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RESEARCH ARTICLE

# Going, going, gone: evidence for loss of an endemic species pair of threespine sticklebacks (*Gasterosteus aculeatus*) with implications for protection under species-at-risk legislation

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**Abstract** Genomic extinction occurs when the unique combination of genetic traits that characterize distinct phenotypes are eliminated by introgressive hybridization even if population size is greater than zero. Benthic and limnetic threespine sticklebacks (*Gasterosteus aculeatus*) constitute reproductively isolated undescribed biological species that have evolved independently in several lakes in southwestern British Columbia, Canada (known as "species pairs" in each lake). Here we investigated whether the two species that comprise the pair from Enos Lake, southeastern Vancouver Island, remain as two distinct gene pools. Multi-season samples (>1200 fish) obtained over two years from throughout the lake and assayed for variation in morphological traits characteristic of the two species (i.e., body depth, dorsal spine count, gill raker counts) and at 12 microsatellite DNA loci consistently indicated the existence of only a single group of sticklebacks. There was no consistent evidence of two groups in any morphological trait, and mean gill raker counts were consistently intermediate (20–21) to those of known benthics (~18) and limnetics (~24) which together comprised strikingly bimodal distributions in historical samples. Genetic analyses employing model-based clustering also consistently indicated the presence of only a single genetic group of sticklebacks. Compared to historical samples and to benthics and limnetics from other lakes, no Enos Lake fish could be identified confidently as a pure benthic or limnetic. Our results provide the strongest evidence yet that the Enos Lake sticklebacks now consist of a single morphological and genetic population of sticklebacks, that the unique combination of genetic and morphological traits that characterized benthic and limnetic sticklebacks no longer exist, and that their current status under Canada's *Species-at Risk Act* as Endangered should be re-evaluated.

**Keywords** Genomic extinction · Microsatellites · Morphology · Threespine sticklebacks · Species-at-Risk Act · Species pairs

## Introduction

Extinction can be defined as the disappearance of a species, i.e., that moment in time when the last remaining individual no longer exists and population size is zero (e.g., Standards and Subcommittee 2010). Confirming the absence of a species can be difficult, especially for widespread and naturally rare taxa, but even in confined systems rigorous sampling is needed for confidence in declaring a taxon extinct. A conservation status of "Extinct" is used in various international and national programs to assess species and provisions are often in place to encompass the uncertainty that sometimes is present when considering the probability of extinction (e.g., Standards and Subcommittee 2010). For instance, the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) acts as the arms-length advisory panel to the Canadian government to assess the conservation status of wildlife in Canada under the federal *Species at Risk Act* (SARA 2003). Guidelines used by COSEWIC define

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an "Extinct" species as one that no longer exists, and an "Extirpated" species as one that no longer exists in Canada, but occurs elsewhere. The assessments by COSEWIC recognize the uncertainty that may be associated with a status of Extinct/Extirpated by considering three guidelines when considering assigning a species Extinct/Extirpated, i.e., when: (i) there exists no remaining habitat for the wildlife species and there have been no records of the wildlife species despite recent surveys; (ii) 50 years have passed since the last credible record of the wildlife species, despite surveys in the interim; or (iii) there is sufficient information to document that no individuals of the wildlife species remain alive (COSEWIC 2016).

Freshwater fishes are, as a group, highly threatened worldwide and, in Canada, southwestern British Columbia has one of the highest proportions of at risk freshwater fishes (Dextrase and Mandrak 2006; Dudgeon et al. 2006; Austin et al. 2008). The so-called "benthic" and "limnetic" threespine sticklebacks found in sympatry in several lakes in southwestern British Columbia are a component of the *Gasterosteus aculeatus* species "complex" (sensu McPhail 2007); they are behaviourally, ecologically, morphologically, and genetically distinct in sympatry and, therefore, behave as two distinct biological species even if they are currently un-recognized as such taxonomically (McPhail 1984; Bentzen and McPhail 1984; Ridgway and McPhail 1984; Bentzen et al. 1984). These sympatric sticklebacks are known as "species pairs" and are part of a complex of forms that are endemic to British Columbia (and therefore Canada) and have arisen independently in each lake were they occur (McPhail 1994; Taylor and McPhail 2000; Jones et al. 2012). Sympatric stickleback species pairs have become well-established in the scientific realm as an example of rapid speciation in action (McKinnon and Rundle 2002; Culotta and Pennisi 2005). Further, their limited distribution in small lakes coupled with actual and potential threats to these fishes and their habitats has resulted in each pair being listed as Threatened or Endangered under Canada's *Species at Risk Act* (e.g., COSEWIC 2013). More recently, the species pair found in Enos Lake on southeastern Vancouver Island illustrates the devastating effects of invasive species may have on native diversity—the benthics and limnetics appear to have collapsed into a hybrid swarm following the appearance of the American signal crayfish (*Pascifiasticus leniusculus*) in the 1980s (Kraak et al. 2001; Taylor et al. 2006; Behm et al. 2010; Malek et al. 2012; COSEWIC 2013).

Species are required to be reassessed by COSEWIC at least every 10 years and in 2012 the Enos lake benthic and limnetic sticklebacks were reassessed. During the reassessment, and despite the evidence for the existence of a hybrid swarm, a status of Endangered was retained because the definition of "Extinct" requires that no *individual* benthic or limnetic fish remain in Enos Lake. Previous studies that had documented the presence of a hybrid swarm were not designed a priori to assess the level of hybridization (admixture) in Enos Lake fish, rather they were either retrospective analysis of pre-existing limited data (Taylor et al. 2006) or represented single season analyses of admixture in relatively small samples of fish (Malek et al. 2012). Further, studies of variation in Enos Lake sticklebacks typically did not sample from all habitats of the lake (e.g. Taylor et al. 2006; Malek et al. 2012). Consequently, COSEWIC assigned a status of Endangered as a conservative measure until more data were collected.

Obtaining robust estimates of the extent of admixture in Enos Lake stickleback has important implications because under a status of Endangered, the federal government is legally required by SARA to complete Recovery Plan and Action Plan documents within timelines and these documents then guide recovery efforts. If, however, the pair is actually Extinct, then recovery actions are not required by law and such efforts may be better placed with other wildlife species where greater potential for recovery exists. In short, the whole machinery of species recovery may be misplaced in terms of Enos Lake sticklebacks if, in fact, they are Extinct and not Endangered. Here, we report the results of an exhaustive morphological and genetic study within the lake to better ascertain the likelihood that benthic and limnetic sticklebacks, as originally described by McPhail (1984), still exist in Enos Lake.

## Materials and methods

### Fish sampling

Under permits issued by the British Columbia Ministry of Environment (NA13-87360), the Fisheries and Oceans Canada Species at Risk program (282-TS), and Fairwinds Real Estate Management Inc, sticklebacks were collected from Enos Lake at four times: May 26–28th, June 27–29th, October 12–14th, 2013, and September 16–18th, 2014. Enos Lake is located on southwestern Vancouver Island, British Columbia (49°16′50″N, 123°09′23″W). It has a surface area of 18 ha, a maximum depth of 15 m, an elevation of 53 m above sea level, and is 1.5 km long × 0.2 km maximum width (see Supplementary Figure 1). Fish were collected using baited minnow traps set along transects on the bottom of the littoral zone and, using a canoe, in open water habitats. Open water traps were set on the bottom and also at depths of between 1 and 3 m below the lake's surface by suspending them from floats. Traps were set along transects to sample the entire lake basin (see Supplementary Figure 1). Typically, one-half of the lake (eight locations/transects and ~30 traps) was sampled over a single

24 h period with overnight trap sets and the other half of the lake was sampled with similar effort the next day. Transects ranged in length from 40 to 180 m depending on location in the lake. Typically, four to eight traps were set across each transect.

McPhail (1984) reported observing and sampling schools of limnetics while searching the lake at night with a nightlight in the autumn of 1977. Consequently, during the October 2013 and September 2014 sampling periods, the entire lake was surveyed by canoe during the day and at night to search for schools of fish using a Coleman lamp and flashlight during the night surveys. Furthermore, the lake was searched during the day for young-of-the-year (YOY) sticklebacks which, when encountered, were sampled using a 1.5 m pole seine. The number of American signal crayfish collected in each trap was recorded and crayfish were sampled haphazardly. All fish were stored in 95% non-denatured ethanol for subsequent morphological and DNA analyses.

We also used a sample (N = 100) of Enos Lake sticklebacks collected in September and October 1977, a time period when the species pair was first discovered (UBC Fish Collection BC83-95 and BC83-96). Finally, we examined two samples of sticklebacks from Murdo-Fraser Pond in North Vancouver, British Columbia, that are descendants of fish introduced from Enos Lake. These introduced fish comprised 445 lab-reared limnetics (produced from crosses of parents captured from the wild) introduced to Murdo-Fraser Pond in September of 1988. This population was supplemented by the further introduction of 150 wild adult limnetics in May of 1989 (S. Miller and D. Schluter, UBC Zoology, personal communication). We examined 35 fish sampled from Murdo-Fraser Pond in 1991 (two to three generations after introduction) and 20 fish sampled in 2014 (~9–13 generations after introduction).

## Morphological analyses

McPhail (1984) described the key morphological traits that distinguish benthic and limnetic sticklebacks from each other in Enos Lake. Among the largest differences were mean body depth [at a common standard length (SL) of 50 mm, benthics = 10.7, limnetics = 8.4 mm], mean number of dorsal spines (benthics = 3.03, limnetics = 2.70), and mean gill raker counts (benthics = 18.5, limnetics = 25.9). Such differences are temporally stable (samples across several years) and at least, in part, genetically determined and hybrids show intermediate trait values consistent with their reflecting inherited differences between benthics and limnetics (McPhail 1984). These differences are also found to distinguish benthic-limnetic species pairs in the three other lakes were they occur (McPhail 1992; Schluter and McPhail 1992; Gow et al. 2008). These measurements and counts, therefore, provide relatively simple phenotypic measures of the distinctiveness of benthics and limnetics and, hence, their relative abundance in Enos Lake. Consequently, we measured SL and body depth (BD) at the first dorsal spine and also counted the number of dorsal spines (DSN) and gill rakers (GRN) on the first gill arch on all samples.

Recently, McGee et al. (2013) reported that epaxial width (EPW), the cross-sectional distance taken near the posterior edge of the operculum, was highly distinctive between benthics and limnetics. Moreover, McGee et al. (2013) demonstrated that this measure was strongly associated with the ability to generate suction during feeding, and that epaxial width was consistently greater in benthic sticklebacks, which is likely advantageous while feeding on invertebrates buried in the littoral zone and attached to vegetation. Consequently, we also measured epaxial width on the 1977 samples of Enos Lake threespine sticklebacks, on all Murdo-Fraser Pond sticklebacks and on a subset of the samples collected from Enos Lake in 2014.

Before taking measurements and counts, ethanol-stored fish were rinsed in water for 48 h and then fixed in 10% formalin for 48 h. After formalin fixation, fish were stored in 45% isopropyl alcohol. Gill rakers on the first arch were counted after staining fish with alizarin red (0.5% in 0.5% potassium hydroxide, KOH) in for 4 h and washed several times over 24 h in 0.5% KOH. The first gill arch was removed by dissection and counted under a stereo microscope.

## Genetic analyses

Tissue samples were subject to DNA extraction using the Qiagen Animal Tissue extraction kit. The DNA was then genotyped at 12 microsatellite loci: *Stn*46, 64, 76, 90, 170, 175, 238, 278, 301, 329, 332 and 389 (Kitano et al. 2008). These loci were selected from hundreds available as they appear to be selectively neutral and are distributed across multiple linkage groups and represent a broad survey of genetic composition of each fish (Kitano et al. 2008). We also assayed a subset of fish at *Stn*43, a locus with allele frequencies that are strongly associated with differences in male nuptial colouration in male threespine sticklebacks in Enos Lake (red throats in limnetic males, overall black colouration in benthic males, Malek et al. 2012). Microsatellite loci were amplified using the polymerase chain reaction (PCR) in 15 µl volumes using the Qiagen Multiplex PCR kit with one primer for each locus labeled with a fluorescent dye. Labelled microsatellite alleles were size fractioned and detected using the Beckman-Coulter CEQ 8000 detection system (Taylor et al. 2006).

### Statistical analyses

Body depth scaled positively with SL, so BD measurements were adjusted to the overall mean SL of all samples compared at one time using the "allometric versus standard" option in the Transform procedure in Past (version 2.94, Hammer et al. 2001). We did not examine males and females separately because McPhail (1992) showed that sexual dimorphism in gill raker and dorsal spine counts was not significant and sexual differences in body depth within benthics and limnetics were only 11–14% of those found between them. Further, the vast majority of our samples we taken outside the breeding season when external signs of maturation were not evident (e.g., nuptial colouration) and any differences between males and females were likely at their minimum.

Size-adjusted BD, and DSN and GRN were examined with histograms. Evidence for more than one mode of measures and counts was assessed using Mixture analysis in Past. The Mixture analysis is a maximum-likelihood method for determining the most likely number of univariate normal distributions (and their statistical parameters) contained within a single, pooled univariate sample when there is no independent information on group membership (Hammer et al. 2001). The suitability of different models for the number of morphological groups of fish were evaluated using the Akaike Information Criterion (AIC) in Past. Specific comparisons of means between or among sets of samples were made with two-sample *t* tests, or Analysis-of-Variance (or their non-parametric equivalents when necessary), respectively, using Past.

After verifying that there was no evidence of scoring errors, large allele dropout, or null alleles using MICRO-CHECKER (Van Oosterhout et al. 2004), the microsatellite data were examined for departures from Hardy–Weinberg or linkage disequilibrium locus-by-locus as such departures can signal the presence of two or more gene pools. Next, the genetic clustering program STRUCTURE version 2.3.3 (Falush et al. 2003) was used to determine the number of distinct genetic clusters (*K*) best represented by the total sample of sticklebacks. STRUCTURE uses a Bayesian clustering method to assign individuals to genetic clusters based on their genotypes. An individual may be assigned to more than one cluster if its genotype indicates admixture of two or more genetic groups. A Markov chain Monte Carlo method (MCMC) was used to estimate posterior probability distributions for each possible number of clusters. Simulations in STRUCTURE were performed using values of *K* between one and five which brackets the null hypothesis of one population, the historical occurrence of two populations, and a reasonable number (3) of other groups that may have been undetected previously in this small lake. The analysis used parameters that allowed admixture and correlated allele frequencies. Each run consisted of a 500,000 step burn-in followed by an additional 1,000,000 steps. Five iterations were run for each value of *K*.

## Results

### Fish collections and observations

A total of 315, 161, 524, and 269 (total = 1269) sticklebacks were collected in the May, June, October 2013 and September 2014, respectively (Table 1). Virtually all of the fish collected were adult or sub-adult fish (i.e., >30 mm SL). The exception was the October 2013 sample which also included 125 YOY sticklebacks. The average SL of fish (not including juveniles) was 52.6 mm (SD = 7.75), 53.5 mm (SD = 5.61), 61.4 mm (SD = 4.40), and 48.3 mm (0.14) SL in May, June, October 2013, and September 2014, respectively. The average SL of the juveniles in the October 2013 sample was 26.4 mm (SD = 3.00). Although catch per trap varied considerably among areas/transects, adult fish were sampled from all areas of the lake and about 30% (378 of 1269) were caught in the minnow traps set in mid-water (1–3 m below the surface of the lake). All of the juvenile fish captured were seined from the extreme southeastern portion of the lake amongst aquatic vegetation in <1 m water depth. American signal crayfish were also captured throughout the lake, especially in the extreme southeastern and northwestern transects and around the island. American signal crayfish catches exceeded 50 in some traps and varied in size from 30 to 40 mm total length to over 100 mm total length. In three nights of canoeing around the lake (about 3 h total observation), we never observed any sticklebacks, in schools or otherwise, swimming about the lake.

### Morphology

Across all samples, the evidence for more than one morphological group of threespine sticklebacks was equivocal at best; Mixture analyses supported single modes for traits across all time periods (six of ten comparisons). No single sample point demonstrated support for two modal groups based both on BD or GRN. In only one of five comparisons did GRN suggest two modes, but the differences in mean counts between modes in this single case was only 10% compared to a >30% difference reported between benthics and limnetics by McPhail (1984).

For instance, in the May 2013 sample, Mixture analysis suggested the existence of two BD groups of fish, one with a mean size adjusted (to 52.6 mm SL) BD of 10.1 mm and the other with a mean BD of 11.9 mm. Only 7 fish of the 221 measured, however, fell into the large BD group.

**Table 1** Summary of samples and metrics (mean, SD) for standard length (SL), body depth at first dorsal spine (BD), epaxial width (EPW), dorsal spine number (DSN), and gill raker number on the first arch (GRN) for threespine sticklebacks (*Gasterosteus aculeatus*) collected from Enos Lake, southeastern Vancouver Island in 1977, 2013, and 2014

| Date | N | SL | BD | EPW | DSN | GRN |
|---|---|---|---|---|---|---|
| 09-10/1977[a] | 50 | 54.1 (4.04) | 9.40 (0.63) | 4.65 (0.58) | 2.98 (0.14) | 18.3 (1.19) |
| 09-10/1977[b] | 50 | 47.9 (2.21) | 8.53 (0.40) | 3.61 (0.29) | 2.73 (0.43) | 24.7 (1.36)[c] |
| 1984-B | 25 | 50 (SL) | 10.0 (0.40) | NM | 3.00 (0.0)[1] | 19.0 (1.2) |
| 1984-B×L | 43 | 50 (SL) | 9.65 (0.73) | NM | 2.95 (0.21) | 22.6 (1.25) |
| 1984-L×B | 37/16 | 50 (SL) | 9.54 (0.35) | NM | 2.88 (0.34) | 23.3 (1.80) |
| 1984-L | 25 | 50 (SL) | 8.55 (0.48) | NM | 2.67 (0.45) | 25.3 (1.03) |
| 05/2013 | 315 | 52.6 (7.75) | 10.3 (0.65) | NM | 2.97 (0.20) | 20.3 (1.51) |
| 06/2013 | 161 | 53.3 (5.61) | 10.4 (0.51) | NM | 2.93 (0.25) | 21.2 (1.53) |
| 10/2013[d] | 399 | 61.4 (4.56) | 12.1 (0.71) | NM | 2.98 (0.24) | 20.3 (1.37) |
| 10/2013[e] | 125 | 26.4 (3.02) | 4.91 (0.23) | NM | 2.94 (0.28) | 20.6 (1.20) |
| 09/2014 | 269 | 43.9 (5.12) | 8.68 (1.13) | 4.42 (1.01) | 3.00 (0.14) | 19.9 (1.32) |
| MF1991 | 35 | 45.5 (6.90) | 7.99 (1.24) | 3.71 (0.60) | 2.99 (0.24) | 22.9 (1.29) |
| MF2014 | 20 | 49.6 (4.38) | 9.61 (1.22) | 4.37 (0.50) | 3.00 (0.00) | 22.0 (2.00) |
| Comparisons at a common standard length of 48 mm | | | | | | |
| 09-10/1977[a] | 50 | 48 | 9.30 (0.56)[1] | 4.20 (0.42)[1] | NA | NA |
| 09-10/1977[b] | 50 | 48 | 7.55 (0.64)[2] | 3.61 (0.21)[2] | NA | NA |
| 09/2014 | 61 | 48 | 9.63 (0.52)[1] | 4.58 (0.44)[3] | NA | NA |
| MF1991 | 35 | 48 | 8.52 (0.44)[3] | 3.91 (0.25)[4] | NA | NA |
| MF2014 | 20 | 48 | 9.31 (0.50)[1] | 4.25 (0.24)[5] | NA | NA |

Samples listed as 1984 are from McPhail (1984), but no date of collection was given. The 1984 samples refer to laboratory raised crosses of benthics (B), benthic (male)×limnetic (female) first generation hybrids, limnetic (male)×benthic (female) first generation hybrids, and limnetics (L). Comparisons at a common standard length of 48 mm not accompanied by the same superscript numeral are significantly different from one another (Tukey test, $P<0.05$). The value 37/16 for the N of the 1984-LxB sample refers to the sample sizes for measures and counts, respectively

*NM* not measured, *NA* not applicable (see non-size adjusted values above)

[a]Littoral zone sample (UBC83-95)

[b]Open-water sample (UBC83-96)

[c]Overall mean of both littoral and open-water samples = 21.6 mm (SD = 3.43)

[d]Adult and sub-adult sample

[e]Juvenile sample (young-of-the-year)

Further, the mean BD of both groups of fish is much larger than the 8.7 mm reported for limnetics (adjusted to 50 mm SL) and both are similar to the mean BD of 10.6–10.8 mm (also adjusted to 50 mm SL) reported for wild-caught benthics by McPhail (1984). Mixture analysis suggested only a single group of fish based on GRN with a mean of 20.3 (range 16–25, Fig. 1), a value intermediate to the values of 18.5 and 24.5 reported for benthics and limnetics, respectively, by McPhail (1984). In the May sample, fish with 24 or more gill rakers constituted only 2% of the total sample. Dorsal spine counts were almost uniformly three, with only 18 of 351 (5.1%) fish having two dorsal spines and one fish having four (mean = 2.96, SD = 0.200). There were no significant difference in mean GRN between fish caught in open water, benthic traps (mean = 20.3, N = 207) and those caught in mid-water traps (mean = 20.8, N = 28, $P>0.10$).

In the June 2013 sample, Mixture analysis suggested a single BD group of fish (mean = 10.4 mm, SD = 0.51, size-adjusted to 53.3 mm SL). By contrast to the May sample, mixture analysis suggested two groups of fish based on GRN, one with a mean of 21.9 (SD = 1.32) and the other with a mean of 19.9 (SD = 0.89), although a model invoking a single group also received support (ΔAIC was 2.7, Fig. 1). Fish with 24 or more gill rakers constituted only 7% of the total sample. Again, very few fish (10/161, 6.1%) had two dorsal spines (overall mean = 2.93, SD = 0.253). Fish sampled from benthic-set traps (N = 87) had significantly deeper bodies (10.6 vs. 10.2 mm at 53.3 mm L, $P<0.001$), fewer gill rakers (20.3 vs. 21.8, $P<0.001$), but no difference in DSN (2.94 vs. 2.92, $P>0.2$), compared to fish sampled with mid-water set traps (N = 71). Again, however, and despite these small differences, all fish tended to have characteristics similar to benthics, or hybrids, and not limnetics as described by McPhail (1984).

In the October 2013 sample, the most outstanding feature was the much larger average length of the fish sampled (mean = 61.4 mm, SD = 4.40) with several fish over 75 mm SL. Mixture analysis indicated the presence of

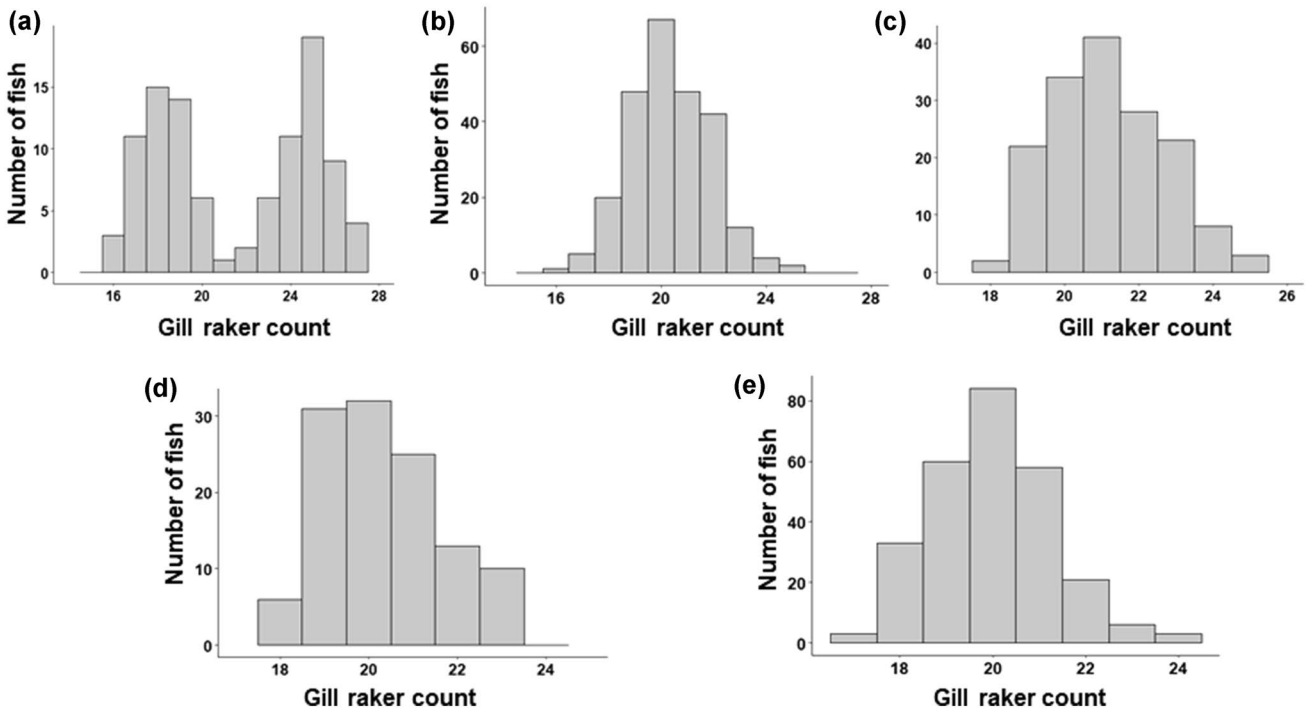


**Fig. 1** Histograms of the distribution of gill raker counts on the first gill arch in threespine sticklebacks (*Gasterosteus aculeatus*) collected from Enos Lake, southeastern Vancouver Island in 1977 (**a**), 2013 (**b** May, **c** June, **d** October), and 2014 (**e** September)

two groups of fish based on BD (adjusted to 61.4 mm TL, group 1 mean = 12.1 mm, SD = 0.55; group 2 = 14.4 mm, SD = 0.29), but again only 6 of 399 fish examined fell into the second group. The average DSN was 2.97 (SD = 0.25) and only 17/399 (4.3%) fish had a few as two dorsal spines. Gill raker counts averaged 20.3 (1.37), very similar to the typical GRN observed in the May and June samples. A model of one group of fish based on GRN was the best supported, although a model of two groups of fish also received some support (ΔAIC was 1.9). Amongst the 125 juveniles examined morphologically, a single univariate distribution of BD which averaged 4.91 mm (SD = 0.23, adjusted to 26.4 mm TL) mm was best supported. 5 of 125 juvenile fish had two dorsal spines (4%) and the average DSN in the sample was 2.94 (SD = 0.27). In juveniles, GRN averaged 20.6 (1.20). A model of one group of fish based on GRN was the best supported, and a model of two groups of fish received much less support (ΔAIC was 4.1).

The average length of fish collected in September of 2014 was 43.9 mm (SD = 5.12) SL. Mixture analysis indicated that two groups of fish existed based on BD (adjusted to 43.9 mm SL, group 1 mean = 8.53 mm, SD = 0.31 mm, N = 227; group 2 = 9.16 mm, SD = 0.52, N = 42). The average DSN was 3.00 (SD = 0.14) and only 2/269 (0.7%) fish had a few as two dorsal spines. Mean GRN was 19.9 (1.32), similar to the GRN observed in the 2013 samples (Fig. 1). A model of one group of fish based on GRN was the best supported, although a model of two groups of fish also received some support (ΔAIC was 1.9).

When all samples with gill raker counts were pooled into fish caught in benthic traps (N = 716) or limnetic traps (N = 325), there was a slight, but significant difference in mean GRN (20.2 vs. 20.8, respectively, $P < 0.001$). The best Mixture model was two GRN groups compared to one (ΔAIC = 15) with means of 19 and 22 gill rakers each, and a greater proportion of fish caught in limnetic traps were in the higher gill raker group (0.16 vs. 0.06, 2 × 2 contingency test, $P < 0.001$). Fish from limnetic traps, however, also had a lower mean DSN (2.94 vs. 2.98, P = 0.01), but were statistically indistinguishable in mean adjusted (to 52.3 mm) BD (10.25 vs. 10.19 mm, P = 0.19) compared to fish caught in benthic traps.

## Comparison to historical samples

The samples from 1977 showed some support for two groups of fish based on BD, but only marginally (ΔAIC < 2) compared to a single group; the respective means in size-adjusted BD (to 51.1 mm SL) were 9.4 (SD = 0.76) and 8.5 mm (SD = 0.18) for benthics and limnetics (as identified by McPhail 1984), respectively (Table 1). By contrast, the 1977 samples clearly displayed two modes in GRN (mode 1 at about 18, mode 2 at 24) and mixture analysis strongly supported a model of two clusters of fish based

on GRN (AIC = 286.2 vs. 342.9 for one group, Fig. 1). In these samples, fish with 24 or more gill rakers constituted 43% of the total sample. Dorsal spine counts were slightly lower than in 2013/14 (mean = 2.88, SD = 0.33) with 12 of 97 (12.4%) fish having two dorsal spines; all but one of the 1977-sampled fish with two dorsal spines were sampled in the surface waters in the limnetic zone of the lake.

### Comparison to Murdo-Fraser Pond fish

Because the 1977, 2014 samples from Enos Lake and Murdo-Fraser Pond sticklebacks (of Enos Lake limnetic ancestry) were all measured for the same set of traits (i.e., epaxial width was not measured in the 2013 samples), we compared these fish for all traits at a common size of 48 mm SL. Here, there was significant among sample variation in size-adjusted BD (ANOVA, $P < 0.0001$): the 2014 Enos Lake samples had the deepest bodies, followed by the Murdo-Fraser Pond 2014 samples, the 1977 Enos Lake benthic samples and the Murdo-Fraser Pond 1991 samples. The 1977 Enos Lake limnetic samples had the shallowest bodies (Table 1). Similarly, there was significant variation in EPW (ANOVA, $P < 0.001$). In fact, the Enos Lake 2014 samples and the Murdo Fraser Pond 2014 sticklebacks (established from Enos Lake limnetics in 1988) tended to have the greatest body depths and epaxial widths, while the Enos Lake 1977 limnetic fish had the shallowest bodies and narrowest epaxial widths (Table 1; Fig. 2).

### Microsatellite analyses

There were no indications of problems with the microsatellites associated with stutter bands, large allele dropout, or null alleles using MICRO-CHECKER so all loci were retained for subsequent analyses. There were several instance of departures from Hardy–Weinberg (four in 24 tests total) and linkage disequilibrium (10 in 132 tests), but none were concentrated on specific loci or locus pairs across the samples so all loci were retained for subsequent analyses.

In the May samples, the most likely number of genetic groups ($K$) inferred from the STRUCTURE analysis was one (Table 2). Plots of the individual fish admixture values when enforcing a model of two genetic groups on the data indicated no evidence of differences in proportional membership of one genetic group or the other amongst individual fish; all fish were composed of approximately an equal (~50%) proportional composition of both genetic groups indicating no segregation amongst individual fish into one genetic group or another. Indeed, the distribution of admixture scores when imposing a $K = 2$ followed a unimodal distribution (Fig. 3) with no hint of fish segregating across two genetic groups (i.e., one with admixture scores between 0 and 0.2 and another with admixture scores between 0.8 and 1.0 as is evident in limnetic and benthic sticklebacks in intact species pair lakes, e.g., Gow et al. 2006, 2008). In the June samples, the most likely number of genetic groups ($K$) inferred from the STRUCTURE analysis was

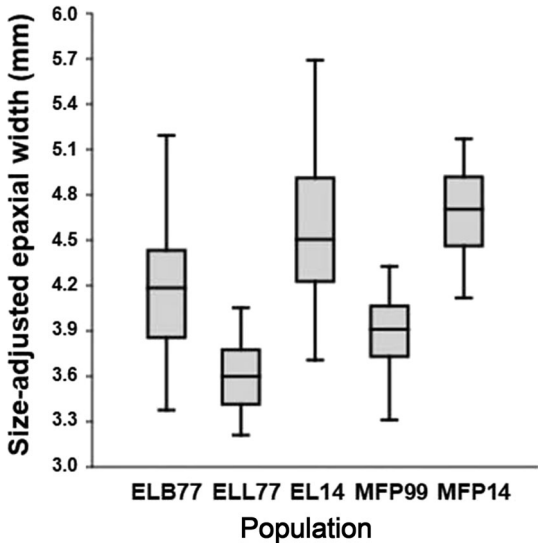


**Fig. 2** Boxplot of size adjusted (to 48 mm standard length) epaxial width in samples of threespine stickleback (*Gasterosteus aculeatus*). *ELB77* Enos Lake benthics sampled in 1977 (N = 50), *ELL77* Enos Lake limnetics sampled in 1977 (N = 50), *EL14* Enos Lake sticklebacks sampled in 2014 (N = 61), *MFP91* Murdo-Fraser Pond sampled in 1991 (N = 35), *MFP14* Murdo-Fraser Pond sampled in 2014 (N = 20). The 25–75 percent quartiles are shown with a box. The *horizontal line* inside the box represents the median and the minimum and maximum values are shown with *short horizontal lines*

**Table 2** Mean (SD, N = 5 replicates) natural logarithm of the probability of the $K$ genetic groups given the observed allele frequencies from assays of 12 microsatellite DNA loci in threespine stickleback (*Gasterosteus aculeatus*) sampled from Enos Lake, southeastern Vancouver Island

| Sample | $K$ | ln P($K$) |
|---|---|---|
| 05/2013 | **1** | **−12,262.3 (0.15)** |
| | 2 | −12,358.9 (47.5) |
| | 3 | −12,542.4 (71.3) |
| 06/2013 | **1** | **−5772.1 (0.40)** |
| | 2 | −5788.3 (3.73) |
| | 3 | −5876.5 (58.4) |
| 10/2013 | **1** | **−13,767.6 (0.15)** |
| | 2 | −13,826.9 (12.1) |
| | 3 | −14,240.5 (112.9) |
| 09/2014 | 1 | −9748.1 (0.23) |
| | **2** | **−9746.5 (3.5)** |
| | 3 | −10,009.6 (109.02) |

The most likely number of genetic groups in indicated in boldface

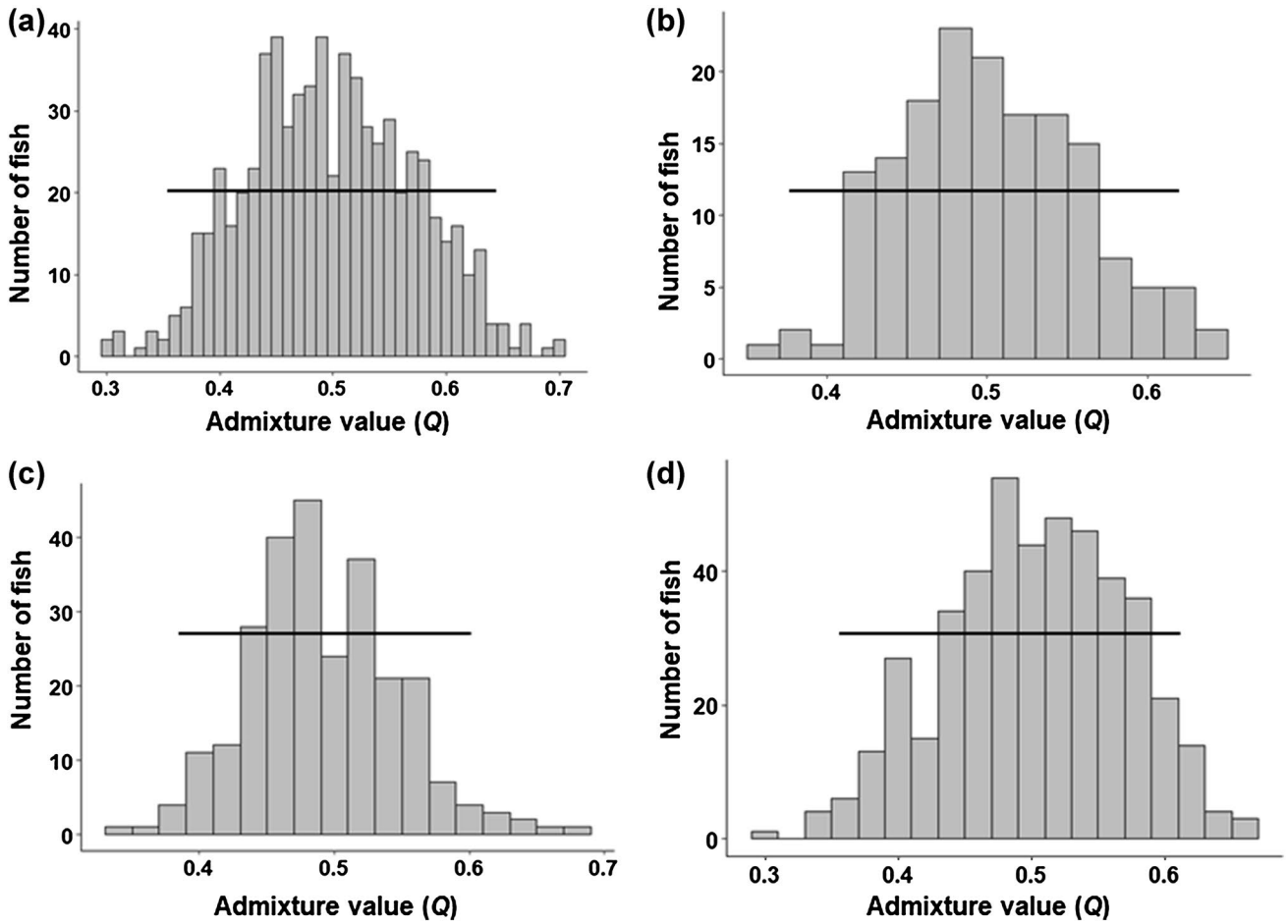


**Fig. 3** Histograms of admixture scores ($Q$) for individual threespine sticklebacks (*Gasterosteus aculeatus*) collected from Enos Lake, southeastern Vancouver Island in **a** May 2013, **b** June 2013, **c** October 2013, and **d** September 2014 when a model of $K=2$ genetic populations is assumed. The $Q$ values were inferred using the Bayesian model clustering algorithm of STRUCTURE version 2.3.3 (Falush et al. 2003) applied to variation at 12 microsatellite DNA loci and are the means from five replicate runs. The *thick horizontal black bars* indicate the range of $Q$ values for fish collected from minnow traps set in mid-water (1–3 m below the surface of the lake) in transects in open water

also one (Table 2). Plots of the individual fish admixture values when forcing a model of two genetic groups on the data indicated no evidence of differences in proportional membership of one genetic group or the other amongst individual fish; all fish were composed of approximately an equal (~50%) proportional composition of both genetic groups indicating no segregation amongst individual fish into one genetic group or another. Similar to the results for the May sample, the distribution of admixture scores followed a unimodal distribution (Fig. 3). In the October samples, the most likely number of genetic groups ($K$) inferred from the STRUCTURE analysis was also one (Table 2). Plots of the individual fish admixture values when forcing a model of two genetic groups on the data indicated no evidence of differences in proportional membership of one genetic group or the other amongst individual fish; all fish were composed of approximately an equal (~50%) proportional composition of both genetic groups indicating no segregation amongst individual fish into one genetic group or another. Similar to the results for the May and June samples, the distribution of admixture scores followed a unimodal distribution (Fig. 3).

By contrast to all other samples, the STRUCTURE analysis suggested that the September 2014 samples comprised two genetic groups, but the difference in likelihood from that of $K=1$ was very small (Table 2) and plots of the individual fish admixture values under $K=2$ genetic groups indicated no evidence of differences in proportional membership of one genetic group or the other amongst individual fish (Supplementary Figure 2). As in other samples, all fish were composed of approximately an equal (~50%) proportional composition of both genetic groups indicating no segregation amongst individual fish into one genetic group or another. Similar to the results from the 2013 samples,

the distribution of admixture scores followed a unimodal distribution (Fig. 3). In all 2013–2014 samples, there was no indication that fish captured in benthic traps constituted a genetic group distinct from those captured in limnetic traps (Fig. 3, Supplementary Figure 2).

A subsample of 192 fish collected from Enos Lake in 2014 (collected without regard to male colour which was not expressed at the time of sampling) exhibited the *Stn*43 135 bp allele (much more common in limnetic than benthic males, Malek et al. 2012) at a frequency of 0.19. The frequency of this allele was significantly lower (contingency test, P = 0.0007) than the frequency of 0.37 found in a sample of 47 Murdo-Fraser Pond fish sampled in 2014. The 1991 Murdo Fraser pond samples could not be assayed confidently presumably owing to a longer period of time that has elapsed since formalin fixation and alcohol storage.

## Discussion

### Evidence for a single gene pool in Enos Lake

Extinction can be difficult to confirm in cases of naturally rare, cryptic, or extremely widespread taxa. Even in cases of relatively small ranges and simple habitats, exhaustive surveys may still be required to declare extinction likely (e.g., the case of the Bramble Cay melomys—Gynther et al. 2016). For instances like the Enos Lake benthic and limnetic sticklebacks, the situation is complicated by the fact that it is genomic extinction (sensu Allendorf et al. 2004) that is the issue at hand; threespine sticklebacks are still abundant in the lake, but evidence for the presence of a hybrid swarm make it unclear whether or not the unique genotypic combinations represented by benthic and limnetic phenotypes are still present (COSEWIC 2013).

Both our morphological and genetic data point to the contemporary existence of a single group of threespine sticklebacks in Enos Lake compared to the two morphological, genetic, and behavioural groups that occurred historically (this study; McPhail 1984; Bentzen and McPhail 1984; Ridgway and McPhail 1984; cf.; Taylor et al. 2006; Cooper et al. 2011). Morphologically, all the current gill raker data conformed to a unimodal population of sticklebacks, and gill raker counts were consistently intermediate to the historical values for benthic and limnetic sticklebacks. In fact, McPhail (1984) used artificial lab-reared crosses between Enos Lake benthics and limnetics to characterize hybrids and most of the traits studied in the current analysis have hybrid-like intermediate values or tend to approach benthic values. There is no indication in any of the morphological traits of limnetic-like values. For gill raker counts, which represent perhaps the most striking difference between "classic" benthics and limnetics, only five fish out of 838 with gill raker counts had as many as 25 gill rakers (0.6%). By contrast, 25 of 100 fish (25.5%) in the 1977 sample had 25 or more gill rakers.

The apparent absence of limnetics in Enos Lake is also suggested by the lack of any observations of schools of limnetics in the open water portions of the lake. These searches were completed by canoe and included thorough daytime surveys of the lake across a total of 9 days. In addition, three nighttime searches, replicating the time and methods of McPhail (1984) used to sample limnetics (a light and dipnet) also resulted in no observations of schooling fish. The only schools of fish observed were those of YOY fish in the extreme southeastern sector of the lake in shallow water in the daytime in October. These YOY fish, however, also consisted of a unimodal group of fish morphologically and genetically.

The microsatellite DNA analyses also suggested that Enos Lake contains a single breeding population of sticklebacks; there was no evidence of two distinct groups within the lake using a variety of analyses. As with the morphological analyses, even segregating samples into those captured in mid-water traps and those in benthic or littoral traps revealed no evidence of distinction.

Microsatellite DNA surveys of three other lakes with benthic-limnetic pairs (Priest, Paxton, and Little Quarry lakes) have been completed using 11–13 loci some of which were selected for their ability to differentiate between high (limnetics) and low (benthics) gill raker types (See Gow et al. 2006, 2008). In these assays, the presence of two genetic populations was strongly supported in each of these three lakes. Further, within each lake, the majority of fish (78% in Little Quarry Lake, 90% in Priest Lake, and 88% in Paxton Lake) had admixture coefficients of either >0.99 (indicating virtually complete composition by one genetic group with low gill raker counts—benthics) or <0.01 (indicating virtually complete composition by the other genetic group with high gill raker counts—limnetics) (Gow et al. 2006, 2008). The 2013–2014 samples from Enos Lake were not surveyed at the same set of loci as Gow et al. (2006, 2008), so the results of these studies are not directly comparable, but a sample of 184 Enos Lake fish collected in 2003 was also assayed by Gow et al. (2006). Similar to our 2013–2014 samples, these Enos Lake 2003 samples comprised a single genetic population (Gow et al. 2006; Taylor unpublished data), but if a $K = 2$ is imposed on the data, no single fish had an admixture coefficient exceeding 0.76 (or <0.24). Similarly, if a $K = 2$ is enforced on the 2013–2014 samples, no single fish (of >1200) had an admixture coefficient greater than 0.68 (or <0.32). Consequently, if one uses the other "intact" species pair lakes as a standard and pure benthics and limnetics are characterized by admixture coefficients of 0.99 and 0.01, respectively, then no such pure individuals appear to exist in Enos Lake.

In summary, the analysis conducted for samples obtained in 2013 and 2014 greatly extend the results of previous more limited studies that first suggested the collapse of formally highly distinctive benthic and limnetic sticklebacks into a single breeding population in Enos Lake (Kraak et al. 2001; Taylor et al. 2006; Behm et al. 2010; Malek et al. 2012).

The Murdo-Fraser Pond fish appear, morphologically, to be very similar to benthics from Enos Lake even though the population was founded by limnetics. That these fish have a limnetic ancestry is supported by our assays which showed a relatively high frequency of the *Stn*43-135 base pair (bp) allele, an allele known to be of much higher frequency in limnetic males compared to benthic males (0.59 vs. 0.12 in 17 fish of each type, Malek et al. 2012). The 2014 Murdo-Fraser Pond samples were even more benthic-like morphologically than the 1991 samples suggesting that the intervening 24 generations selected strongly for benthic trophic characters in this small, shallow pond (<100 m long × ~1 m depth) environment. Even by 1991, only two generations after founding, the Murdo-Fraser Pond sticklebacks had developed trait values more similar to (body depths and epaxial widths), or rapidly approaching (gill raker counts), those of benthics than their limnetic ancestors. In addition to natural selection, phenotypic plasticity also likely played a role in the phenotypic changes observed, although at least for the traits we examined, plasticity tends to be a relative minor factor compared to genetically based changes in sticklebacks (Day et al. 1994; Leaver and Reimchen 2012). These results support the observation that the characteristics of lake environments are critical factors influencing the evolution of phenotypic variation in sticklebacks (e.g., Lavin and McPhail 1985; Schluter and McPhail 1992; Nosil and Reimchen 2005). The dramatic changes in phenotype experienced by the Murdo-Fraser Pond fish following transplantation is consistent with rapid phenotypic changes observed in other sticklebacks introduced to new environments or experiencing fluctuating environments (e.g., Klepaker 1993; Bell et al. 2004; Kitano et al.2008; Barrett et al. 2011; Leaver and Reimchen 2012). Further, such rapid phenotypic change supports the idea that other examples of ecological change, such as the appearance of American signal crayfish in Enos Lake, could result in changes in phenotype induced by elevated hybridization between benthics and limnetics or other effects of crayfish (e.g., altered selective regimes or breeding behaviour, Behm et al. 2010; Velema et al. 2012).

## Conservation implications

Some uncertainty remains about the processes that have driven the collapse of the Enos Lake sticklebacks (i.e., elevated hybridization, selection for intermediate phenotypes, or a combination of both, see discussion in Taylor et al. 2006; Velema et al. 2012). Our data, consisting of over 1200 fish sampled from all areas of the lake, across multiple seasons, life-history stages and two years, however, argue that the benthics and limnetics in Enos Lake, as characterized historically by the unique combinations of characters in Enos Lake and to intact species pairs in other lakes, no longer exist. Of course, more exhaustive sampling using whole genome sequencing will provide the ultimate answer to the question of the continued existence of benthics and limnetics in Enos Lake, but conservation decisions often must be made under uncertainty. In the balance remains the potential reassessment and listing of Enos Lake benthic and limnetic threespine sticklebacks as Extinct under Canada's SARA. A reassessment of Extinct would mean that recovery actions for this species pair would not be justified because there are no limnetics or benthics left to recover. Alternatively, it could be argued that the individual alleles that differentiate benthic and limnetic sticklebacks still exist in Enos Lake, they are just highly admixed with each other. The lake clearly still provides good habitat for sticklebacks in general, especially given the large size and good growth of fish observed over the summer of 2013. Habitat restoration combined with complete removal of American signal crayfish could perhaps restore the original selective environment that promoted a high degree of disruptive natural selection and assortative mating associated with the evolution of benthics and limnetics (McPhail 1993; Rundle et al. 2000). Unfortunately, baseline limnological conditions (i.e., pre-crayfish) of Enos Lake and the feasibility of crayfish removal and aquatic vegetation restoration remain unknown. Further, even if the disturbance that led to the collapse of the species pair could be removed, and original habitat quality restored, modelling results suggest that the likelihood of re-emergence of the benthic and limnetic species pair is probably low given the extensive admixture across many generations of disturbance (Gilman and Behm 2011).

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