	744	0
	Total	3047	0

preserved the spines in ethanol. The last 744 stickleback released into Scout Lake could not be spine clipped due to time constraints. Stickleback dorsal spines deter predation by fishes (Hoogland *et al.*, 1957; Reimchen, 1991) and birds (Reimchen, 1994), but the sport fish released into the lakes were too small to eat adult anadromous stickleback even after the first spine had been clipped (Reimchen, 1991), and there were few piscivorous birds on the lakes after they were treated with rotenone. Thus, removing a dorsal spine should not have significantly reduced viability of the released stickleback.

We used an overdose of MS222 to euthanize a random sample of 210 specimens from Rabbit Slough over several days of capture in 2009 (Table 1). The right pectoral fin was removed and placed in a numbered tube of 95% ethanol for genetic analysis, and the remainder of the specimen was tagged with a corresponding number, fixed in 10% buffered formalin, stored in 50% isopropyl alcohol, and stained with Alizarin Red S (e.g. Bell *et al.*, 2004, 2010) to visualize superficial bones.

Sampling Cheney and Scout Lakes for resident threespine stickleback

Several of us sampled stickleback in Cheney and Scout Lakes, but we all followed the same general procedures: Gee minnow traps (mesh size 6.35 or 3.175 mm) were set within 5 m of shore at less than 2 m depth for up to 24 hours (2966Appendix.txt). We euthanized threespine stickleback at the lake with an overdose of MS222 and immediately washed them and placed them into ethanol or 10% buffered formalin. Invasive Alaska blackfish were counted, euthanized, and discarded at Cheney Lake but were absent from Scout Lake. Salmonids were counted and released at both lakes (data not reported).

Size and sex of preserved 2009 Rabbit Slough sample

Standard length (SL, i.e. distance from the tip of the upper jaw to the end of the last vertebra) was measured with digital calipers, and sex was determined by gonadal

examination (Wootton, 1976) in 210 specimens sampled from Rabbit Slough in 2009. Sexing by gonadal examination was quick and accurate because the fish were sexually mature.

Lateral plate morph composition of stickleback samples

Formalin-fixed, Alizarin-stained specimens from Rabbit Slough in 2009 and Cheney Lake in 2010, 2011, and 2015 (2966Appendix.txt) were available to score lateral plate morphs (*sensu* Hagen and Gilbertson, 1972; see also Bell, 1981; Bell and Foster, 1994; Bell *et al.*, 2004) by inspection with or without magnification, depending on size. Most preserved specimens were in use for other studies or preserved in ethanol for DNA analysis and could not be phenotyped. Complete morphs have an uninterrupted row of modally 33 plates per side running continuously from the head onto the caudal peduncle. Partial morphs have anterior and posterior plate rows separated by an unplated area at least two body segments wide. Low morphs have ≤ 10 anterior plates per side. Although partials may grade into completes, lows are easy to distinguish.

Catch per unit effort

We usually recorded the number of traps and the times that they were set and removed from the lakes. The number of specimens in each sample was counted at the lake or in the laboratory. Catch per unit effort (CPUE, trap-hours) is the number of specimens captured (N) divided by the product of the number of traps and the length of time in hours (h) that they were in the water:

$$\text{CPUE [trap-hours]} = N/(\text{trap} \times \text{time [h]}).$$

CPUE was plotted against days since the populations were founded. We use CPUE to estimate relative abundance of stickleback and Alaska blackfish. Most samples were made in early spring (2966Appendix.txt) and are comparable, but CPUE cannot be compared with late-summer samples that are dominated by abundant young of the year.

Parasite sampling and identification

We transported live stickleback to the University of Alaska Anchorage for parasite identification. Specimens from Rabbit Slough were examined in June 2009, the year that stickleback were released from it into Cheney Lake. Those from Cheney Lake (2010) and Scout Lake (2012) were examined for parasites during June, one year after stickleback were released into each lake. Stickleback were lightly anaesthetized with MS222 and euthanized by decapitation. One of us (G.A.B.) examined 10 specimens from each sample under a dissection microscope, and some organs (e.g. gall bladder) were inspected under a compound microscope. The integument, gastrointestinal tract, and all internal organs were examined. Hoffman (1999), Lom and Dykova (1992), and Moles (2007) were used to identify parasites, and Olsen (1974) was used to determine parasite life cycles.

Separately, we dissected preserved stickleback to determine whether they were infected by *Schistocephalus solidus*, a large, conspicuous cestode. Infection prevalence is the number of infected fish divided by the number of fish examined.

RESULTS

Preserved threespine stickleback samples

Ethanol-preserved specimens were stored in 70% ethanol and formalin-fixed specimens in alcohol solutions (2966Appendix.txt). Some formalin-fixed specimens were stained with Alizarin Red S to visualize the lateral plates. The formalin-fixed specimens are in the collectors' laboratories while the ethanol-preserved samples are in David M. Kingsley's laboratory at Stanford University School of Medicine (2966Appendix.txt). May and June samples from Cheney Lake average 192 specimens per year (the two preservatives combined) and range up to 703 specimens, while samples from Scout Lake average 56.4 and range up to 445 specimens per year. All specimens appeared to have anadromous phenotypes when they were removed from traps or counted in the lab, but none of them were large enough to have been anadromous stickleback that we had released into the lakes.

Size and sex ratio of the Rabbit Slough sample

A sample of 210 formalin-fixed, anadromous stickleback from Rabbit Slough in 2009 has a standard length of 61–77 mm, except for two small females (Fig. 1). Mean standard length was 69.3 mm for females and 65.7 mm for males. There were 127 (60.5%) females and 83 (39.5%) males.

Lateral plate morph variation

Only formalin-fixed samples in M.A. Bell's lab were stained with Alizarin Red S to score lateral plate morphs. The 2009 Rabbit Slough ($N = 210$) and 2010 Cheney Lake samples ($N = 343$) were monomorphic for the complete morph. However, we examined five samples from Cheney Lake in 2011 ($N = 1128$), one of which ($N = 427$, MAB11-6E,

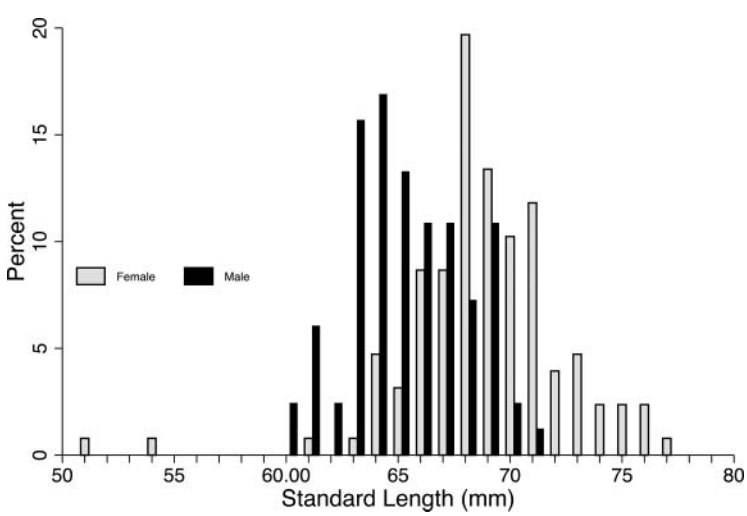

Fig. 1. Size-frequency distribution of anadromous threespine stickleback from Rabbit Slough in 2009.

2966Appendix.txt) included three low morphs (0.7%). By August 2015, low morph frequency in three samples had increased to an average of about 20% ($N = 68$ of 314, 41 of 223, and 25 of 123 specimens, respectively).

Catch per unit effort

Threespine stickleback were reintroduced into Cheney and Scout Lakes in different years, so we report temporal change in the catch per unit effort (CPUE) by days or years after reintroduction. CPUE in both lakes varied considerably among trap sets within years, but their range of values formed a pattern among years (Figs. 2, 3; 2966Appendix.txt). One year after release into Cheney Lake, we obtained maximal CPUE, averaging 2.4 stickleback per trap-hour. It declined to 1.1 in year 2 and 0.34 in year 3. Despite 1751 trap-hours (2966Appendix.txt), virtually no stickleback were caught in Cheney Lake in years 4 (mean CPUE = 0.012 per trap-hour) and 5 (mean CPUE = 0 per trap-hour). CPUE recovered to 0.2 fish per trap-hour in year 6, and increased later that summer to 1.5, mostly small stickleback born a few months earlier. Mean CPUE did not vary among years as much in Scout Lake as in Cheney Lake, the bottleneck was earlier, but the general pattern of temporal change was similar. CPUE was maximal the first year, averaging 2.0 stickleback per trap-hour, dropped to about 0.1 in the second and third years, and increased to 0.75 in year 4, the last year it was sampled (2015). We observed numerous fry in the shallows of both lakes in June 2015 and captured large samples of young in Cheney Lake in August 2015 (2966Appendix.txt).

Alaska blackfish do not occur in Scout Lake but have become abundant in Cheney Lake since 2012. Blackfish averaged about 0.003 per trap-hour in 2010 and 2011. Since then,

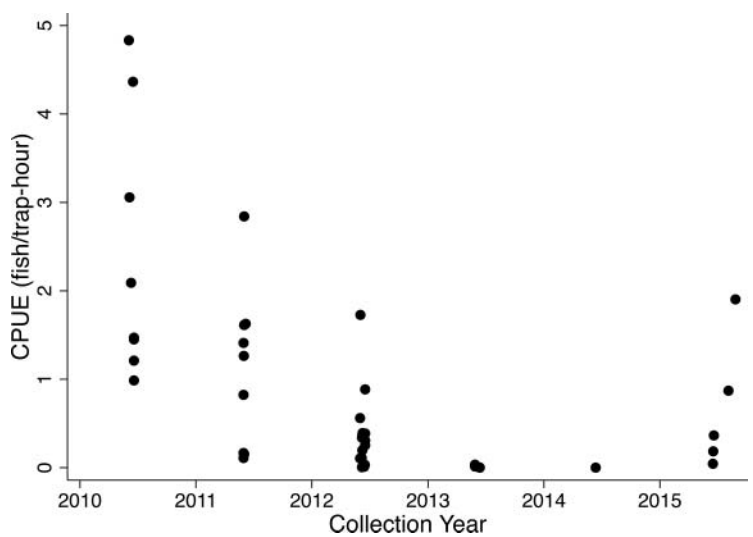

Fig. 2. Temporal change in catch per unit effort (CPUE) of threespine stickleback from Cheney Lake, beginning the year after introduction: 2010. Points were plotted by elapsed days since introduction, though only years are indicated in the axis label. Some points overlap and are not visible. See 2966Appendix.txt for sample details.

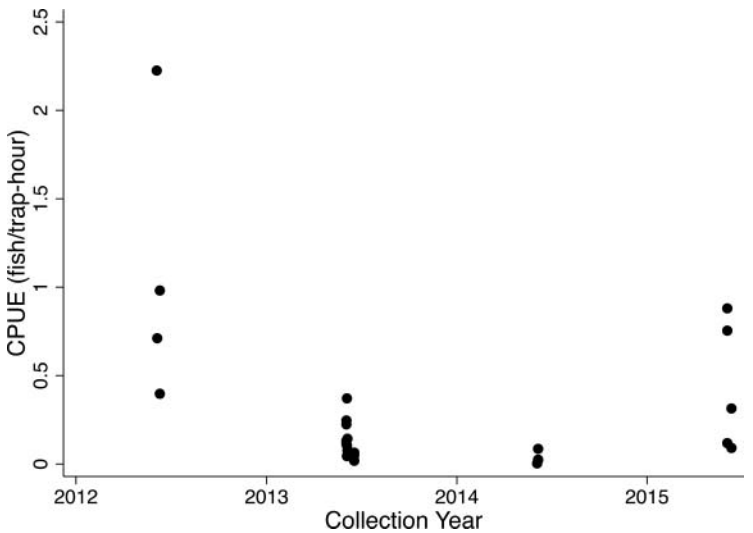

Fig. 3. Temporal variation in catch per unit effort (CPUE) of threespine stickleback from Scout Lake, beginning the year after introduction: 2012. Points were plotted by elapsed days since introduction, though only years are indicated in the axis label. Some points overlap and are not visible. See 2966Appendix.txt for sample details.

annual average blackfish CPUE has increased to 0.21, 0.079, 0.11, and 0.27 in June samples between 2012 and 2015.

Stickleback parasites

Seventeen parasite taxa (Table 2), including 13 species, three additional genera, and unidentified digenean metacercaria cysts ('black spot') were observed in the stickleback samples. Five species are unicellular eukaryotes, 10 belong to the Platyhelminthes, one species is an acanthocephalan, and one is a mollusc. Five of the platyhelminths are trematodes. Ten of the parasite taxa have complex life cycles (i.e. at least two hosts), including three from Rabbit Slough, seven from Cheney Lake, and three from Scout Lake. Six parasite taxa infected Rabbit Slough stickleback, the Cheney Lake sample produced 12 parasite taxa, including 10 identified species, and the Scout Lake sample had only four parasite species plus one assigned only to genus. Two species infected all three populations, two infected two populations, and 13 (nine from Cheney Lake) infected only one population. Four of the Cheney Lake parasite species occurred in the Rabbit Slough sample and two of the parasite species from Scout Lake occurred in the other two samples. About half of the parasite species are generalist fish parasites, and the other half specialize on sticklebacks. There are no obvious differences among populations for the frequency of stickleback specialists. Nine of the parasite species have not been reported previously from Alaska (Table 2) (Moles, 2007).

The prevalence of plerocercoids of the cestode, *Schistocephalus solidus*, exceeded 60% (unweighted mean) in Cheney Lake stickleback during the three years sampled (2010: 207 infected/464 dissected; 2011: 733/926; 2012: 381/605), but prevalence averaged less than 2% in Scout Lake stickleback (2012: 6/446; 2013: 12/496; 2014: 0/54).

Table 2. Presence (+) and absence (–) of threespine stickleback parasites from Rabbit Slough in 2009, Cheney Lake in 2010, and Scout Lake in 2012, the years after the lake populations were founded using anadromous stickleback from Rabbit Slough

Parasite	Rabbit Slough	Cheney Lake	Scout Lake	Host D	Host I^1	Host I^2	Class	Breadth
*Trichodina tenuidens**	+	+	–	G	—	—	Ciliophora	S
Trichodina domerguei	–	+	–	G	—	—	Ciliophora	Ge
*Goussia aculeata**	–	+	–	G	—	—	Conoidasida	TS
*Myxobilatus gasterostei**	–	+	–	G	I	—	Myxosporea	S
*Sphaerosphora elegans**	–	+	–	G	I	—	Myxosporea	TS
*Apatemon gracilis**	+	+	+	B	Mo	G	Trematoda	Ge
*Crepidostomum metoecus**	–	+	–	G	Mo	C	Trematoda	Ge
Crepidostomum farionis	–	–	+	G	Mo	C	Trematoda	Ge
Diplostomum sp.*	–	+	–	B	Mo	G	Trematoda	
Digenea sp. (blackspot)**	–	+	–				Trematoda	
*Gyrodactylus alexanderi**	+	+	+	G	—	—	Monoginea	TS
Dactylogyrus sp.	–	–	+	G	—	—	Monoginea	
Dilepis sp.*	+	–	–	B	Mo	G	Cestoda	
Schistocephalus solidus	–	+	–	B	G	P	Cestoda	TS
Dyphyllobothrium dendricum	–	–	+	Ma	P	G	Cestoda	Ge
Neoechinorhynchus rutili	+	–	–	G	C	—	Eoacanthocephala	Ge
Anodonta beringiana	+	+	–	G	—	—	Bivalva	Ge
Total taxa	6	12	5					

Note: Abbreviations for *Host* column: D, definitive host in which the parasite reproduces; I^1, first intermediate host; I^2, second intermediate host; and host taxa, G, *Gasterosteus*; I, insect; Ma, mammal; Mo, mollusc; B, bird; P, planktonic crustacean; C, benthic crustacean. A long dash (—) in an intermediate host column indicates that there is no primary or secondary intermediate host. If the only entry is in the definitive host column, the parasite has a direct life cycle on *G. aculeatus*. *Breadth* refers to specificity for the fish host and abbreviations are: Ge, generalist; S, use of threespine and ninespine sticklebacks; and TS, use of only threespine stickleback.
* Not reported in Moles (2007), new records for Alaska. ** Unidentified melanized digenean metacercaria.

DISCUSSION

Use of the Rabbit Slough population to found lake populations

Many populations could have been chosen to re-establish threespine stickleback in Cheney and Scout Lakes. The Rabbit Slough anadromous population offered several advantages (Frankham *et al.*, 2009) and also provided an opportunity to study adaptation of anadromous stickleback to freshwater conditions. In Rabbit Slough, we could capture numerous sexually mature individuals that had not yet reproduced. They are easy to transport, and the population has tolerated intensive sampling before (Aguirre *et al.*, 2008; Furin *et al.*, 2012). Anadromous females produce larger clutches than smaller freshwater females (Baker, 1994; Baker *et al.*, 2008; Karve *et al.*, 2013; Kurz *et al.*, 2016), maximizing the potential number of progeny per introduced female. Anadromous populations, including Rabbit Slough, are genetically diverse compared with resident freshwater populations (e.g. Withler and McPhail, 1985; Taylor and McPhail, 1999, 2000; Aguirre, 2007), and they contain numerous freshwater-adapted alleles (Jones *et al.*, 2012). Anadromous stickleback have founded innumerable freshwater populations since

Holarctic deglaciation (e.g. Lindsey, 1962; McPhail and Lindsey, 1970; Bell, 1976; Schluter and Conte, 2009), including well-documented recent colonization events (Bell and Aguirre, 2013; Lescak *et al.*, 2015), indicating that our introductions would succeed. Reintroduced individuals should be adapted to the recipient habitat (Frankham *et al.*, 2009), but anadromous stickleback are not well adapted to lakes. However, stickleback introduced from another lake might have cryptic, maladaptive phenotypes [e.g. for immunity (see Scharsack *et al.*, 2007)] and limited genetic variation to adapt. Thus, we chose to use a genetically diverse but poorly adapted anadromous stickleback from Rabbit Slough.

First-generation reproductive output

We can make a crude but instructive estimate of the number of eggs produced by the Rabbit Slough stickleback that we released into Cheney Lake in 2009. Kurz's (personal communication, 4 October 2015) regression equation for clutch size (CS) on SL for Rabbit Slough stickleback in 2009 is (Kurz *et al.*, 2016):

$$\text{CS (eggs)} = -358.014 \text{ eggs} + (8.674 \text{ eggs} \times \text{SL}).$$

Using this equation and the mean SL of Rabbit Slough females in 2009 (i.e. 69.3 mm), we estimate that each female released into Cheney Lake produced an average of 243 eggs per clutch, which is similar to an estimate for a nearby anadromous population (Karve *et al.*, 2013). Female stickleback in Cook Inlet lakes spawn about every 4 days (Brown-Peterson and Heins, 2009) and stop breeding in late June (Heins *et al.*, 1999). Rabbit Slough females were gravid until 23 June in 2005 and 2006 (M.A. Bell, unpublished data), confirming this date for persistence of breeding by Rabbit Slough fish. The weighted mean date of release of stickleback into Cheney Lake in 2009 was 31 May (Table 1), allowing an average female to breed at least six times and produce 1458 eggs. We released 2964 stickleback into Cheney Lake, of which about 60.5% were females. Thus, 2,614,515 stickleback eggs could have been produced in Cheney Lake in 2009. This number should be viewed with caution, but despite predation (Reimchen, 1994) and cannibalism (Foster, 1994; Whoriskey and FitzGerald, 1994) of eggs and fry, hundreds of thousands to a few million progeny could have been produced. This crude estimate is consistent with our capture of numerous one-year-old stickleback in Cheney Lake in 2010 (Fig. 2; 2966Appendix.txt). A similar number of eggs should have been produced in Scout Lake, although the delayed introduction of some individuals may have reduced egg production. The large number of eggs produced in each lake to form the F_1 generation has important implications for contemporary evolution.

Parasite diversity and *Schistocephalus* prevalence

Stickleback parasites differ among habitats (Wegner *et al.*, 2003; MacColl, 2009) and should play a major role in both the ability of species to colonize (Scharsack *et al.*, 2007; MacColl, 2009; MacColl and Chapman, 2010) and their subsequent adaptation to a new habitat (Haldane, 1949; Anderson and May, 1982; Milinski, 2006). Anadromous and lake-resident stickleback should be infected by different parasite species because their habitats contrast for salinity and availability of other hosts in the life cycle (Barber, 2007). Stickleback acquire many parasites from their prey (Barber, 2007; Rybkina *et al.*, 2016), which must influence infection in different habitats. This difference appears to be a cost to anadromous stickleback colonizing fresh water (MacColl and Chapman, 2010). Lake stickleback experience greater parasite diversity than river populations (Scharsack *et al.*, 2007),

and sympatric planktivorous stickleback are infected by different parasite species than benthic ones (MacColl, 2009). Lake populations are more resistant than river populations to experimental infection (Scharsack *et al.*, 2007), and experimental infection imposes strong directional selection on major histocompatibility complex (MHC) alleles (Eizaguirre *et al.*, 2012a, 2012b). Thus, we expected anadromous Rabbit Slough stickleback to initially suffer greater parasite infection in Cheney and Scout Lakes than in their native habitat but to have more allelic variation than lake fish to adapt to their new lake habitats. We also anticipate rapid contemporary evolution of MHC and other alleles related to immunity in these populations.

The numbers of stickleback populations and parasite taxa are too small for meaningful statistical comparison, but the Scout Lake and Rabbit Slough samples had roughly equal parasite diversity and *Schistocephalus* prevalence. In contrast, the Cheney Lake sample was infected by at least twice as many parasite taxa and had 30 times greater *Schistocephalus* prevalence. Parasite taxonomic diversity in our samples might be suspect because we examined only 10 fish per population, but the sample sizes were equal among populations, and the Cheney Lake population appears to suffer much greater parasitism, which was comparable to that observed by Rybkina *et al.* (2016), who used much larger samples. The difference in numbers of parasite species between the two lake populations is probably real, and it is unlikely to reflect heritable differences for host resistance, because we assessed parasitism during their first generation in each lake.

Elevated stickleback parasitism in Cheney Lake may have resulted from how we performed the introductions or several ecological differences between the lakes. We introduced stickleback to Cheney Lake only 7 months after the original population was exterminated, whereas the Scout Lake introduction was not performed until 19 months after the lake was treated. If short-lived infected hosts had died before the stickleback were released, the delay of the Scout Lake reintroduction could have interfered with complex life cycles of stickleback parasites (Table 2) (Olsen, 1974). However, the proportion of parasites with complex life cycles in the Cheney Lake sample is no higher than that in the other two samples. Alaska blackfish persisted in Cheney Lake, but it was probably not a reservoir that caused increased parasitism in the Cheney Lake stickleback because they were not enriched with generalist parasite species that could have infected blackfish. Specifically, elevated prevalence of *S. solidus* in Cheney Lake stickleback could not have resulted from persistence of blackfish because *S. solidus* is a threespine stickleback specialist (Orr *et al.*, 1969). Threespine stickleback acquire *S. solidus* by eating infected planktonic copepods (Barber, 2013), and sympatric planktivorous (i.e. limnetic) stickleback are more likely than benthic-feeding stickleback in lakes to be infected by it (MacColl, 2009). This should cause greater infection by *S. solidus* in the deeper, more limnetic Scout Lake, yet the opposite was observed. Relatively eutrophic conditions in Cheney Lake may have favoured elevated parasite prevalence. Unfortunately, we have only two reintroduced populations to compare, and they have numerous ecological differences. Consequently, we cannot attribute elevated parasitism in the Cheney Lake population to any one of several potential causes, but there appear to have been real difference in parasitism. If they persist, they could cause divergence of immunological traits between the two reintroduced populations (Eizaguirre *et al.*, 2012a, 2012b).

Demography of the introduced populations

Despite the uncertainty of our estimate of the reproductive output of anadromous stickleback released into Cheney Lake, several hundred thousand to a few million stickleback eggs were produced there in 2009. Our CPUE estimates are also crude but consistent with this large estimate (Figs. 2, 3; 2966Appendix.txt). Reproductively mature stickleback that were born in both lakes were abundant a year after stickleback were introduced (Kurz *et al.*, 2016). However, both populations experienced demographic bottlenecks. The bottleneck in Cheney Lake occurred two years later and was more severe than in Scout Lake. The released anadromous stickleback normally breed in fresh water, and they apparently produced numerous progeny that grew and survived well in both lakes. Perhaps only rare individuals with sets of multiple freshwater-adapted alleles (see below) reproduced in later generations, and the population growth apparent in 2015 is due to their increased frequencies and elevated reproductive success.

Although both populations were established with about 3000 adults from the same source, they experienced somewhat different demographic histories. Several ecological differences between the lakes might be responsible. Scout Lake is larger and deeper than Cheney Lake. It may be more suitable for anadromous phenotypes (see Spoljaric and Reimchen, 2007) and expose them to different parasites (MacColl, 2009). Greater parasite infection in Cheney Lake may have depressed population growth (Heins *et al.*, 1999, 2010). Cheney Lake is an urban lake with greater anthropogenic influence. It is more eutrophic than Scout Lake, and its greater turbidity could have reduced feeding efficiency (Sohel, 2015) and mate detection (Courchamp *et al.*, 2008). Cheney Lake contains Alaska blackfish, which eat threespine stickleback and may compete with them for prey (Eidam *et al.*, in press). Thus, several ecological differences may have contributed to demographic differences between the two populations and may select for different traits.

Contemporary evolution in threespine stickleback: source of genetic variation

Both the Cheney and Scout Lake populations resemble anadromous stickleback that developed in fresh water (see Wund *et al.*, 2008, 2016). They began to adapt to freshwater conditions quickly, suggesting that adaptation is based on existing allelic variation and not on new mutations (Barrett and Schluter, 2007). This genetic variation could have originated among surviving resident freshwater stickleback or in the introduced Rabbit Slough fish. Rotenone was at lethal concentrations for an extended period in both lakes, and caged fish in Scout Lake died for some time after treatment. No stickleback were captured between treatment and release of the Rabbit Slough stickleback in either lake. Both lakes lack inlet or outlet streams, and it is unlikely that small stickleback could disperse up the long drainage pipe from Cheney Lake into Chester Creek and enter the lake through the fine mesh cap. Thus, it is very likely that in both populations freshwater-adapted alleles were carried by the introduced Rabbit Slough stickleback.

When oceanic stickleback colonize fresh water, they convergently evolve typical freshwater phenotypes based on freshwater-adapted alleles carried at low frequencies by ancestral populations (Colosimo *et al.*, 2005; Schluter and Conte, 2009; Jones *et al.*, 2012; Bell and Aguirre, 2013). However, colonizing populations of most species may be small, and intense directional selection may further reduce effective population size (N_e), exacerbating loss of adaptive variation through genetic drift (Falk *et al.*, 2012; Tobler *et al.*, 2013). Accordingly, ancestral genetic

variation segregating at low frequencies in oceanic populations may be lost during or soon after colonization due to founder effects, genetic drift, or the winnowing effects of intense directional selection (Lescak *et al.*, 2015), but these effects have not prevented freshwater-adapted alleles at many loci from being recycled between oceanic and freshwater populations for millions of years (Colosimo *et al.*, 2005; Jones *et al.*, 2012).

Recently, Jones *et al.* (2012) identified 242 genomic regions with separate monophyletic freshwater and oceanic sub-clades in their gene trees. Thus, these freshwater sub-clades must each have evolved from one common ancestral sequence, been carried as rare variants in oceanic populations, and formed the basis for adaptation during colonization of fresh water. Instead of being scattered throughout the genome as separate loci (Schluter and Conte, 2009), however, many of them form groups of linked freshwater-adapted alleles (Hohenlohe *et al.*, 2012; Jones *et al.*, 2012; Roesti *et al.*, 2013; Miller *et al.*, 2014) that increase fitness in fresh water. There is also limited evidence that these rare freshwater-adapted alleles are partially recessive (Bell and Aguirre, 2013). At low frequencies in oceanic populations, they will rarely be homozygous and be expressed, and they should experience weak purifying selection. Recessive freshwater-adapted alleles could have started accumulating at least 10 million years ago (e.g. Bell and Foster, 1994; Bell *et al.*, 2009) and been maintained in oceanic populations in a gene flow-selection equilibrium (Levene, 1953; Hedrick, 2006). After millions of years, oceanic threespine stickleback populations have accumulated hundreds of them.

Although this pool of freshwater-adapted alleles facilitates rapid adaptation to fresh water, there is evidence that some of them are lost during colonization. Lescak *et al.* (2015) studied several freshwater populations that were derived from oceanic ancestors since 1964 but have evolved typical freshwater phenotypes based on different sets of alleles. Similarly, Leinonen *et al.* (2012) described boreal lake populations with reduced lateral plate coverage, but this reduction resulted from reduced plate size rather than the more typical reduced plate number. They argued that small plate size evolved in the absence of Ectodysplasin alleles (eda^L) for low plate number (Colosimo *et al.*, 2004), reducing the fitness costs of high plate coverage in fresh water (Barrett, 2010). Thus, there is evidence that some freshwater-adaptive alleles of oceanic ancestors may be lost when they found freshwater populations.

Even if they are not lost, recessive freshwater-adapted alleles will initially be at low frequencies (p), and homozygotes that express them should be much rarer during the first generation (F_1) in the lake [p^2, assuming random mating (Barrett and Schluter, 2007)]. Although this could have been true of the Cheney and Scout Lake stickleback populations, both of them were founded with about 3000 adults, which had a good probability of including heterozygotes at many loci with freshwater-adapted alleles at frequencies as low as tenths of a percent. They produced hundreds of thousands to a few million F_1 progeny during the first generation, and homozygotes for recessive freshwater phenotypes should have occurred at appreciable frequencies in the F_1 and subsequent generations, even before selection increased their frequencies. The effects of intense directional selection on genetic variation are difficult to predict and could have eroded genetic diversity (Falk *et al.*, 2012; Tobler *et al.*, 2013), but the populations reintroduced into Cheney and Scout Lakes should contain ample genetic variation for adaptation. Preliminary results are consistent with this expectation.

Contemporary evolution: Cheney and Scout Lakes

Two concurrent studies compared stickleback phenotypes in Cheney and Scout Lakes to those in Rabbit Slough grown under common rearing conditions, and a third compared phenotypes of field-caught specimens. In Cheney Lake, body shape, which usually differs between anadromous and resident freshwater populations (Walker and Bell, 2000; Aguirre *et al.*, 2008; Aguirre and Bell, 2012; but see Spoljaric and Reimchen, 2007), likely evolved significantly towards typical freshwater phenotypes after a single generation (Wund *et al.*, 2016). The distribution of shape phenotypes in the Cheney Lake fish in this experiment suggests that divergence (Wund *et al.*, 2016) depended on alleles at many loci, which is consistent with a quantitative trait locus (QTL) analysis (Albert *et al.*, 2007). After only two generations in Scout Lake, lab-reared stickleback exhibited enhanced low-salinity tolerance compared with Rabbit Slough controls, but the genetic basis for this divergence was unclear (Divino *et al.*, 2016). Similarly, field-caught stickleback from Cheney and Scout Lakes contrasted with their Rabbit Slough ancestor for four of five female life-history phenotypes studied (Kurz *et al.*, 2016). Although phenotypic plasticity probably contributed to this life-history divergence, these phenotypes also may have evolved.

The Rabbit Slough ancestor is monomorphically completely plated (Aguirre *et al.*, 2008), and most lake stickleback populations from the Cook Inlet basin (Bourgeois *et al.*, 1994) and western North America (Hagen and Gilbertson, 1972; Bell, 1984; Barrett, 2010) are monomorphic or almost mononmorphically low plated. Thus, we expect the low morph to increase in frequency in the reintroduced populations if there is genetic variation for plate morphs. Low morphs were absent or virtually absent the first two years in the introduced Cheney Lake samples, but within six generations (i.e. 6 years) and after a two-year bottleneck, the frequency of lows had increased to about 20%. This increase resembles that in the Loberg Lake population 3–8 years after it was founded by anadromous stickleback (Bell *et al.*, 2004). Le Rouzic *et al.* (2011) obtained similar results during the first six years after a lake population was founded using equal numbers (i.e. 250) of specimens from monomorphic low and monomorphic completely plated lake populations. Plate morph expression depends strongly on the *eda* genotype, and there is limited phenotypic plasticity, so appearance of a high frequency of low morphs must represent evolution. The eda^L allele appears to be very rare in the Rabbit Slough population [i.e. 1 eda^L allele in 1554 *eda* alleles (Bell *et al.*, 2010)], but its frequency may have been underestimated because there is recombination between the marker used and the causal nucleotide in eda^L (O'Brown *et al.*, 2015). Analysis of diverse traits in four separate studies suggests that the Cheney and Scout Lake populations diverged rapidly, which is consistent with the presence of freshwater-adapted alleles in the reintroduced populations and expectations from other studies (Bell *et al.*, 2004; Kristjánsson, 2005; Barrett *et al.*, 2008; Arif *et al.*, 2009; Le Rouzic *et al.*, 2011; Aguirre and Bell, 2012).

Future research value of the Cheney and Scout Lake populations

We have described the source, introduction methods, and early demography, parasitology, and contemporary evolution of anadromous threespine stickleback populations that we founded in Cheney and Scout Lakes as a background for future studies of contemporary evolution in these populations. Loberg Lake was colonized by anadromous stickleback in the 1980s (Bell *et al.*, 2004), and it is in the same drainage as Rabbit Slough. Since it was first sampled, every typically divergent morphological trait measured in that population has

evolved towards the freshwater condition (Bell *et al.*, 2004; Arif *et al.*, 2009; Aguirre and Bell, 2012). Our limited results for lateral plate morph evolution and evidence from other concurrent studies (Divino *et al.*, 2016; Kurz *et al.*, 2016; Wund *et al.*, 2016) suggest that the earliest stages of divergence in the Cheney and Scout Lake populations are comparable to those in the Loberg Lake population. However, it will be possible to study these earliest changes using existing samples from Cheney and Scout Lakes (2966Appendix.txt).

Will other phenotypes and frequencies of freshwater-adapted alleles evolve rapidly? Will freshwater-adapted alleles evolve along the same trajectory as in the Loberg Lake population or diverge from one another as have those reported by Lescak *et al.* (2015)? Are alleles linked tightly to other adaptive alleles (Hohenlohe *et al.*, 2012; Roesti *et al.*, 2013) or alleles initially at higher frequencies less likely to be lost or to evolve faster than more isolated or more rare alleles (Tobler *et al.*, 2013)? We do not know exactly when the Loberg Lake population was founded or its ancestor's identity, but that population had started to diverge before we started sampling it (Arif *et al.*, 2009; Aguirre and Bell, 2012), and we lack DNA samples for the first 10 years of observation. These deficiencies have been remedied in our samples from Cheney and Scout Lakes.

Hardwick *et al.* (2015) discussed several problems in the interpretation of experimental field studies of selection, and they will apply to studies of contemporary evolution in threespine stickleback. However, these problems are limited in lake stickleback. Large samples are needed (Kingsolver *et al.*, 2001), and lake stickleback are abundant enough to make very large samples. Sampling during the wrong part of the life cycle may miss important periods of selection, but the short breeding season in Cook Inlet stickleback (Heins *et al.*, 1999) produces large cohorts that can be sampled periodically, especially during the first year of the typical two-year life cycle (Baker *et al.*, 2008). Selection and evolution may differ between the sexes, but threespine stickleback can be sexed by gonadal inspection (Wootton, 1976) or using genetic markers (Withler *et al.*, 1986; Peichel *et al.*, 2004), so large samples can be obtained and assigned to sex. Spatial variation of selection and evolution is another potential problem, but analysis of spatial variation within (e.g. Bell *et al.*, 2004) and among lakes is feasible for threespine stickleback. Hardwick *et al.* (2015) were concerned about distinguishing the effects of past and current evolution, but anadromous stickleback populations have limited genomic and phenotypic variation over moderately large geographical distances [e.g. Cook Inlet and the adjacent north Pacific (Hohenlohe *et al.*, 2012)], so contemporary evolution of their descendants starts from a common genotypic point.

Another problem that cannot be eliminated but can be understood in threespine stickleback is the effects of genetic covariance (Hohenlohe *et al.*, 2012; Jones *et al.*, 2012; Roesti *et al.*, 2013). Stickleback samples can be large enough to assess phenotypic covariances for numerous traits, and genetic covariances can be studied using linkage analyses. For example, many phenotypes map to the *eda* locus (Colosimo *et al.*, 2005; Albert *et al.*, 2007; Miller *et al.*, 2014), and alleles over large linkage distances may not respond to selection independently (Tobler *et al.*, 2013). In addition, plate morph, which is strongly influenced by *eda,* influences growth rate (Marchinko and Schluter, 2007; Barrett *et al.*, 2009), and there is good reason to expect that size influences selection based on both survival (e.g. Shoup and Wahl, 2011) and reproductive fitness (Rowland, 1994; Baker *et al.*, 2008; Furin *et al.*, 2012). While this correlation between plate morph and growth rate has been recognized, it would have been cryptic without careful analysis, and similar unrecognized correlations may exist. This pitfall is limited in stickleback but cannot be eliminated.

The final challenge noted by Hardwick *et al.* (2015) is deviation from natural conditions. Lakes treated with rotenone might exhibit lingering ecological effects, but contemporary

stickleback evolution in Loberg Lake, which was treated with rotenone in 1982, has been consistent with divergence among older populations (Bell *et al.*, 2004; Arif *et al.*, 2009; Aguirre and Bell, 2012). In addition, lakes formed by human activities (Klepaker, 1993) or natural events (Lescak *et al.*, 2015) and never treated with rotenone can be studied, and young freshwater resident stickleback populations that evolved in such lakes exist in Cook Inlet basin (M.A. Bell *et al.*, unpublished data). Thus, the pitfalls enumerated by Hardwick *et al.* (2015) are manageable using the populations in Cheney and Scout Lakes and other young Cook Inlet populations.

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