Running Title: ECOLOGICAL SELECTION AGAINST WILD HYBRIDS

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 stickleback (*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, providing compelling evidence that extrinsic selection plays an important role in maintaining species divergence and supporting a role for ecological speciation in sticklebacks.

Key words: admixture, *Gasterosteus aculeatus*, microsatellites, natural selection, extrinsic post-zygotic reproductive isolation.

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 pre-zygotic barriers such as habitat and temporal isolation, immigrant inviability, sexual isolation and post-mating pre-zygotic 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, and 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

58 in the wild is needed to better understand the role of extrinsic post-zygotic isolation in the
59 formation of species and the maintenance of their integrity, as well as to improve our
60 understanding of the mechanisms that underlie reduced hybrid fitness.
61
62 The recently derived post-glacial sympatric species pairs of benthic and limnetic threespine
63 sticklebacks (*Gasterosteus aculeatus*) are one of the most extensively studied systems for
64 investigating the role of ecologically-based divergent selection in the evolution of both pre- and
65 post-zygotic reproductive isolation (reviewed in McKinnon & Rundle, 2002; Nosil *et al.*, 2005;
66 Rundle & Nosil, 2005). These sticklebacks are among few species to have had the ecological
67 component of hybrid fitness experimentally assessed. Phenotypically intermediate hybrids show
68 reduced foraging efficiency relative to the parental types in their respective specialised habitats,
69 which are the littoral zone for the bottom-dwelling benthics, and the pelagic zone for the open-
70 water limnetics (Schluter, 1995). Although these laboratory-reared F_1 hybrids experienced a
71 growth disadvantage in field transplant enclosure experiments, they experienced no fitness
72 disadvantage under benign laboratory conditions (Schluter, 1995; Hatfield & Schluter, 1999).
73 An ecological basis for this post-zygotic isolation was confirmed by a reciprocal transplant
74 experiment using hybrid backcrosses which controlled for intrinsic genetic incompatibilities
75 (Rundle, 2002). These elegant experiments on growth rate in hybrids, however, took place under
76 semi-natural conditions in field enclosures and over only a short time period of three weeks.
77 Furthermore, little is known about the impacts of other fitness components, such as disease
78 resistance and predator avoidance (but see Vamosi & Schluter, 2002). Thus, the effects of
79 admixture on fitness in free-ranging benthic and limnetic sticklebacks over the duration of their
80 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, British Columbia, 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 sacrificed 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. Whilst 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 ten *G. aculeatus* dinucleotide microsatellite loci (Supplementary material 1). 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) according to Gow *et al.* (2006).

Admixture analysis across stickleback life-history stages

The program STRUCTURE (Pritchard *et al.*, 2000) was used firstly 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 frequencies 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 $h_i = 0.5 - \left| 0.5 - q_b^{(i)} \right|$ (*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.

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 both methods at each sample point, and the association between hybrid frequency and life-history stage was assessed by one-tailed chi-squared (χ^2) 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. $>> 0$ and $<< 1$.

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 species pairs, respectively) of individuals show evidence of mixed ancestry greater than 10% (Fig. 3).

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 to 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 out of ten of these intra-generation comparisons between consecutive life-history stages

showed a qualitative decline in mean h_i and five out of eight overall intra-generation comparisons were significant (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, hybrid frequency and mean h_i (Fig. 4) fluctuated more amongst juvenile samples, while those at the adult level were lower and relatively consistent.

Given the level of polymorphism (Supplementary material 1) 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, although the most inclusive method of defining a population's level of genetic intermediateness (mean individual h_i compared to hybrid frequency estimates) was the most statistically powerful (Supplementary material 2).

The overrepresentation 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 out

of four intra-generation comparisons (Fig. 5). The results were also robust to the distribution of any missing genotypes (Supplementary material 1) and when we excluded each locus in turn (Supplementary material 3).

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 4).

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 life span. Indeed, our estimates exclude some fitness parameters, such as pre-juvenile 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 utilises the same area (Vamosi & Schluter, 1999).

Our study covered a greater portion of the stickleback's life span 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.

Whilst 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 remains 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), whilst 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 variation in the effectiveness of pre-zygotic 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 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, British Columbia, 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). Pre-zygotic 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, whilst an increase would indicate a hybrid advantage.

287 Although we have presented evidence for selection against hybrids, the processes driving the
288 demise of natural hybrids remain speculative. Whilst comparative and experimental work
289 strongly implicate divergent selection caused by interspecific resource competition (Bentzen &
290 McPhail, 1984; Schluter & McPhail, 1992; Schluter, 1993, 1994, 1995, 2003) in driving the
291 divergence of the species pair, other aspects such as predation (Vamosi & Schluter, 2002, 2004;
292 Rundle *et al.*, 2003) and parasitism may also contribute and deserve further attention. Although
293 hybridity declines throughout the life-cycle, the low but persistent level of admixed individuals
294 that remain in the adult population identifies a potential role for sexual, as well as natural
295 selection to maintain benthic-limnetic species integrity in the face of some gene flow. Indeed,
296 sexual selection against hybrid males has been implicated by field mating trials (Vamosi &
297 Schluter, 1999).
298
299 To improve our understanding of the ecological mechanisms underlying the selection against
300 hybrids, future research can now focus on morphological and diet analyses of the hybrids
301 identified in this study, as well as a more extensive genetic characterisation of them that would
302 enable precise identification of their status e.g. F_1, F_2, backcrosses etc. (Gow et al. 2006).
303 Continued monitoring of the species pairs may also yield spatial and temporal variations in
304 patterns of hybrid frequency that may prove valuable in identifying environmental factors
305 affecting relative hybrid fitness. Furthermore, now that a battery of genetic and genomic tools is
306 available for threespine stickleback (Peichel *et al.*, 2001; Kingsley & Peichel, 2007), we may be
307 able to identify the genetic basis of post-zygotic reproductive barriers and tease apart the fitness
308 consequences associated with different performance measures of individuals in the wild.
309

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References

Anderson, E.C. & Thompson, E.A. 2002. A model-based method for identifying species hybrids using multilocus genetic data. *Genetics* **160:** 1217-1229.

Bentzen, P. & McPhail, J.D. 1984. Ecology and evolution of sympatric sticklebacks (*Gasterosteus*): specialization for alternative trophic niches in the Enos Lake species pair. *Can. J. Zool.* **62:** 2280-2286.

Bentzen, P., Ridgway, M.S. & McPhail, J.D. 1984. Ecology and evolution of sympatric sticklebacks (*Gasterosteus*): spatial segregation and seasonal habitat shifts in the Enos Lake species pair. *Can. J. Zool.* **62:** 2436-2439.

Bert, T.M. & Arnold, W.S. 1995. An empirical test of predictions of two competing models for the maintenance and fate of hybrid zones: both models are supported in a hard-clam hybrid zone. *Evolution* **49:** 276-289.

Boughman, J.W. 2001. Divergent sexual selection enhances reproductive isolation in sticklebacks. *Nature* **411:** 944-948.

Campbell, D.R. & Waser, N.M. 2007. Evolutionary dynamics of an *Ipomopsis* hybrid zone: confronting models with lifetime fitness data. *Am. Nat.* **169:** 298-310.

341 Carney, S.E., Gardner, K.A. & Rieseberg, L.H. 2000. Evolutionary changes over the fifty-year
342 history of a hybrid population of sunflowers (*Helianthus*). *Evolution* **54:** 462-474.
343
344 Coyne, J.A. & Orr, H.A. 2004. *Speciation.* Sinauer Associates, Sunderland.
345
346 Cruzan, M.B. & Arnold, M.L. 1994. Assortative mating and natural selection in an *Iris* hybrid
347 zone. *Evolution* **48:** 1946-1958.
348
349 Dowling, T.E. & Moore, W.S. 1985. Evidence for selection against hybrids in the family
350 Cyprinidae (genus *Notropis*). *Evolution* **39:** 152-158.
351
352 Duvernell, D.D., Schaefer, J.F, Hancks, D.C., Fonoti, J.A, & Ravanelli, A.M. 2007.
353 Hybridization and reproductive isolation among syntopic populations of the topminnows
354 *Fundulus notatus* and *F. olivaceus*. *J. Evolution. Biol.* **20:** 152-164.
355
356 Evanno, G., Regnaut, S. & Goudet, J. 2005. Detecting the number of clusters of individuals
357 using the software STRUCTURE: a simulation study. *Mol. Ecol.* **14:** 2611–2620.
358
359 Gow, J.L., Peichel, C.L. & Taylor, E.B. 2006. Contrasting hybridization rates between sympatric
360 threespine sticklebacks highlight the fragility of reproductive barriers between evolutionarily
361 young species. *Mol. Ecol.* **15:** 739-752.
362

363 Grant, P.R., Grant, B.R., Markert, J.A., Keller, L.F. & Petren, K. 2004. Convergent evolution of
364 Darwin's finches caused by introgressive hybridization and selection. *Evolution* **58:** 1588–1599.
365
366 Hatfield, T. & Schluter, D. 1999. Ecological speciation in sticklebacks: environment dependent
367 hybrid fitness. *Evolution* **53:** 866–873.
368
369 Howard, D.J., Waring, G.L., Tibbets, C.A. & Gregory, P.G. 1993. Survival of hybrids in a
370 mosaic hybrid zone. *Evolution* **47:** 789-800.
371
372 Johansen-Morris, A.D. & Latta, R.G. 2006. Fitness consequences of hybridization between
373 ecotypes of *Avena barbata*: hybrid breakdown, hybrid vigor, and transgressive segregation.
374 *Evolution* **60:** 1585–1595.
375
376 Kingsley, D.M. & Peichel, C.L. 2007. The molecular genetics of evolutionary change in
377 sticklebacks. In: *Biology of the Three-Spined Stickleback* (S. Östlund-Nilsson, I. Mayer & F.
378 Huntingford, eds), pp. 41-81. CRC Press, Boca Raton.
379
380 Kocher, T.D. & Sage, R.D. 1986. Further genetic analyses of a hybrid zone between leopard
381 frogs (*Rana pipiens* complex) in central Texas. *Evolution* **40:** 21-33.
382
383 Linn, C.E., Jr., Dambroski, H.R., Feder, J.L., Berlocher, S.H., Nojima, S. & Roelofs, W.L. 2004.
384 Postzygotic isolating factor in sympatric speciation in *Rhagoletis* flies: reduced response of
385 hybrids to parental host-fruit odors. *Proc. Natl. Acad. USA* **101:** 17753-17758.

386

387 McKinnon, J.S. & Rundle, H.D. 2002. Speciation in nature: the threespine stickleback model
388 systems. *Trends. Ecol. Evol.* **17:** 480-488.

389

390 McPhail, J.D. 1984. Ecology and evolution of sympatric sticklebacks (*Gasterosteus*):
391 morphological and genetic evidence for a species pair in Enos Lake, British Columbia. *Can. J.*
392 *Zool.* **62:** 1402-1408.

393

394 McPhail, J.D. 1992. Ecology and evolution of sympatric sticklebacks (*Gasterosteus*): evidence
395 for a species pair in Paxton Lake, Texada Island British Columbia. *Can. J. Zool.* **70:** 361-369.

396

397 Nagel, L. & Schluter, D. 1998. Body size, natural selection, and speciation in sticklebacks.
398 *Evolution* **52:** 209–218.

399

400 Nosil, P., Vines, T.H. & Funk, D.J. 2005. Perspective: reproductive isolation caused by natural
401 selection against immigrants from divergent habitats. *Evolution* **59:** 705-719.

402

403 Peichel, C.L., Nereng, K.S., Ohgi, K.A., Cole, B.L.E., Colosimo, P.F., Buerkle, C.A., Schluter
404 D. & Kingsley, D.M. 2001. The genetic architecture of divergence between threespine
405 stickleback species. *Nature* **414:** 901-905.

406

407 Pritchard, J.K., Stephens, M. & Donnelly, P. 2000. Inference of population structure using
408 multilocus genotype data. *Genetics* **155:** 945-959.

Pritchard, J.K. & Wen, W. 2003. Documentation for STRUCTURE software: version 2 (Available from http://pritch.bsd.uchicago.edu).

Ridgway, M.S. & McPhail, J.D. 1984. Ecology and evolution of sympatric sticklebacks (*Gasterosteus*): mate choice and reproductive isolation in the Enos Lake species pair. *Can. J. Zool.* **62:** 1813-1818.

Rogers, S.M. & Bernatchez, L. 2006. The genetic basis of intrinsic and extrinsic post-zygotic reproductive isolation jointly promoting speciation in the lake whitefish species complex (*Coregonus clupeaformis*). *J. Evolution. Biol.* **19:** 1979-1994.

Rundle, H.D. 2002. A test of ecologically dependent postmating isolation between sympatric sticklebacks. *Evolution* **56:** 322-329.

Rundle, H.D. & Nosil, P. 2005. Ecological speciation. *Ecol. Lett.* **8:** 336-352.

Rundle, H.D., Vamosi, S.M. & Schluter, D. 2003. Experimental evidence of predation's effect on divergent selection during character displacement in sticklebacks. *Proc. Natl. Acad. Sci. USA* **100:** 14943-14948.

Schluter, D. 1993. Adaptive radiation in sticklebacks: size, shape and habitat use efficiency. *Ecology* **74:** 699–709.

Schluter, D. 1994. Experimental evidence that competition promotes divergence in adaptive radiation. *Science* **266:** 798–800.

Schluter, D. 1995. Adaptive radiation in sticklebacks: trade-offs in feeding performance and growth. *Ecology* **76:** 82–90.

Schluter, D. 2000. *The Ecology of Adaptive Radiation.* Oxford University Press, Oxford.

Schluter, D. 2001. Ecology and the origin of species. *Trends. Ecol. Evol.* **16:** 372-380.

Schluter, D. 2003. Frequency dependent natural selection during character displacement in sticklebacks. *Evolution* **57:** 1142-1150.

Schluter, D. & McPhail, J.D. 1992. Ecological character displacement and speciation in sticklebacks. *Am. Nat.* **140:** 85–108.

Seehausen, O. 2006. Conservation: losing biodiversity by reverse speciation. *Curr. Biol.* **16:** R334-R337.

Taylor, E.B., Boughman, J.W., Groenenboom, M., Sniatynski, M., Schluter, D. & Gow, J.L. 2006. Speciation in reverse: morphological and genetic evidence of the collapse of a three-spined stickleback (*Gasterosteus aculeatus*) species pair. *Mol. Ecol.* **15:** 343-355.

Taylor, E.B. & McPhail, J.D. 1999. Evolutionary history of an adaptive radiation in species pairs of threespine sticklebacks (*Gasterosteus*): insights from mitochondrial DNA. *Biol. J. Linn. Soc.* **66:** 271–291.

Taylor, E.B. & McPhail, J.D. 2000. Historical contingency and ecological determinism interact to prime speciation in sticklebacks, *Gasterosteus*. *P. Roy. Soc. B-Biol. Sci.* **267:** 2375–2384.

Vähä, J.-P. & Primmer, C.R. 2005. Efficiency of model-based Bayesian methods for detecting hybrid individuals under different hybridization scenarios and with different numbers of loci. *Mol. Ecol.* **15:** 63-72.

Vamosi, S.M. & Schluter, D. 1999. Sexual selection against hybrids between sympatric sticklebacks: evidence from a field experiment. *Evolution* **53:** 874–880.

Vamosi, S.M. & Schluter, D. 2002. Impacts of trout predation on fitness of sympatric sticklebacks and their hybrids. *P. Roy. Soc. B-Biol. Sci.* **269:** 923-930.

Vamosi, S.M. & Schluter, D. 2004. Character shifts in the defensive armor of sympatric sticklebacks. *Evolution* **58:** 376-385.

Wilhelm, R. & Hilbish, T.J. 1998. Assessment of natural selection in a hybrid population of mussels: evaluation of exogenous vs endogenous selection models. *Mar. Biol.* **131:** 505-514.

Figure 1. The most probable number of genetic clusters for each of the species pairs of threespine stickleback 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.

Figure 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.

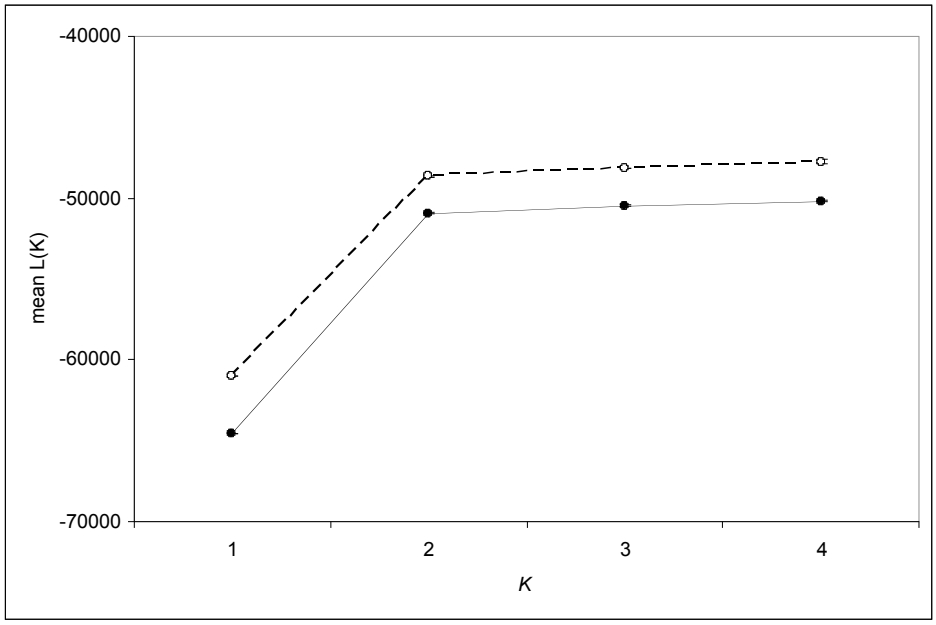
Figure 3. Proportion of pure benthic, pure limnetic and admixed threespine sticklebacks for each of nine sample points spanning four life-cycles for (A) Paxton and (B) Priest Lake species pairs. Individuals classified by admixture value, $q_b^{(i)}$, according to Fig. 2 as either benthic (black bars), 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.

Figure 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

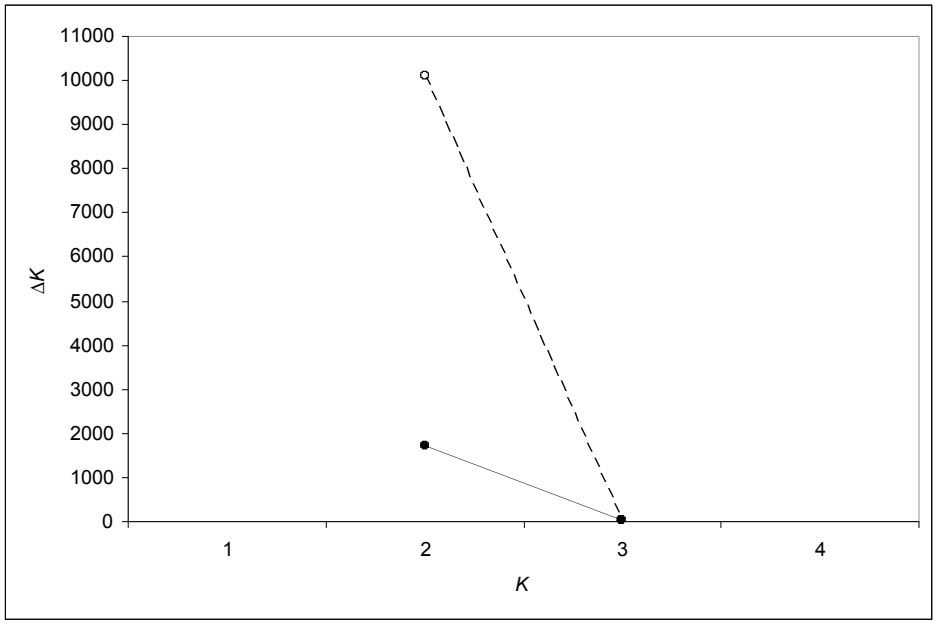
501 according to life-history stage (juv, juvenile; sub, sub-adult; ad, adult). Results are given for
502 Kruskal Wallis one-way ANOVAs: *, significant; error bars illustrate ± variance. Refer to Fig. 2
503 for more sampling details.
504
505 **Figure 5.** Proportions of admixed individuals among three life-history stages spanning four life-
506 cycles for (A) Paxton and (B) Priest Lake species pairs. Mean individual hybridity (h_i) given for
507 each of nine sample points. *P* values given for Kruskal Wallis one-way ANOVAs or Mann-
508 Whitney *U* tests within each generation: *, significant; *NS*, non significant. Sample names are
509 composed of life-history stage abbreviation (juv, juvenile; sub, sub-adult; ad, adult) followed by
510 sampling year; sample sizes are given in parentheses; life-cycles are separated by dashed vertical
511 lines.
512

Figure 1.

(A)

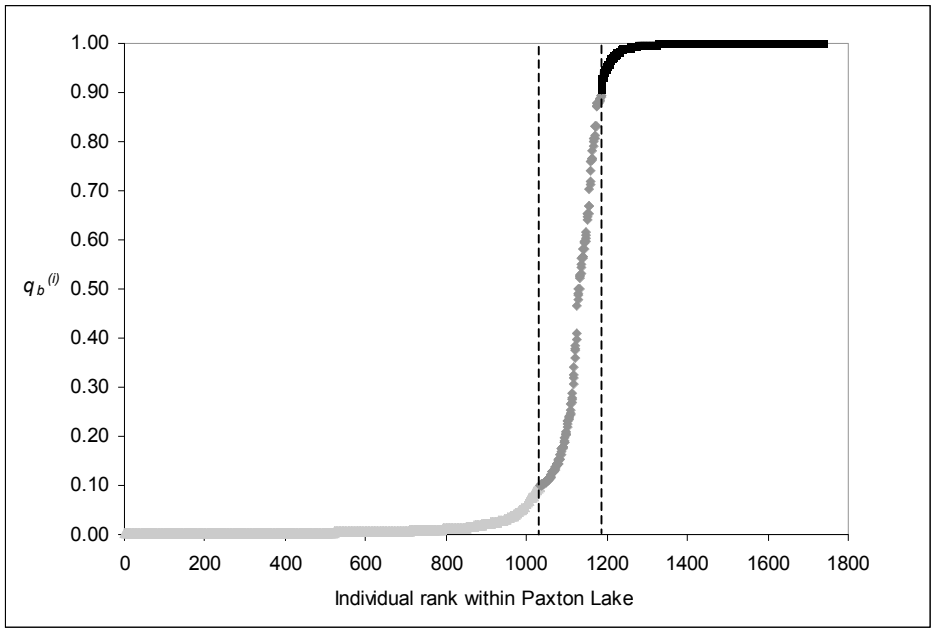

-40000
-50000
-60000
-70000
mean L(K)
1
2
3
4
K

(B)


11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
ΔK
1
2
3
4
K

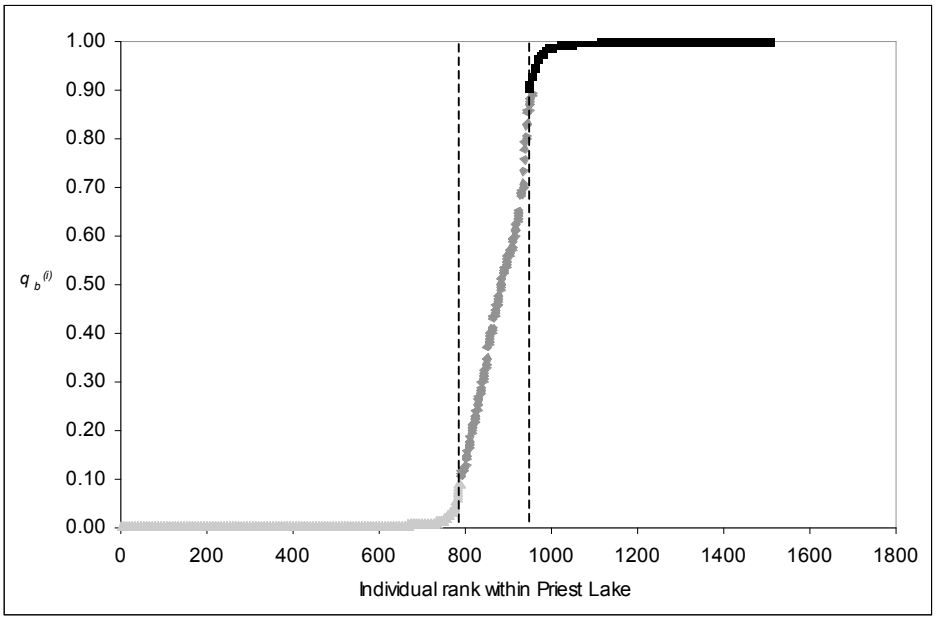
519 **Figure 2.**

520 (A)



521

522 (B)

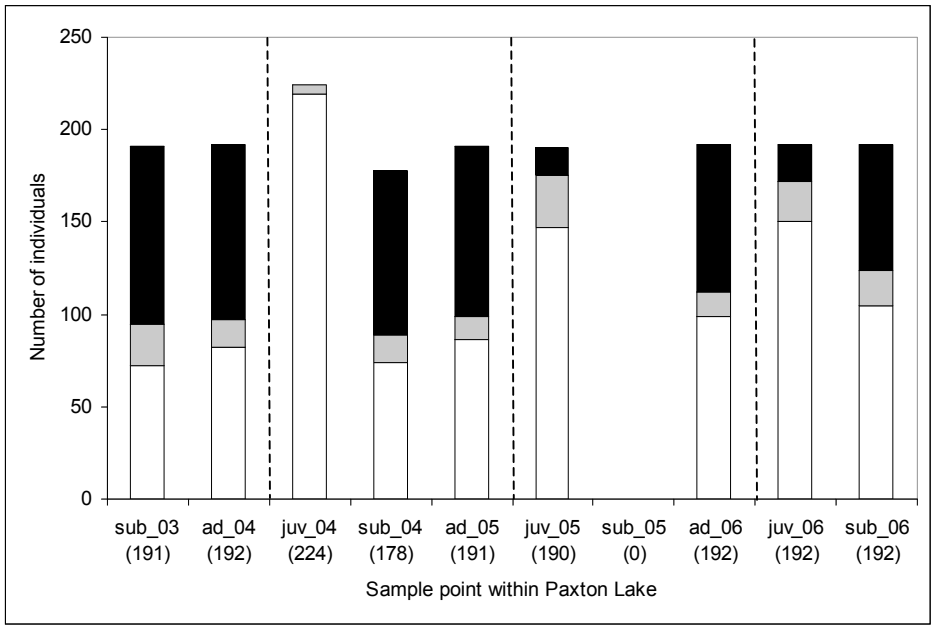


523

524

Figure 3.

(A)


250
200
150
100
50
0
Number of individuals
sub_03 (191)
ad_04 (192)
juv_04 (224)
sub_04 (178)
ad_05 (191)
juv_05 (190)
sub_05 (0)
ad_06 (192)
juv_06 (192)
sub_06 (192)
Sample point within Paxton Lake

(B)

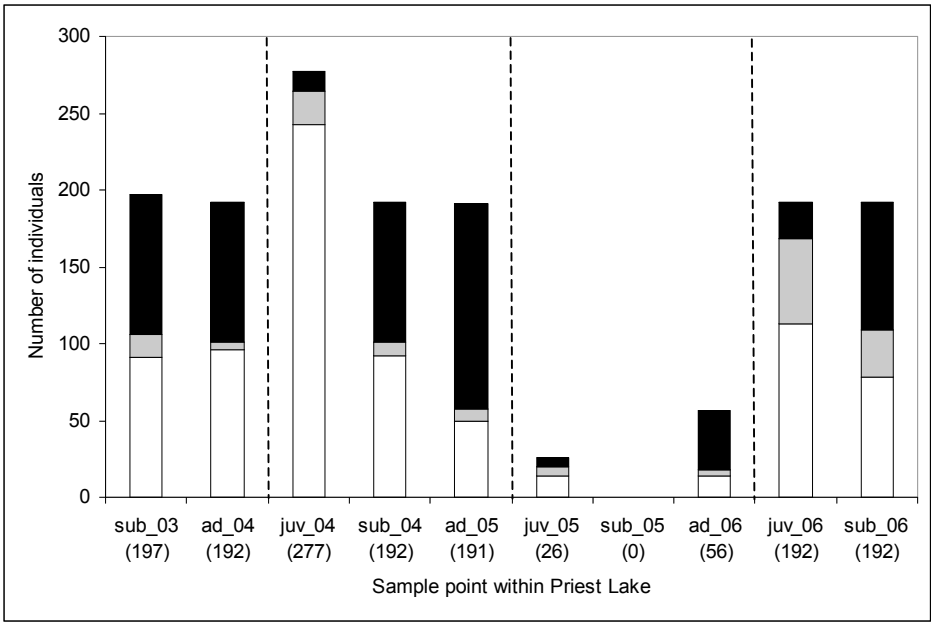
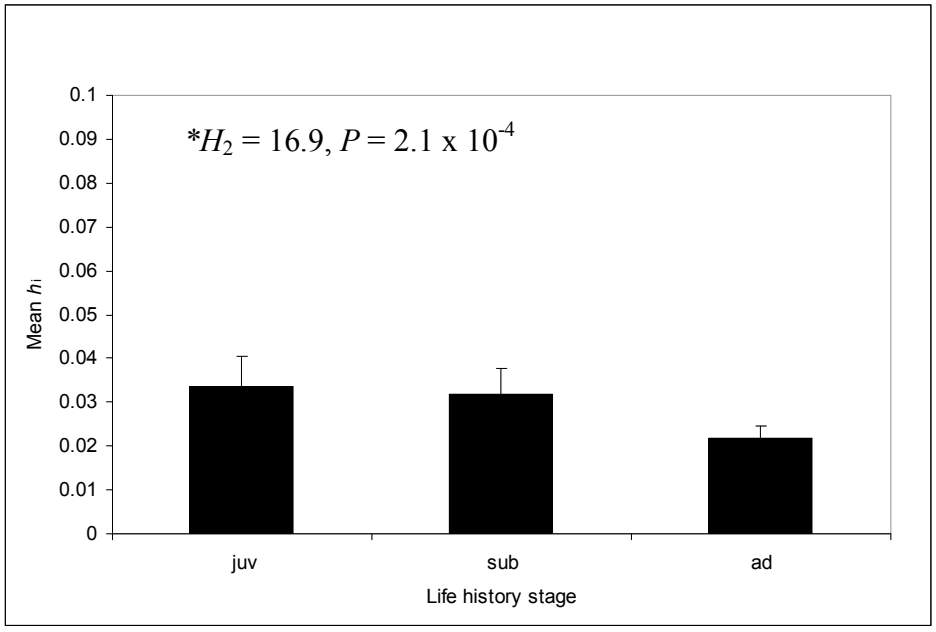

300
250
200
150
100
50
0
Number of individuals
sub_03 (197)
ad_04 (192)
juv_04 (277)
sub_04 (192)
ad_05 (191)
juv_05 (26)
sub_05 (0)
ad_06 (56)
juv_06 (192)
sub_06 (192)
Sample point within Priest Lake

Figure 4.

(A)


*H_2 = 16.9, P = 2.1 x 10^{-4}
Mean h_i
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
juv
sub
ad
Life history stage

(B)

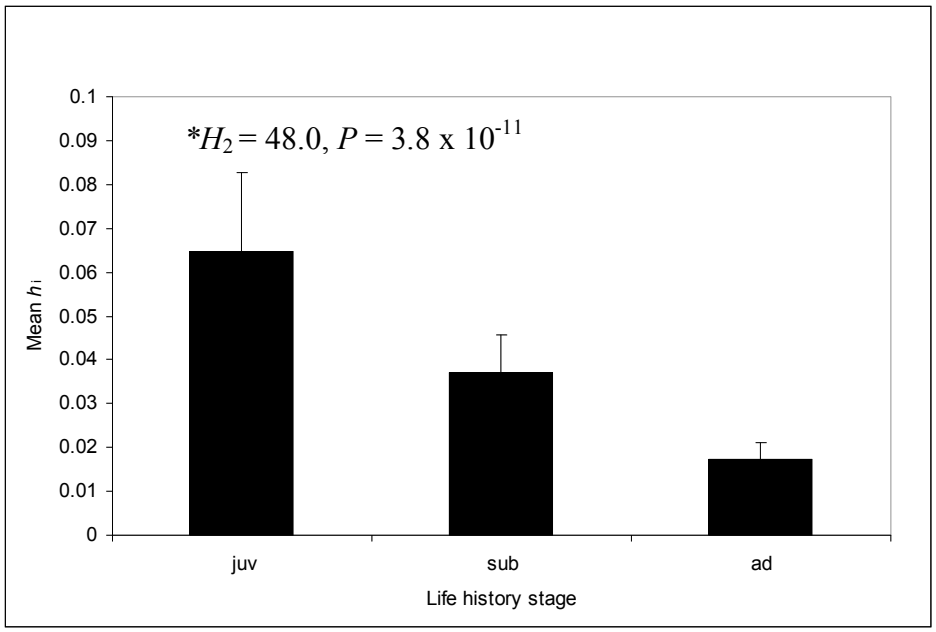
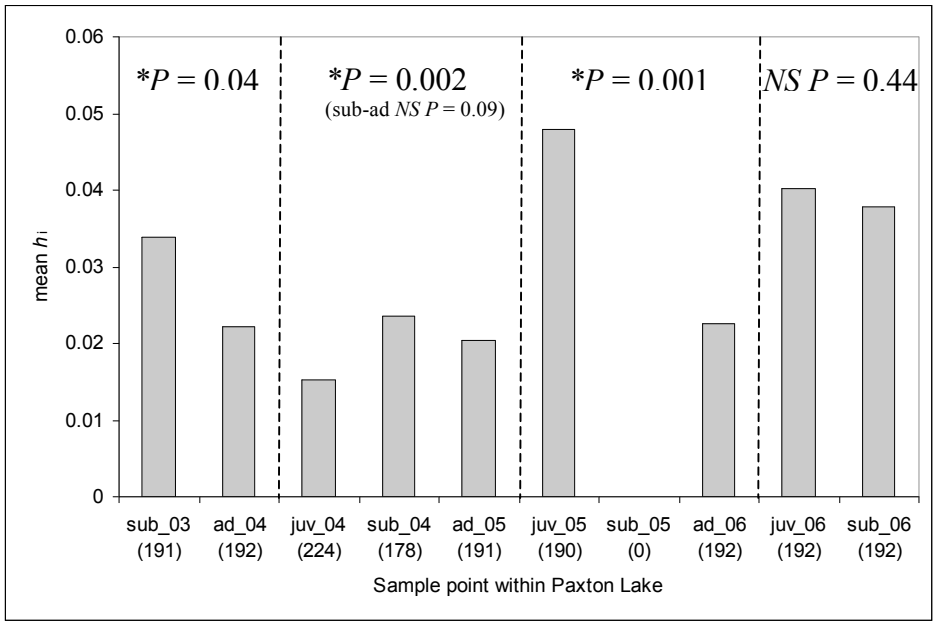
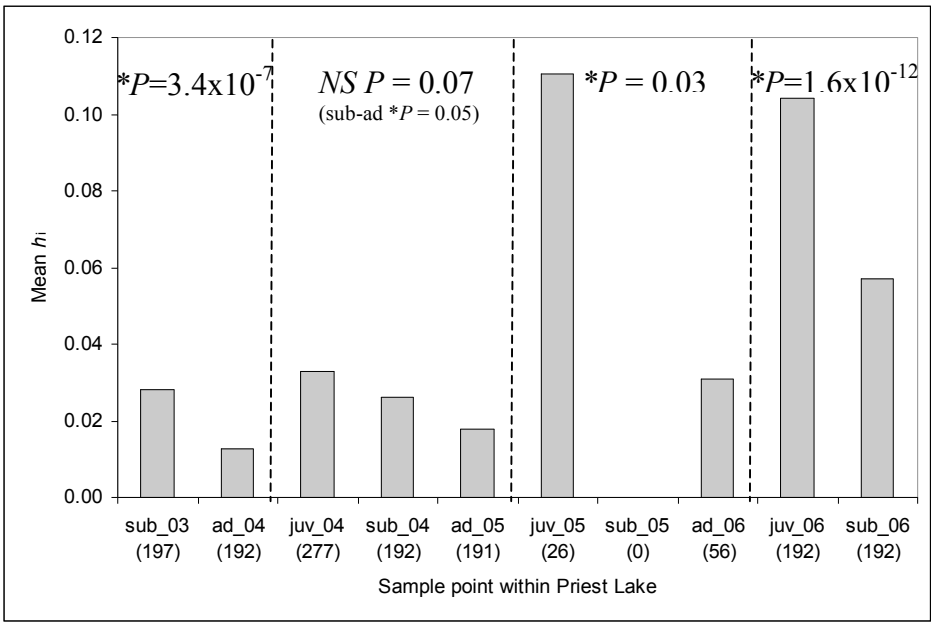

*H_2 = 48.0, P = 3.8 x 10^{-11}
Mean h_i
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
juv
sub
ad
Life history stage

Figure 5.

(A)


*P = 0.04
*P = 0.002
(sub-ad NS P = 0.09)
*P = 0.001
NS P = 0.44
mean h_i
0.06
0.05
0.04
0.03
0.02
0.01
0
sub_03 (191)
ad_04 (192)
juv_04 (224)
sub_04 (178)
ad_05 (191)
juv_05 (190)
sub_05 (0)
ad_06 (192)
juv_06 (192)
sub_06 (192)
Sample point within Paxton Lake

(B)


*P=3.4x10-7
NS P = 0.07
(sub-ad *P = 0.05)
*P = 0.03
*P=1.6x10-12
Mean h_i
0.12
0.10
0.08
0.06
0.04
0.02
0.00
sub_03 (197)
ad_04 (192)
juv_04 (277)
sub_04 (192)
ad_05 (191)
juv_05 (26)
sub_05 (0)
ad_06 (56)
juv_06 (192)
sub_06 (192)
Sample point within Priest Lake

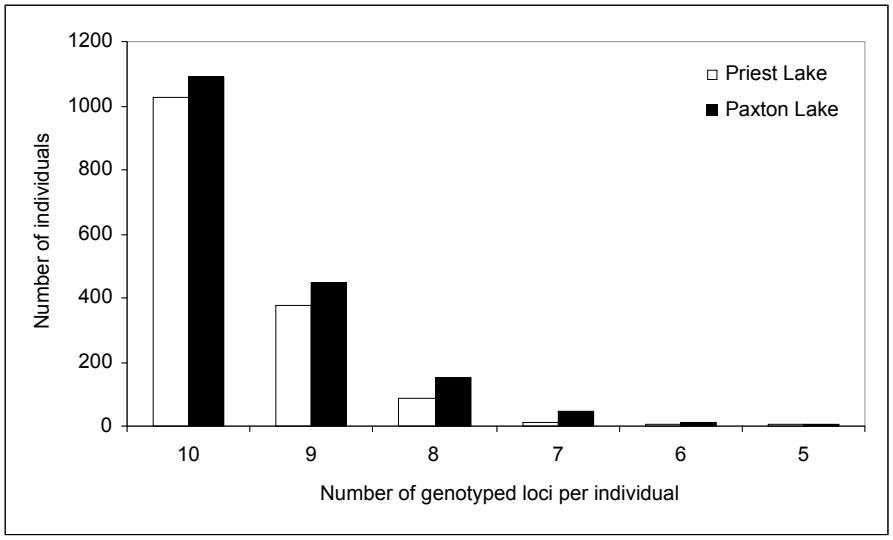
Supplementary material 1

All microsatellites were highly polymorphic and the number of alleles per locus ranged from 20 to 59, with an overall mean of 33 (Table S1). The genotyping fail rate was 4.95 % overall (Table S1). All samples included in the analysis (1742 out of 1747 from Paxton Lake and 1515 out of 1517 from Priest Lake), however, had a minimum of five successfully genotyped loci, with the vast majority (97 % Paxton Lake, 99 % Priest Lake) genotyped at eight or more of the ten loci (Fig. S1A). There was no significant correlation between an individuals' hybridity index and its number of missing genotypes (Fig. S1B; $r_s = 0.00$, $P = 0.99$ for Paxton and $r_s = 0.04$, $P = 0.17$ for Priest Lake species pairs).

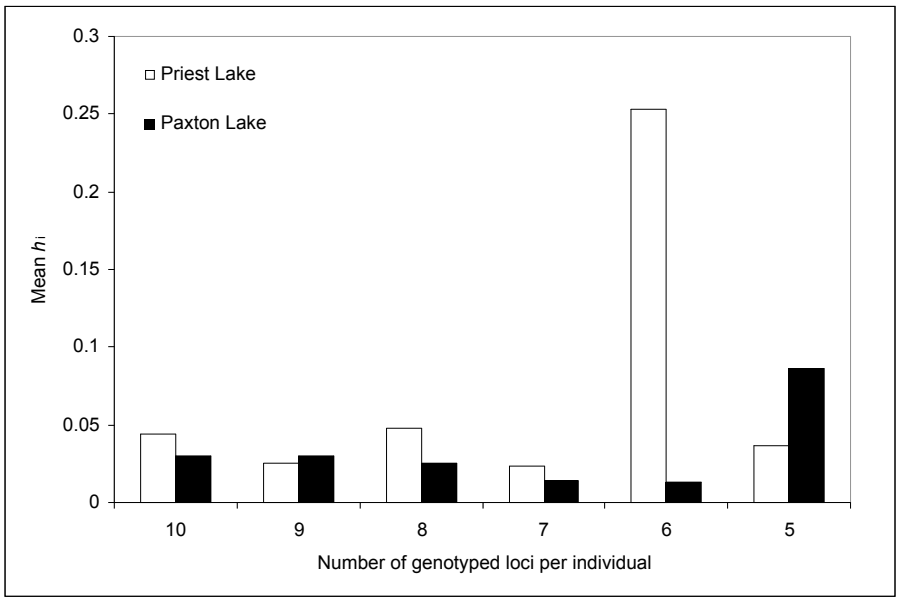
Table S1. Genotyping properties of ten dinucleotide microsatellite loci used to screen 3264 threespine sticklebacks collected from Paxton and Priest Lake species pairs (n = 1517 and 1747, respectively).

	Number of alleles			Alleles (base pairs)			Missing genotypes	
	Priest	Paxton	Both	Lowest	Highest	Range	Number	%
*Stn*216	19	20	25	151	243	92	22	0.67
*Stn*43	22	25	29	124	188	64	128	3.92
*Stn*386	21	19	24	202	259	57	132	4.04
*Stn*388	20	17	20	181	219	38	186	5.70
*Stn*254	24	30	35	203	289	86	110	3.37
*Stn*295	19	28	30	147	221	74	349	10.69
*Stn*142	32	22	34	165	237	72	37	1.13
*Stn*383	30	23	30	164	224	60	313	9.59
*Gac*7	41	38	44	98	198	100	231	7.08
*Cir*51	55	57	59	179	295	116	68	2.08
mean	28.3	27.9	33.0					4.95

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



564
565 (B)



566

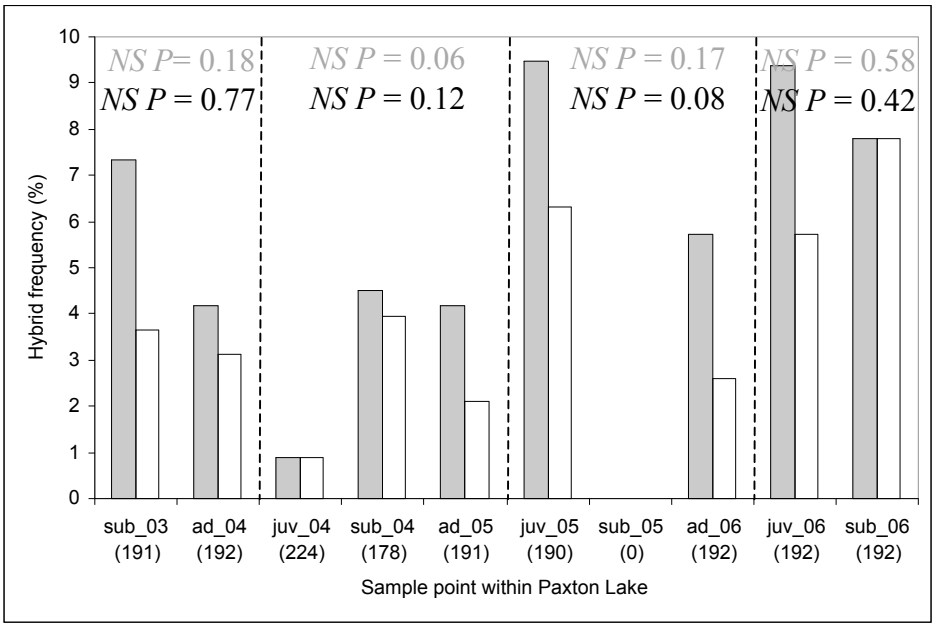
Supplementary material 2

Temporal changes in hybrid frequency derived from two different Bayesian assignments echoed the trends in h_i, with the sole exception of a non-significant shift in trend due to the 2006 Paxton Lake juvenile sample when calculated using the more specific inheritance model implemented in NewHybrids (Fig. S2). Indeed, one-tailed χ^2 tests again found significant overall declines in hybrid frequency across successively older life-history stages in Priest Lake species pair (χ^2_2 = 43.2, $P = 4.2 \times 10^{-10}$ & $\chi^2_2 = 48.8$, $P = 2.6 \times 10^{-11}$ for specific and general inheritance models, respectively), a pattern reflected within generations (Fig. S2). Although this decline in hybrid frequency was not significant within generations of the Paxton Lake species pair, a significant decline from sub-adults to adults was detected with the more specific inheritance model (χ^2_1 = 5.0, $P = 0.025$) when samples were pooled across generations according life-history stage.

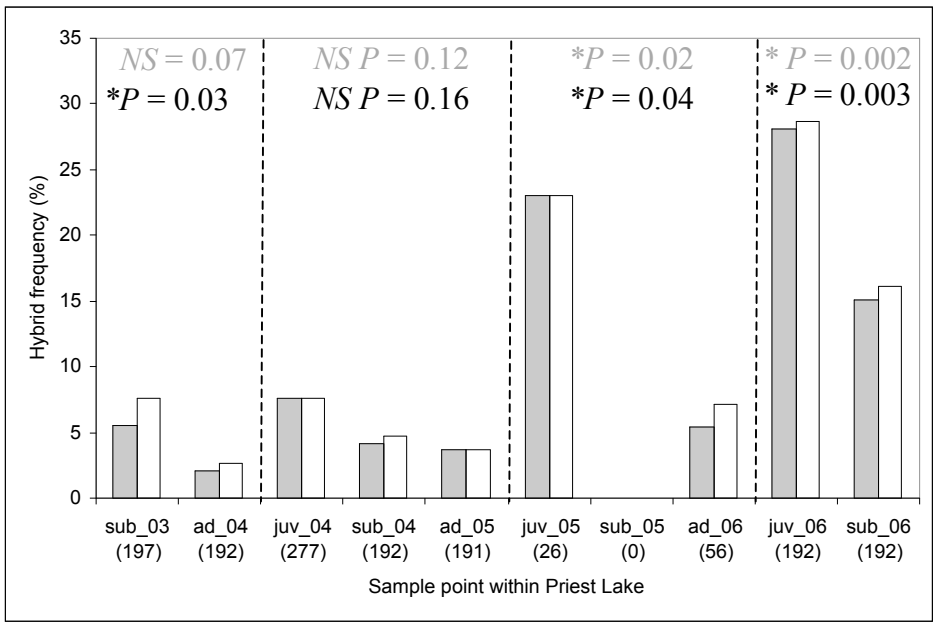
Figure S2. Hybrid frequency among three life history stages spanning four life cycles for (A) Paxton and (B) Priest Lake species pairs. Percentage hybrids calculated using STRUCTURE shown by grey bars, and those using NewHybrids shown by white bars. *P* values given for one-tailed chi-squared (χ^2) tests within each generation: *, significant; *NS*, non significant (grey text for STRUCTURE results, black for NewHybrids). Sample names are composed of life history stage abbreviation (juv, juvenile; sub, subadult; ad, adult) followed by sampling year; sample sizes are given in parentheses; life cycles are separated by dashed vertical lines

Figure S2.

(A)


NS P= 0.18
NS P = 0.77
NS P = 0.06
NS P = 0.12
NS P = 0.17
NS P = 0.08
NS P = 0.58
NS P = 0.42
Hybrid frequency (%)
0
1
2
3
4
5
6
7
8
9
10
sub_03 (191)
ad_04 (192)
juv_04 (224)
sub_04 (178)
ad_05 (191)
juv_05 (190)
sub_05 (0)
ad_06 (192)
juv_06 (192)
sub_06 (192)
Sample point within Paxton Lake

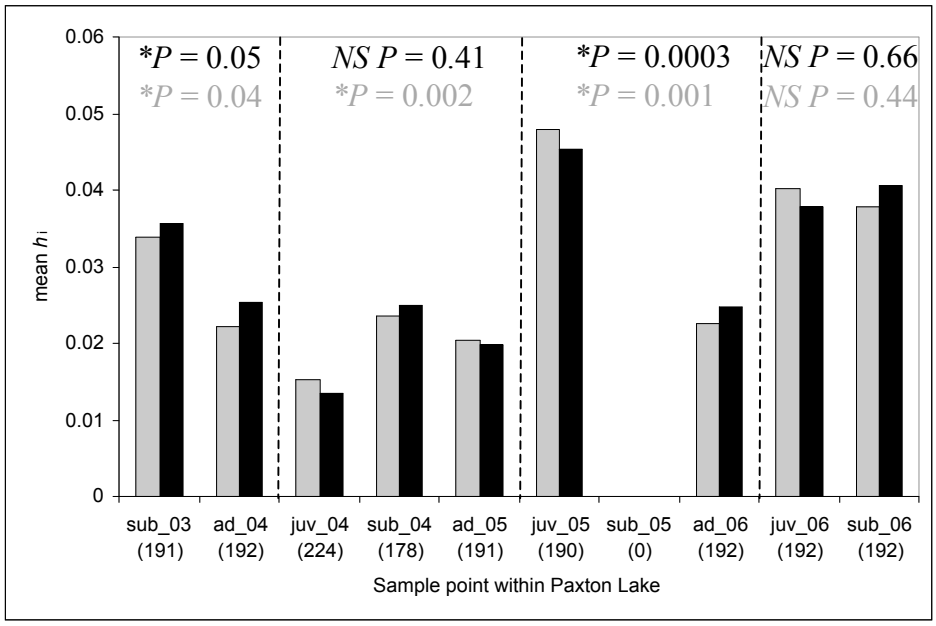
(B)


NS = 0.07
*P = 0.03
NS P = 0.12
NS P = 0.16
*P = 0.02
*P = 0.04
* P = 0.002
* P = 0.003
Hybrid frequency (%)
0
5
10
15
20
25
30
35
sub_03 (197)
ad_04 (192)
juv_04 (277)
sub_04 (192)
ad_05 (191)
juv_05 (26)
sub_05 (0)
ad_06 (56)
juv_06 (192)
sub_06 (192)
Sample point within Priest Lake

Supplementary material 3

The admixture analysis was robust to the exclusion of any particular locus. Reanalysis of the Paxton and Priest Lake species pair data sets after the exclusion of each locus in turn yielded a maximum deviation from the original analysis' mean population $q_b^{(i)}$ of 0.8 % and 1.5 % for the Paxton and Priest Lake species pair, respectively. This resulted in consistently significant declines in hybridity across consecutive life-history stages (Kruskal Wallis one-way ANOVAs all $P << 0.05$) with only one minor discrepancy. When we excluded *Stn*383 from the Paxton Lake species pair data set, sub-adult h_i (0.034) was greater than that for adult h_i (0.023), but also greater than juvenile h_i (0.031). Intra-generation trends, however, remained comparable with the original analysis (Fig. S3).

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 ten microsatellite loci (grey bars) and excluding *Stn*383 (black bars). *P* values given for Kruskal Wallis one-way ANOVAs or Mann-Whitney *U* tests within each generation: *, significant; *NS*, non significant (grey text for analysis of ten loci, black for that excluding *Stn*383). Sample names are composed of life history stage abbreviation (juv, juvenile; sub, subadult; ad, adult) followed by sampling year; sample sizes are given in parentheses; life cycles are separated by dashed vertical lines.



Supplementary material 4

Due to the difficulty of approximating juvenile classification in the field, it was challenging to collect balanced samples. This resulted in the skewing of some juvenile samples towards limnetics (Fig. S4), which could influence hybridity estimates if hybrids are associated with a particular species in nature (Endler. 1986). In particular, a spatial association between hybrids and limnetics in nature could result in the false detection of declining hybridity across the life-cycle. There is, however, little evidence of any such association from experimental trials: foraging behaviour and the pattern of feeding performance to growth rate in hybrids seem most similar to those of benthics (Schluter 1993, 1995), while the composition and prey size of their diet is intermediate but overlapping with both parental species (Schluter 1993; Vamosi *et al.* 2000). There is also no correlation in our data between a sample's proportion of limnetics and hybrid individuals to support the idea of preferential association between hybrids and one or the other parental species ($r_s = 0.05$, $P = 0.89$ for Paxton and $r_s = 0.37$, $P = 0.33$ for Priest Lake species pairs using NewHybrids classifications). Regardless, our findings are robust to the exclusion of juvenile samples from the analysis, with a significant decline from sub-adult to adult life-history stages in three out of four intra-generation comparisons (Fig. 5).

References

Endler, J.A. 1986. *Natural Selection in the Wild*. Princeton University Press, Princeton.

Schluter, D. 1993. Adaptive radiation in sticklebacks: size, shape and habitat use efficiency. *Ecology* **74:** 699–709.

Schluter, D. 1995. Adaptive radiation in sticklebacks: trade-offs in feeding performance and growth. *Ecology* **76:** 82–90.

637 Vamosi, S.M., Hatfield, T. & Schluter, D. 2000. A test of ecological selection against young-of-
638 the-year hybrids of sympatric sticklebacks. *J. Fish. Biol.* **57:** 109-121.

639

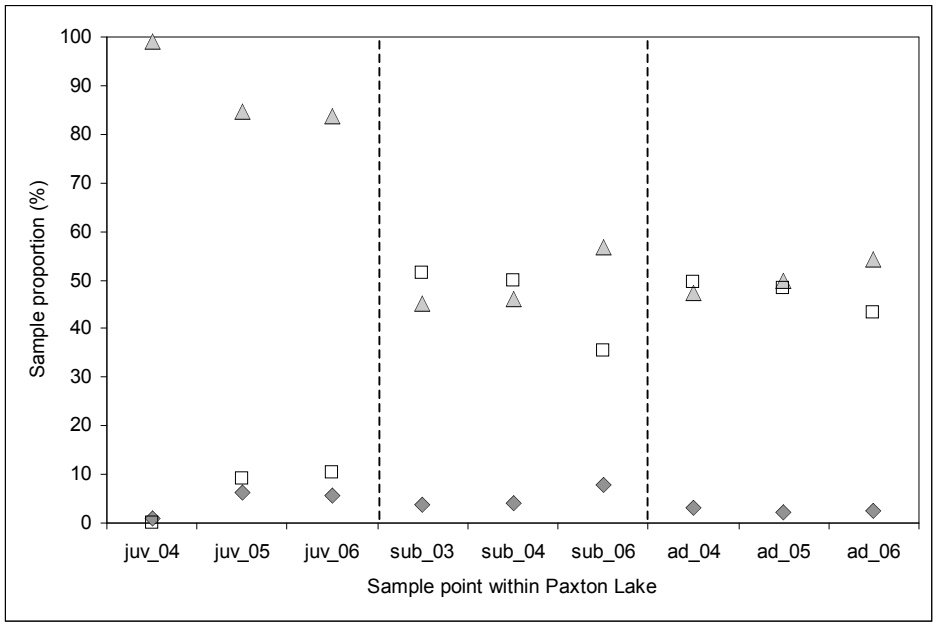
640 **Figure S4.** Proportion of sample assigned as benthic (□), limnetic (△) or hybrid (◈) by
641 NewHybrids for (A) Paxton and (B) Priest Lake species pair. Sample points are arranged
642 chronologically by life-history stage, which are separated by dashed vertical lines. Sample
643 names are composed of life history stage abbreviation (juv, juvenile; sub, subadult; ad, adult)
644 followed by sampling year.

645

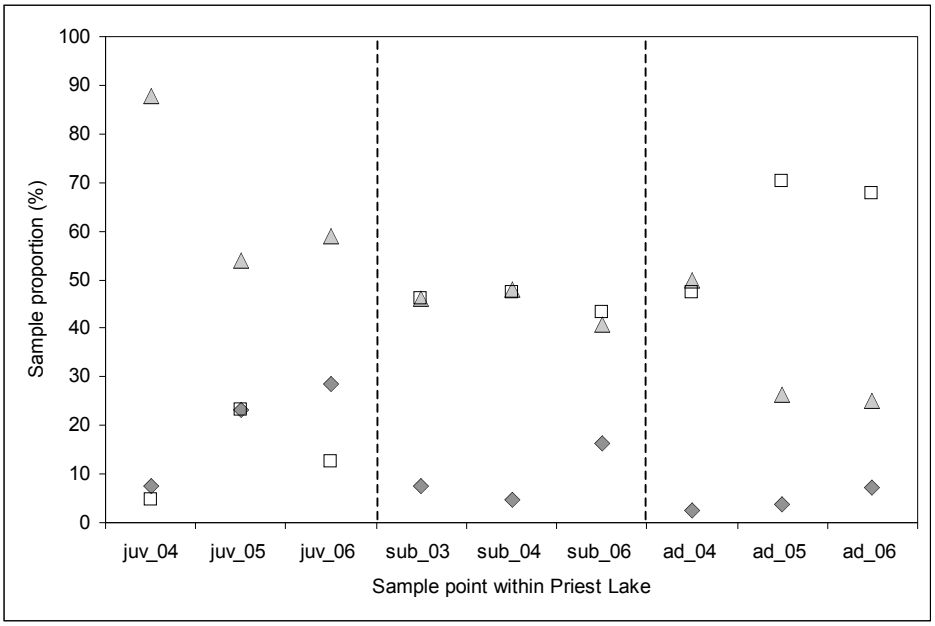
646

Figure S4.

(A)


Sample proportion (%)
100
90
80
70
60
50
40
30
20
10
0
juv_04
juv_05
juv_06
sub_03
sub_04
sub_06
ad_04
ad_05
ad_06
Sample point within Paxton Lake

(B)


Sample proportion (%)
100
90
80
70
60
50
40
30
20
10
0
juv_04
juv_05
juv_06
sub_03
sub_04
sub_06
ad_04
ad_05
ad_06
Sample point within Priest Lake