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Ecological selection against hybrids in natural populations of sympatric threespine sticklebacks

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Abstract

Experimental work has provided evidence for extrinsic post-zygotic isolation, a phenomenon unique to ecological speciation. The role that ecological components to reduced hybrid fitness play in promoting speciation and maintaining species integrity in the wild, however, is not as well understood. We addressed this problem by testing for selection against naturally occurring hybrids in two sympatric species pairs of benthic and limnetic threespine sticklebacks (*Gasterosteus aculeatus*). If post-zygotic isolation is a significant reproductive barrier, the relative frequency of hybrids within a population should decline significantly across the life-cycle. Such a trend in a natural population would give independent support to experimental evidence for extrinsic, rather than intrinsic, post-zygotic isolation in this system. Indeed, tracing mean individual hybridity (genetic intermediateness) across three life-history stages spanning four generations revealed just such a decline. This provides compelling evidence that extrinsic selection plays an important role in maintaining species divergence and supports a role for ecological speciation in sticklebacks.

Introduction

The past decade has witnessed a renewed interest in ecological speciation, the 'process by which barriers to gene flow evolve between populations as a result of ecologically-based divergent selection' (Rundle & Nosil, 2005). This revival has been accompanied by an upsurge in research identifying and measuring reproductive isolation; this knowledge will lead us to a better understanding of the process of speciation (Schluter, 2001; Coyne & Orr, 2004). Indeed, this work is beginning to yield insight into the relative contributions of diverse forms of isolating barriers, including prezygotic barriers such as habitat and temporal isolation, immigrant inviability, sexual isolation and post-mating prezygotic isolation, as well as post-zygotic barriers that can be intrinsic (genetic), ecologically dependent (extrinsic) or due to sexual selection against hybrids (Nosil *et al.*, 2005; Rundle & Nosil, 2005; Rogers & Bernatchez, 2006).

Of these varied categories, extrinsic post-zygotic isolation is unique to ecologically based divergent selection. It arises when the fitness of hybrids (i.e. individuals of mixed ancestry) is reduced relative to parental types because of a mismatch between a hybrid phenotype and its environment (Coyne & Orr, 2004). As long as there is no intermediate environment in which hybrids may thrive, those with intermediate phenotypes that are maladapted to both parental niches are subject to the divergent selection that acts between parental environments (Schluter, 2000). Although some studies have directly estimated the strength of extrinsic post-zygotic isolation, these have been limited to reciprocal transplant experiments with flowering plants (Johansen-Morris & Latta, 2006; reviewed in Campbell & Waser, 2007), phytophagous insects (reviewed in Linn *et al.*, 2004) and threespine sticklebacks under semi-natural conditions (Hatfield & Schluter, 1999; Rundle, 2002). Direct evidence of selection against natural hybrids in the wild is needed to better understand the role of extrinsic

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post-zygotic isolation in the formation of species and the maintenance of their integrity, as well as to improve our understanding of the mechanisms that underlie reduced hybrid fitness.

The recently derived post-glacial sympatric species pairs of benthic and limnetic threespine sticklebacks (*Gasterosteus aculeatus*) are one of the most extensively studied systems for investigating the role of ecologically based divergent selection in the evolution of both pre- and post-zygotic reproductive isolation (reviewed in McKinnon & Rundle, 2002; Nosil *et al.*, 2005; Rundle & Nosil, 2005). These sticklebacks are among few species to have had the ecological component of hybrid fitness experimentally assessed. Phenotypically intermediate hybrids show reduced foraging efficiency relative to the parental types in their respective specialized habitats, which are the littoral zone for the bottom-dwelling benthics, and the pelagic zone for the open-water limnetics (Schluter, 1995). Although these laboratory-reared F_1 hybrids experienced a growth disadvantage in field transplant enclosure experiments, they showed no fitness disadvantage under benign laboratory conditions (Schluter, 1995; Hatfield & Schluter, 1999). An ecological basis for this post-zygotic isolation was confirmed by a reciprocal transplant experiment using hybrid backcrosses which controlled for intrinsic genetic incompatibilities (Rundle, 2002). These elegant experiments on growth rate in hybrids, however, took place under semi-natural conditions in field enclosures and only over a short time period of three weeks. Furthermore, little is known about the impacts of other fitness components, such as disease resistance and predator avoidance (but see Vamosi & Schluter, 2002). Thus, the effects of admixture on fitness in free-ranging benthic and limnetic sticklebacks over the duration of their lives in nature remain to be determined.

If post-zygotic isolation was unimportant in maintaining species divergence, there should be no significant variation in the relative frequency of hybrids found in natural populations across the stickleback life-cycle. On the other hand, if selection against hybrids contributes significantly to reproductive isolation, a decrease in the relative frequency of hybrids across successively older life-history stages is expected. Any such decline in a natural population would give independent support for extrinsic, rather than intrinsic, post-zygotic isolation between benthic and limnetic sticklebacks. An approach that can assess this would complement experimental findings by providing evidence from free-ranging fish over the duration of their lives in nature. The development of diagnostic marker profiles that unambiguously identify benthics, limnetics and their hybrids (Gow *et al.*, 2006) has, indeed, enabled us to test this prediction in the two extant stickleback species pairs. We report here the mean individual hybridity (a measure of genetic intermediateness) across three life-history stages in natural populations spanning four generations.

Materials and methods

Sample collection

We collected tissue samples from three different life stages of the stickleback species pairs found in Paxton and Priest lakes on Texada Island, BC, Canada, over the course of four generations from 2003 to 2006. An average of 192 specimens of juveniles, sub-adults or adults were collected at specific time points from each lake during the stickleback's non-overlapping generations. These were killed with an overdose of MS-222 and preserved in 95% ethanol before DNA extraction. Adults were sampled near the beginning of their discrete breeding season in April (May in 2006), when both species have moved into the littoral zone to mate. Thirty minnow traps distributed approximately evenly along the entire shoreline were used in conjunction with dip-netting to obtain lake-wide samples of both species. Sub-adults were sampled using the same strategy in September before their offshore over-winter migration. Juveniles were dip-netted from along the shoreline in July. Although effort was made to balance the proportions of benthics and limnetics in these collections, we did not selectively exclude indeterminate forms, i.e. fish that appeared to have ambiguous morphology were not discarded.

Microsatellite genotyping

A total of 3264 fish was genotyped at 10 *G. aculeatus* dinucleotide microsatellite loci (Supplementary material; Table S1, Fig. S1). Eight of these comprise a species diagnostic molecular profile for these species pairs (*Stn*388, *Stn*295, *Stn*142, *Stn*383, *Stn*254, *Stn*216, *Stn*386, *Stn*43; Gow *et al.*, 2006) and were used alongside two other microsatellites that are highly polymorphic in these populations (*Gac*7 and *Cir*51, Gow *et al.*, 2006), providing a highly discriminatory tool with which to distinguish between benthics, limnetics and their hybrids (Gow *et al.*, 2006). These loci were amplified by PCR and genotyped using flourescently labelled primers on a CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA) according to Gow *et al.* (2006).

Admixture analysis across stickleback life-history stages

The program STRUCTURE (Pritchard *et al.*, 2000) was used to confirm the number of discrete genetic clusters (K). This Bayesian algorithm, Markov chain Monte Carlo-based approach uses a genetic inheritance model to minimize Hardy Weinberg and linkage disequilibrium within cluster groups. We calculated the probability of there being one to four clusters by running five simulations for each K value, using the admixture and correlated allele frequency models. Simulations began with a

'burn-in' period of 25 000 iterations to minimize the dependence of subsequent parameter estimates on starting values, and parameters were estimated after a further 200 000 iterations. We followed the procedure and guidance of Pritchard & Wen (2003) and Evanno *et al.* (2005) to estimate the number of clusters given the data; the earlier qualitative method, which estimates the real number of clusters as the K value where the 'log probability of data' (L(K)) begins to plateau (Pritchard & Wen, 2003), has been formalized by the *ad hoc* statistic ΔK, which is based on the rate of change in L(K) between successive K values (Evanno *et al.*, 2005).

With the most probable number of clusters being two (Fig. 1), each individuals' admixture proportions between benthic and limnetic gene pools were estimated for each of the five simulations where $K = 2$. Following this, each individual's average proportion of ancestry in the benthic population ($q_b^{(i)}$) was calculated. To assess the modality of admixture for each species pair across their life-cycles, $q_b^{(i)}$ were transformed into hybridity (h_i) values using the formula $h_i = 0.5 - |0.5 - q_b^{(i)}|$ (*sensu* Carney *et al.*, 2000; Duvernell *et al.*, 2007). Ranging from 0 for pure parentals to 0.5 for F_1 hybrids, this value provides a measure of how intermediate an individual's multilocus genotype is on the admixture scale. Differences in mean h_i between life-history stages were tested using Kruskal–Wallis one-way ANOVAS or Mann–Whitney U-tests. Firstly, data for each life-history stage was pooled from different temporal sampling points for each species pair. Differences were then also tested within each generation. Calculation of h_i and global ANOVAS were repeated for each species pair data set, excluding a single locus at a time to ensure that no single locus was biasing results.

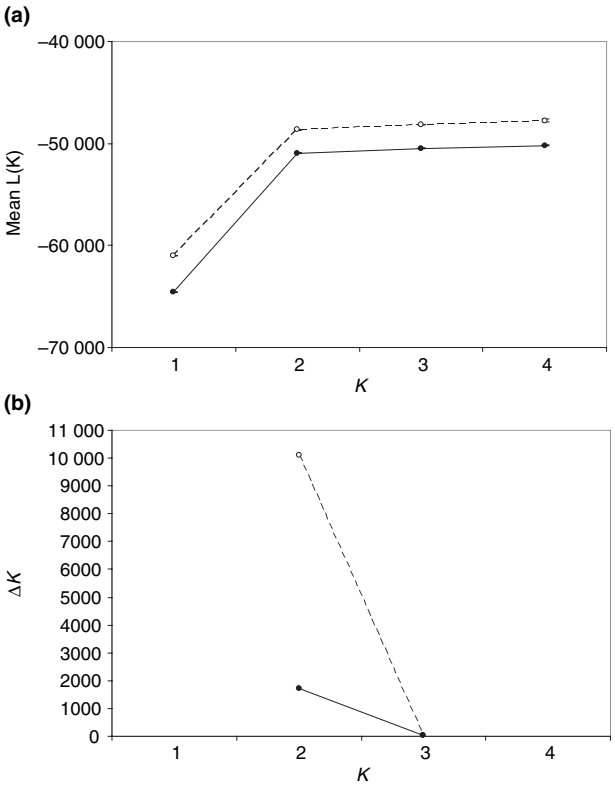

Fig. 1 The most probable number of genetic clusters for each of the species pairs of threespine sticklebacks is estimated to be two. (A) Log probability of data L(K) (Pritchard & Wen, 2003) plateaus at $K = 2$ and (B) ΔK (Evanno *et al.*, 2005) is modal at $K = 2$. Standard deviations for L(K) are too small to visualize but range from 0.08 to 161 and increase with K. Solid circles with solid lines and empty circles with dashed lines represent results for Paxton ($n = 1742$) and Priest Lake ($n = 1515$) species pairs respectively.

For comparative purposes, we explored the STRUCTURE results using an alternative assessment of hybridization, as well as investigating an alternative analysis method. Firstly, individuals were assigned as benthic, limnetic or hybrid based on the 90% posterior probability interval (90% PI) of $q_b^{(i)}$ calculated in STRUCTURE: a benthic had a 90% PI overlapping 1, a limnetic had a 90% PI overlapping 0 and a hybrid had a 90% PI overlapping with neither 0 nor 1. Secondly, we assigned individuals as benthic, limnetic or hybrid using NewHybrids Version 1.1 (Anderson & Thompson, 2002), according to Gow *et al.* (2006). This Bayesian method implements a more specific inheritance model than STRUCTURE. Hybrid frequency was calculated for each sample point using both methods, and the association between hybrid frequency and life-history stage was assessed by one-tailed chi-squared tests for independence.

Results

Bi-modal admixture values indicate strong reproductive isolation within species pairs

The distribution of individual admixture values within a population (ranging from 0 to 1 between two parental types) indicates the proportion of individuals that are of mixed parental ancestry. If reproductive isolation is strong, hybridization will be rare and the distribution of admixture values is expected to be bi-modal, with most individuals having values near 0 or 1. By contrast, if reproductive barriers are weak and hybridization is more common, the distribution of these values will tend towards uni-modality, with more individuals having admixed values between the parental extremes, i.e. $\gg 0$ and $\ll 1$.

Having been assigned by their average proportion of ancestry in the benthic population ($q_b^{(i)}$), individual sticklebacks' admixture values exhibit a strongly bimodal frequency distribution within each species pair (Fig. 2). The majority of these samples, which represent three life-history stages spanning four generations (Fig. 3), were assigned to a parental species (91.2% and 89.1% for Paxton and Priest Lake species pairs respectively). Only a minority (8.8% and 10.9% for Paxton and Priest Lake

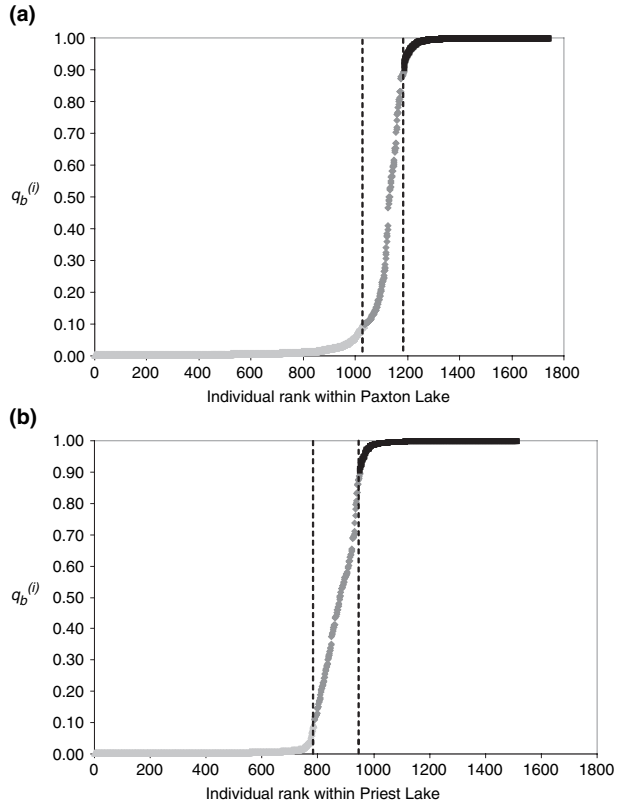

Fig. 2 Frequency distribution of individual sticklebacks' average proportion of ancestry in the benthic population ($q_b^{(i)}$) estimated by STRUCTURE ($K = 2$) for all samples collected from (A) Paxton ($n = 1742$) and (B) Priest Lake ($n = 1515$) species pairs from 2003 to 2006. The proportion of parental and admixed individuals is illustrated by plotting $q_b^{(i)}$ values against their rank. A threshold $q_b^{(i)}$ value of 0.1 divides parental (benthic, ■; limnetic △) and admixed individuals (◆), which are separated by dashed vertical lines.

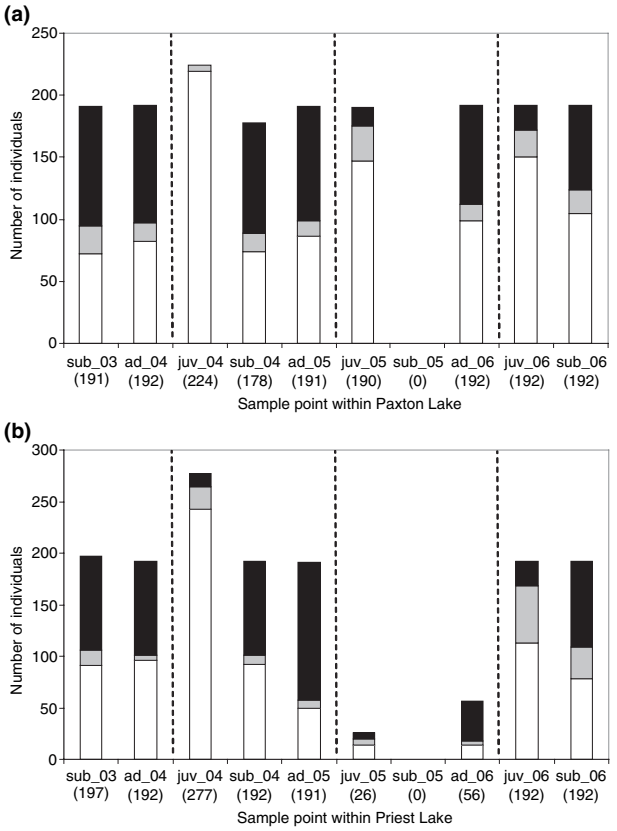

Fig. 3 Proportion of pure benthic, pure limnetic and admixed threespine sticklebacks for each of nine sample points spanning four life-cycles for (A) Paxton and (B) Priest Lake species pairs. Individuals classified by admixture value, $q_b^{(i)}$, according to Fig. 2 as either benthic (black bars), limnetic (white bars) or admixed (grey bars). Sample names are composed of life-history stage abbreviation (juv, juvenile; sub, sub-adult; ad, adult) followed by sampling year; sample sizes are given in parentheses; life-cycles are separated by dashed vertical lines.

species pairs respectively) of individuals show evidence of mixed ancestry greater than 10% (Fig. 2).

Levels of hybridity decline across successive life-history stages

Our summary statistics of the overall degree of admixture within species pairs are a useful indicator of differentiation between benthic and limnetic sticklebacks; however, they obscure any changes that may be occurring throughout the stickleback life-cycle. Having transformed individuals' $q_b^{(i)}$ into hybridity values (h_i), we were able to assess any deviation in the level of genetic intermediateness in populations across life-history stages.

A comparison of mean h_i revealed a consistent pattern of decreasing hybridity across life-history stages for both species pairs. Indeed, Kruskal–Wallis one-way ANOVAS found significant differences among life-history stages in both species pairs when samples were combined across generations and pooled according to life-history stage (Fig. 4). That is, there is lower mean h_i amongst successively older life-history stages compared with younger ones. This global pattern is reflected within each generation: the highest mean h_i tends to occur among juveniles and declines as they reach the sub-adult stage, with the lowest values when they are adults. Indeed, nine of 10 of these intra-generation comparisons between consecutive life-history stages showed a qualitative decline in mean h_i and five of eight overall intra-generation comparisons declined significantly (Fig. 5).

Overall, greater than an 80% decline in hybrid frequency (based on assignment using the 90% posterior probability interval of $q_b^{(i)}$) was observed in the Priest Lake species pair from juveniles (mean 19.6%, SD 10.7) to adults (mean 3.7%, SD 1.6). The overall decrease in hybrid frequency within the Paxton Lake species pair from juveniles (mean 6.58%, SD 4.9) to adults (mean 4.7%, SD 0.9) was smaller, at about 30%. In both lakes,

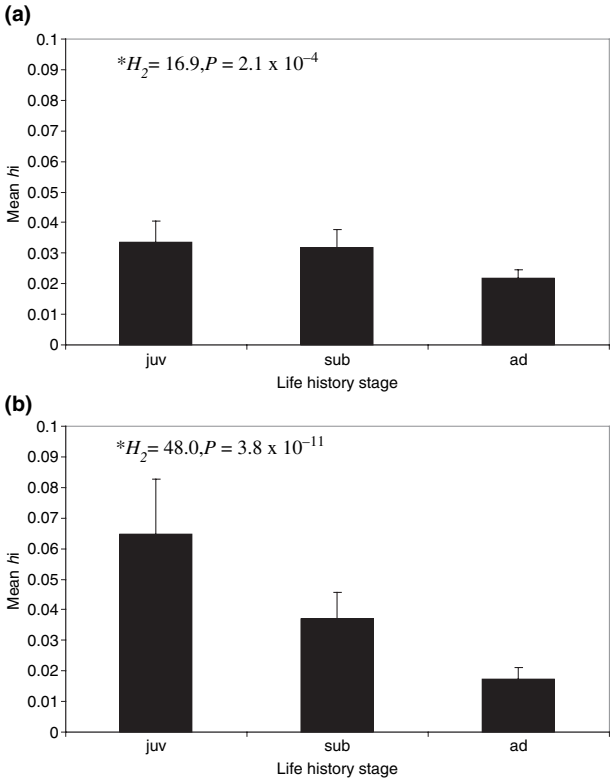

Fig. 4 Proportions of admixed individuals among three life-history stages for (A) Paxton and (B) Priest Lake species pairs. Mean individual hybridity (h_i) was calculated across generations according to life-history stage (juv, juvenile; sub, sub-adult; ad, adult). Results are given for Kruskal–Wallis one-way ANOVAS: *, significant; error bars illustrate ± variance. Refer to Fig. 2 for more sampling details.

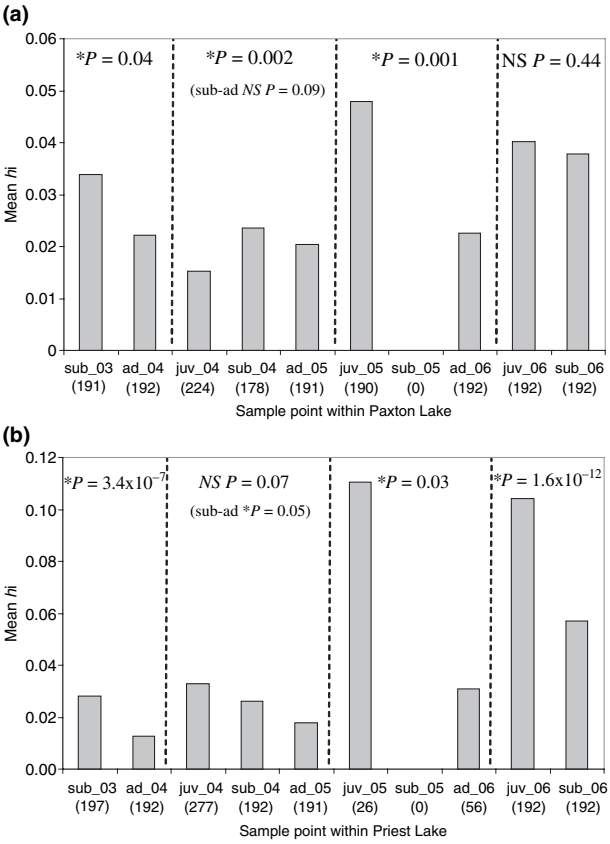

Fig. 5 Proportions of admixed individuals among three life-history stages spanning four life-cycles for (A) Paxton and (B) Priest Lake species pairs. Mean individual hybridity (h_i) given for each of nine sample points. P values given for Kruskal Wallis one-way ANOVAS or Mann–Whitney U-tests within each generation: *, significant; *NS*, nonsignificant. Sample names are composed of life-history stage abbreviation (juv, juvenile; sub, sub-adult; ad, adult) followed by sampling year; sample sizes are given in parentheses; life-cycles are separated by dashed vertical lines.

hybrid frequency and mean h_i (Fig. 4) fluctuated more amongst juvenile samples, whereas those at the adult level were lower and relatively consistent.

Given the level of polymorphism (Supplementary material; Table S1, Fig. S1) and divergence ($F_{ST} = 0.27$ between benthics and limnetics in both species pairs, unpublished data from Gow *et al.*, 2006) in our data, efficiency of both model-based Bayesian methods in estimating the proportion of hybrid individuals in a population is expected to exceed 95% (Vähä & Primmer, 2005; Gow *et al.*, 2006). Indeed, our methodological comparison was robust to an alternative assessment of hybridization, as well as to the application of models differing in the specificity of their genetic inheritance (Supplementary material; Fig. S2).

The over-representation of limnetics in some juvenile samples (Fig. 3) did not influence our conclusion; a significant decline occurred from sub-adult to adult life-history stages in three of four intra-generation comparisons (Fig. 5). The results were also robust to the distribution of any missing genotypes (Supplementary material; Table S1, Fig. S1) and when we excluded each locus in turn (Supplementary material; Fig. S3).

Discussion

Mean juvenile hybrid frequencies of 7% and 20% in Paxton and Priest Lake species pairs, respectively, illustrate that hybridization continues between benthic and limnetic sticklebacks, despite strong assortative mating (Ridgway & McPhail, 1984; Nagel & Schluter, 1998; Boughman, 2001). Although not every comparison was significant, the overall consistent decline in genetic intermediateness (assessed by mean individual hybridity and hybrid frequency) across successive life-history stages strongly supports the prediction that selection against such hybrids contributes significantly to reproductive isolation between benthic and limnetic sticklebacks in the wild. Given that all stickleback adults share the littoral zone during the breeding season, with benthics and limnetics varying only in microhabitat

preference (Bentzen *et al.*, 1984), we are confident that we consistently sampled hybrids throughout their life-cycle and that the consistent trend of decreasing hybridity with age in both lakes is not a sampling artefact (Supplementary material; Fig. S4).

The impressive overall declines in the proportion of hybrids (about 30% and 80% in Paxton and Priest Lake species pairs respectively) yield insight into the strength of post-mating isolation and also how it accumulates over the stickleback lifespan. Indeed, our estimates exclude some fitness parameters, such as prejuvenile survival and adult breeding success, such that the overall decline in hybridity across the stickleback life-cycle may be even greater than we documented here. Sexual selection against hybrid adult males has, in fact, been implicated by field mating trials in which F_1 hybrid males suffered a reduced mating success in their preferred nesting habitat relative to the parental limnetic species that utilizes the same area (Vamosi & Schluter, 1999).

Our study covered a large portion of the stickleback's lifespan across multiple generations within two independently derived species pairs (Taylor & McPhail, 1999, 2000) and used hybridity to infer reduced survival of hybrids. These novel aspects of our study should provide a more direct estimate of fitness than short-term growth rate, and a natural, parallel context to earlier investigations of trophic segregation and performance in sticklebacks (McPhail, 1984, 1992; Schluter, 1993, 1995; Hatfield & Schluter, 1999; Rundle, 2002). Given the previous evidence for extrinsic, rather than intrinsic, post-zygotic processes in stickleback reproductive isolation, it is highly likely that there is a strong ecological component to the selection that we have provided evidence for in wild sticklebacks.

Although our understanding of the genetic basis of traits associated with post-zygotic isolation advances (Coyne & Orr, 2004), the fates of hybrid individuals and consequences of post-zygotic isolation in the wild remain poorly understood. Our results clearly show that hybrid sticklebacks are less likely to contribute to subsequent generations. There are few accounts of selection against hybrid individuals in natural populations. Some extrinsic selection against hybrids was inferred from static cohort analyses of irises (Cruzan & Arnold, 1994) and bivalves (Bert & Arnold, 1995; Wilhelm & Hilbish, 1998), whereas dynamic cohort analysis suggested intrinsic (Kocher & Sage, 1986) and extrinsic (Howard *et al.*, 1993) selection against hybrids in leopard frogs and ground crickets, respectively. Dowling & Moore's (1985) study of a cyprinid fish hybrid zone showed consistent selection against hybrids relative to both parental types over multiple cohorts but could not distinguish between intrinsic or extrinsic processes.

Hybrid frequency can oscillate with environmental conditions (Grant *et al.*, 2004; Taylor *et al.*, 2006). Our finding that levels of genetic intermediateness deviated most amongst juvenile stages implies inherent annual fluctuations in stickleback hybridization rates. In our study, this is brought about by a variation in the effectiveness of prezygotic and very early post-zygotic isolation between breeding seasons. Furthermore, adult populations seem to converge on a lower, relatively consistent level of hybrids (mean hybrid frequency of 5% for Paxton Lake and 4% for Priest Lake species pairs), suggesting that extrinsic selection within these species pairs is pivotal in maintaining their distinct gene pools in sympatry. This scenario has changed, however, in the other extant species pair in Enos Lake on Vancouver Island, BC, where a single admixed population now exists (Gow *et al.*, 2006; Taylor *et al.*, 2006). This speciation reversal is associated with human-induced environmental change, a phenomenon of growing concern to biodiversity loss (Seehausen, 2006). Prezygotic reproductive barriers that control the number of hybrids produced clearly must have diminished within Enos Lake; however, the fate of admixed individuals relative to parental types remains unclear. A study similar to the present one could tackle this question; no significant variation in the genetic intermediateness in Enos Lake across the stickleback life-cycle would support the prediction that selection against hybrids is no longer contributing to reproductive isolation within this endangered species pair, whereas an increase would indicate a hybrid advantage.

Although we have presented evidence for selection against hybrids, the processes driving the demise of natural hybrids remain speculative. Although comparative and experimental work strongly implicates divergent selection caused by interspecific resource competition (Bentzen & McPhail, 1984; Schluter & McPhail, 1992; Schluter, 1993, 1994, 1995, 2003) in driving the divergence of the species pair, other aspects such as predation (Vamosi & Schluter, 2002, 2004; Rundle *et al.*, 2003) and parasitism may also contribute and deserve further attention. Although hybridity declines throughout the life-cycle, the low but persistent level of admixed individuals that remain in the adult population identifies a potential role for sexual, as well as natural selection to maintain benthic–limnetic species integrity in the face of some gene flow. Indeed, sexual selection against hybrid males has been implicated by field mating trials (Vamosi & Schluter, 1999).

To improve our understanding of the ecological mechanisms underlying the selection against hybrids, future research can now focus on morphological and diet analyses of the hybrids identified in this study, as well as a more extensive genetic characterization of the hybrids to enable precise identification of their status, e.g. F_1, F_2, backcrosses, etc. (Gow *et al.*, 2006). Continued monitoring of the species pairs may also yield spatial and temporal variations in patterns of hybrid frequency that may prove valuable in identifying environmental factors affecting relative hybrid fitness. Furthermore, now that a

battery of genetic and genomic tools is available for threespine sticklebacks (Peichel *et al*., 2001; Kingsley & Peichel, 2007), we may be able to identify the genetic basis of post-zygotic reproductive barriers and tease apart the fitness consequences associated with different performance measures of individuals in the wild.

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Supplementary material

The following supplementary material is available for this article:

Table S1 Genotyping properties of 10 dinucleotide microsatellite loci used to screen 3264 threespine sticklebacks collected from Paxton and Priest Lake species pairs ($n = 1517$ and 1747 respectively).

Figure S1 Characteristics of the number of microsatellite loci (listed in Table S1) successfully genotyped per individual for the 3257 threespine sticklebacks analysed from Paxton and Priest Lake species pairs ($n = 1742$ and 1515 respectively). (A) Frequency distribution and (B) Plot against mean individual hybridity index (h_i).

Figure S2 Hybrid frequency among three life-history stages spanning four life-cycles for (A) Paxton and (B) Priest Lake species pairs. Percentage hybrids calculated using STRUCTURE shown by grey bars, and those using NewHybrids shown by white bars.

Figure S3 Proportion of admixed individuals among three life-history stages spanning four life-cycles for Paxton Lake species pairs. Mean individual hybridity (h_i) given for each of nine sample points for analysis including all 10 microsatellite loci (grey bars) and excluding *Stn*383 (black bars).

Figure S4 Proportion of sample assigned as benthic (□), limnetic (△) or hybrid (◈) by NewHybrids for (A) Paxton and (B) Priest Lake species pair.

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