

## Research



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# Heterosis counteracts hybrid breakdown to forestall speciation by parallel natural selection

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In contrast to ecological speciation, where reproductive isolation evolves as a consequence of divergent natural selection, speciation by parallel natural selection has been less thoroughly studied. To test whether parallel evolution drives speciation, we leveraged the repeated evolution of benthic and limnetic ecotypes of threespine stickleback fish and estimated fitness for pure crosses and within-ecotype hybrids in semi-natural ponds and in laboratory aquaria. In ponds, we detected hybrid breakdown in both ecotypes but this was counterbalanced by heterosis and the strength of post-zygotic isolation was nil. In aquaria, we detected heterosis in limnetic crosses and breakdown in benthic crosses, which is suggestive of process- and ecotype-specific environment-dependence. In ponds, heterosis and breakdown were three times greater in limnetic crosses than in benthic crosses, contrasting the prediction that the fitness consequences of hybridization should be greater in crosses among more derived ecotypes. Consistent with a primary role for stochastic processes, patterns differed among crosses between populations from different lakes. Yet, the observation of qualitatively similar patterns of heterosis and hybrid breakdown for both ecotypes when averaging the lake pairs indicates that the outcome of hybridization is repeatable in a general sense.

## 1. Introduction

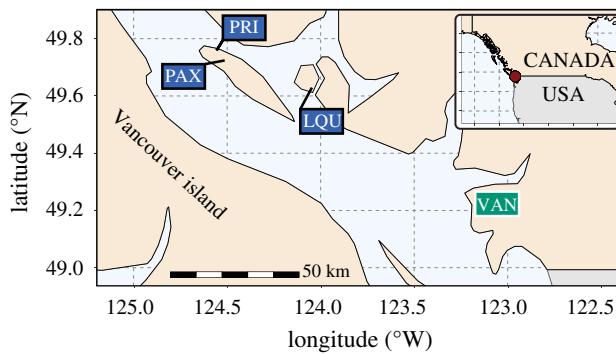
Ecological speciation is the process by which reproductive isolating barriers between lineages evolve as a consequence of adaptation via divergent natural selection between niches or environments [1–4]. Divergent selection can lead to the evolution of post-zygotic isolating barriers when the alleles underlying adaptation to divergent environments function poorly together when combined in hybrids [5]. In many examples of ecological speciation, the fitness of hybrids depends critically on the ecological context [1,3]—termed ‘extrinsic’ post-zygotic isolation [5]—though ‘intrinsic’ or environment-independent barriers can also evolve via divergent natural selection [6,7]. Hybrids formed between ecologically divergent species can have low fitness in the field because there is no niche suited to their intermediate or mismatched phenotypes [8–12], even though they may have high fitness in benign environments [8,13]. The study of ecological speciation has substantially clarified the role of ecology in driving the origin of species.

Compared to speciation by divergent natural selection, speciation by parallel natural selection—where the same phenotype is favoured by selection in different populations—is less studied. Studying speciation by parallel selection is important because it tells us about the role of adaptation by natural selection *per se*, compared to adaptation via divergent natural selection specifically, in speciation [14]. Speciation by parallel natural selection can occur via drift and ‘mutation-order’ processes, when alternative alleles that reduce hybrid fitness arise and are fixed in populations experiencing similar selection pressures [15]. Incompatibility can manifest in F<sub>1</sub> hybrids when there is uniparental inheritance or genetic dominance [16,17], or in recombinant hybrids (e.g. the F<sub>2</sub> generation) when additive alleles segregate. In either case, incompatibilities can be ‘intrinsic’

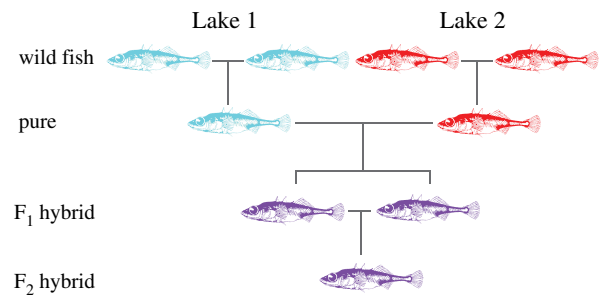
(a) photographs of the extant species pairs



(b) map of study region



(c) cross types



**Figure 1.** System photograph, map and cross schematic. (a) Photographs of the three extant species pairs (benthic–top, limnetic–bottom): Paxton (PAX), Priest (PST) and Little Quarry (LQU) Lakes. (b) Map of the study region, locations of the lakes (blue boxes) and location of our field and laboratory experiments (VAN). (c) Schematic of cross types made within a single ecotype for a single lake pair (no siblings were crossed). (Online version in colour.)

(environment-independent) or ‘extrinsic’, where the combination of opposite-ancestry alleles cause hybrids to express transgressive or mismatched phenotypes that are maladaptive in the parental niche [18–21]. The alternative alleles that cause incompatibility can fix from standing variation if founder populations carried different alleles or had different demographic histories [22], or if distinct *de novo* mutations arise and spread in allopatric populations [23]. The reduction in hybrid fitness caused by the segregation of incompatible alleles in recombinant hybrids is termed ‘hybrid breakdown’ [18].

To test hypotheses about the evolution of post-zygotic barriers by parallel selection, we leveraged the threespine stickleback (*Gasterosteus aculeatus* L.) species pairs (figure 1a). The species pairs are independently derived [24–26] and occur in three lakes in coastal southern British Columbia, Canada (figure 1b). Each lake contains two reproductively isolated stickleback ecotypes—the benthic ecotype is larger and feeds on macroinvertebrates, whereas the limnetic ecotype is smaller and feeds on zooplankton [27–33]. Between-ecotype hybrids are viable and fertile when raised in aquaria, but suffer fitness disadvantages under field conditions [8,29,30,34,35]. The species pairs represent one of the clearest examples of parallel phenotypic evolution in fishes [36]; see [37] for detailed discussion of non-parallel aspects). The species pairs originated <15 kya after anadromous stickleback colonized newly formed post-glacial lakes [38], likely through a process of ‘double invasion’ wherein the original colonists founded the benthic population and a second anadromous population founded the limnetic population [38,39]. Evolution from new mutation is known to occur [40], but a great deal of evolution involved the fixation of alleles from standing genetic variation [26,41].

We conducted experiments in the field and laboratory to investigate the fitness consequences of hybridization after

parallel phenotypic evolution. We tracked the survival and growth of juveniles to estimate fitness. Because post-zygotic isolation in stickleback is typically extrinsic [8,35,42], we expected fitness differences to manifest primarily under field conditions. We anticipated any of three main patterns. First, hybrids have no reduction in fitness, as expected if evolution is highly parallel or if divergent alleles are compatible. Second, hybrids exhibit breakdown, as expected if opposite-ancestry alleles are incompatible. Third, hybrids exhibit heterosis, as expected if heterozygosity is favoured by selection. Prior research indicates a largely additive genetic basis of phenotypic evolution in the species pairs [43,44] that is partially shared among different populations of the same ecotype [37]. Given this, we expected that  $F_1$  hybrids would have similar fitness to pure crosses and that  $F_2$  hybrids might exhibit hybrid breakdown due to the segregation of population-specific QTL [17,19,20]. Theoretical studies using Fisher’s [45] geometric model of adaptation with *de novo* mutation predict that hybrid breakdown is proportional to the magnitude of evolutionary change in parallel from the common ancestor [18,19]. Thus, we predicted that its magnitude would be greater in crosses between populations of the more derived benthic ecotype than in crosses between limnetic populations, which are more ancestor-like [24,26,46]. Our results provide novel insights into the efficacy and predictability of speciation by parallel selection.

## 2. Methods

### (a) Experimental crosses

We made all possible pair-wise within-ecotype crosses using the three extant benthic–limnetic species pairs—Paxton, Priest and Little Quarry Lakes (figure 1c). We made pure within-population

crosses (i.e. 'parental' or 'non-hybrid' crosses) for each ecotype and lake. We also generated  $F_1$  and  $F_2$  within-ecotype hybrids. All crosses were between unrelated families, but some families and individuals were used as parents more than once in separate crosses. Parents of all crosses hatched in the laboratory, and no wild progenitors were collected before 2016. Crosses were made in March 2020. The data underlying the results are drawn from 111 unique crosses.

### (b) Pond experiment

The pond experiment occurred in three semi-natural experimental ponds at the University of British Columbia. Ponds were established from 2008 to 2010, are  $25 \times 15$  m, and contain habitat for both ecotypes (see [43]). Previous research indicated that the diets of benthic and limnetic fish in the ponds were similar to their diets in natural lakes [43]. Except for their use in previous experiments, the ponds are unmanipulated environments. Each pond contained fish with ancestry from only two lakes (pond 4—Paxton and Little Quarry; pond 9—Priest and Little Quarry; pond 19—Priest and Paxton).

The pond experiment included over 3700 individual fish—at least 600 from each ecotype in each of the three ponds (see electronic supplementary material, table S1). Sample sizes were determined using power analyses conducted in advance of the experiment (electronic supplementary material, Methods). For crosses of both ecotypes, we introduced approximately 100 individuals of both pure crosses, 200  $F_1$  hybrids, and 200  $F_2$  hybrids (see electronic supplementary material, figure S1).

Before introduction, fish were weighed to the nearest 0.01 g and then implanted with a sequential coded wire tag (hereafter 'tags'; Northwest Marine Technology, Anacortes, WA, USA). Tags are dissected out of recaptured fish and read to identify individuals (electronic supplementary material, Methods). Tagged fish were approximately 25–35 mm standard length. Fish recovered for 48 h after tagging, then were transported to ponds in coolers filled halfway with water from their tank. At the ponds, we diluted the tank water 50:50 with pond water and released fish into the ponds the following morning. We read the tags of the few fish that perished during this period and removed them from the analysis. Establishment of each pond occurred over approximately two weeks in 2020 (pond 4: 14–28 June; pond 9: 29 June–10 July; pond 19: 11–24 July).

We retrieved surviving fish from each pond using minnow traps and by dip-netting beginning on 14 September 2020. When fish returns slowed to  $\leq 5$  per evening of trapping, we added 2 l of 5% rotenone to each pond. Remaining fish were captured as they swam to the surface. Fish were euthanized with an overdose of MS-222, then we recorded their mass, took a photograph, and froze them at  $-20^\circ\text{C}$  in labelled tubes. Tags were extracted from lightly thawed fish and then read.

### (c) Laboratory experiment

While the pond experiment was ongoing, we conducted an experiment in laboratory aquaria with the goal of inferring whether the patterns observed in ponds were environment-dependent. The laboratory experiment was conducted using 1101 aquaria ( $n=60$ ) in a common recirculating system. Most aquaria contained both ecotypes and all individuals from a given ecotype inhabiting the same tank were from the same family. Some families were unique to the aquarium experiment, and other tanks contained siblings of fish in the pond experiment. Twenty grams of fish were added to each tank ( $n=2151$ ). We recorded the mass of each fish at the experiment onset, but fish were not tagged. Immediately after we ended the pond experiment, we euthanized all surviving fish in aquaria with an overdose of MS-222, and recorded their ecotype

(distinguishable visually) and mass (mean days in aquaria = 74). We estimated growth only in aquaria where all fish of a given ecotype survived. In these aquaria, we assume that the individuals' rank-order of sizes did not change between the beginning and end of the experiment and quantified the difference in mass between the initial and final sampling points as 'growth'. Survival is recorded as the difference in the number of fish between the start and end of the experiment.

### (d) Data analysis

We fitted linear or generalized linear models depending on the fitness component. Response variables were either survival (binary; generalized linear model) or final mass (continuous; linear model). All mass variables were log-transformed ( $\ln[x + 1]$ ) prior to analysis. When comparing cross types (i.e. pure,  $F_1$ ,  $F_2$ ), we fitted models for the benthic and limnetic ecotypes separately because of significant differences in survival and body size, but used parameter estimates to calculate a composite fitness metric that was compared directly between ecotypes (see below). We fitted separate models for pond and aquarium data.

Most models fitted to pond data included 'lake pair' as a random effect, which includes the combined effects of pond and lake pair ('pair-as-random' models). These models included cross type as a fixed effect with three factor levels: pure,  $F_1$  hybrid and  $F_2$  hybrid. Initial mass was a covariate in all models. For models of growth, we included duration—the number of days between the initial and final mass measurement—as a covariate. Survival models also included a duration term, calculated as the difference between the day a fish was introduced into the pond or tank and the first day of trapping (pond) or sampling (aquarium); we do not know on which day an unrecovered fish died. Although we analyse the model output directly, we base our conclusions primarily on a composite fitness metric (described below) that combine survival and growth estimates from models. These pair-as-random models thus estimate the fitness consequences of hybridization for both ecotypes across the three lake pairs.

The fitness consequences of hybridization after parallel phenotypic evolution are determined by chance events during evolution, and we therefore tested whether the fitness consequences of hybridization differed among lake pairs (e.g. Paxton  $\times$  Priest versus Paxton  $\times$  Little Quarry). These analyses fit models to pond data that included lake pair, and the interaction between cross type and lake pair, as fixed effects ('pair-as-fixed' models). Note that the effect of lake pair is confounded by effects that could be specific to a single pond—this is addressed in the Discussion. Estimates of mean survival and growth, which are generated for each cross type for each lake pair for both ecotypes, were used to estimate composite fitness and allowed us to estimate the extent to which patterns of hybrid fitness were not repeatable among lake pairs.

For all analyses (both pond and laboratory experiments), we did not include family (i.e. unique fertilized clutch) or rearing tank (for large families that were split into multiple tanks) as random effects in our analysis. Thus, each individual fish is considered an experimental unit. Because many families were split between two or more tanks, the variance among rearing tanks includes the variance among families. Family accounted for no additional variance when tank was included in a model estimating final mass (model comparison ANOVA;  $p=0.24$ ). All fish from a tank went into the pond at the same time, and the time of introduction of each tank was determined systematically to ensure maximal variation among cross types and ecotypes. We therefore account for family and rearing tank effects by including initial mass and duration (determined by introduction date which is the same for all fish in a rearing tank) in all models fitted to pond data.

All analyses were done using R v. 4.0.3 [47]. Mixed models were fit with the `lme4` package [48] and analysed with `lmerTest` [49] and the Kenward–Roger approximation for the denominator degrees of freedom [50]. *Post hoc* analyses were done using the ‘`emmeans`’ and ‘`pairs`’ functions in `emmeans` [51], with Tukey HSD-corrected *p*-values. Model fits were visualized with `visreg` [52]. Data processing used functions in the `tidyverse` [53].

### (e) Composite fitness metric

We generated a composite fitness metric for each cross type as the product of survival proportion and mean growth (final mass) [54,55]. Because survival and growth cumulatively affect fitness, composite fitness metrics capture group differences more authentically than survival or growth alone. Conclusions of analyses with multiplicative fitness are more conservative than an alternate metric estimating fitness using more assumption-laden estimates of fecundity and overwinter survival (electronic supplementary material, table S2). We estimated fitness of each hybrid cross type and combined pure parents in each pond using estimates of survival and growth from models. Mass estimates were back-transformed from the log scale for fitness calculations [56]. We divided each cross type’s fitness value by the composite fitness of the pure crosses to standardize them. Thus,  $F_1$  and  $F_2$  fitness is relative to the pure crosses, which have a value of 1.

We used bootstrapping for statistical comparisons of composite fitness. Bootstrap iterations resampled survival and growth of individuals of each cross type so that the sample sizes were identical to the authentic dataset. For the pond experiment, resampling was conducted within the individual aquaria that were tagged and released into the ponds to ensure the ‘duration’ covariate was consistent across iterations. Resampling for the aquarium experiment was also conducted within individual aquaria. We analysed the data from each iteration using identical models as conducted above. Because we only had three lake pairs, sample sizes were too low to resample the ‘pair’ random effect. Each iteration returned point estimates for composite fitness. To compute confidence intervals of composite fitness, the bootstrapped fitness estimates were divided by the composite fitness of the relevant pure cross observed in the authentic data. Alternative bootstrap resampling procedures were similar or less conservative (electronic supplementary material, figure S2). We considered comparisons to be statistically significant if the 95% bootstrap confidence interval of the difference excluded 0. We tested all pairwise differences among pure,  $F_1$  hybrid and  $F_2$  hybrid crosses. The pure versus  $F_1$  difference tests for heterosis. We tested for hybrid breakdown by determining if the fitness of  $F_2$  hybrids was less than the midpoint of pure crosses and  $F_1$  hybrids because any effect of heterozygosity that manifests in the  $F_1$  is expected to be halved in the  $F_2$  (these analyses account for uncertainty in the estimate of the midpoint). Post-zygotic isolation is the difference between pure crosses and  $F_2$  hybrids, and we only interpret data from ponds for this metric. In addition to the differences among cross types within each ecotype, bootstrap data are used to compare the magnitude of heterosis and breakdown between benthic and limnetic ecotypes, and to compare pond and aquarium experiments.

## 3. Results

### (a) Summary of pond experiment

We recovered tags from 59.6% of fish that were introduced into ponds. Initial mass and ‘duration’ were both positively associated with final mass (electronic supplementary material, figures S3 and S4). Recapture rate, which we

assume reflects survival, was 78.2% for benthic crosses and 40.9% for limnetic crosses (‘ecotype’ main effect:  $\chi^2_1 = 402.92$ ;  $p < 0.0001$ ). Recaptured benthic fish were on average 3.8× their initial mass and recaptured limnetic fish were on average 2.4× their initial mass.

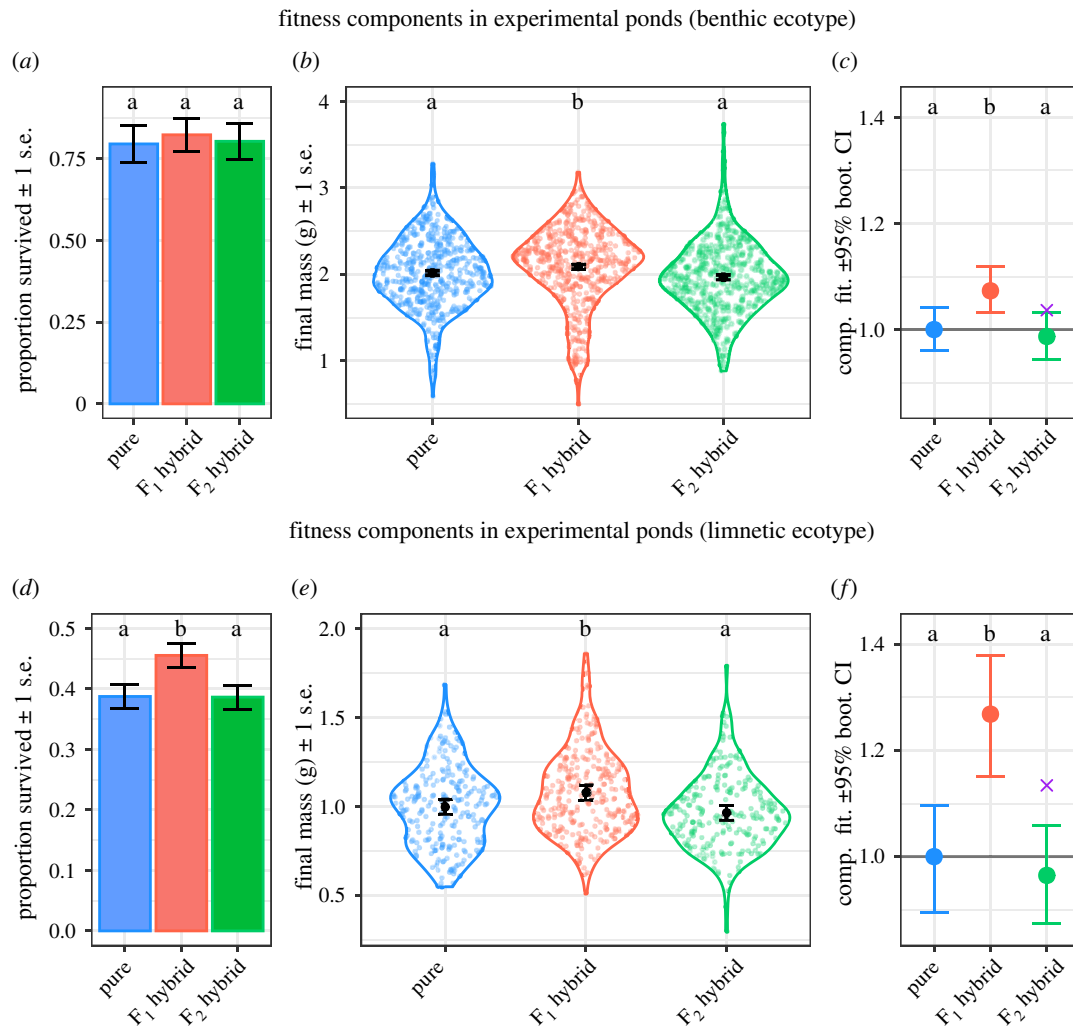
### (b) Relationships among cross types

Cross type (i.e. pure,  $F_1$ , or  $F_2$ ) often had a significant effect on survival and growth in ponds for both ecotypes (figure 2; pair-as-random models). Growth—but not survival—differed among benthic cross types (main effects; survival— $\chi^2_2 = 1.61$ ,  $p = 0.45$ , growth— $F_{2,1437} = 8.72$ ,  $p = 0.0002$ ), and both survival and growth differed among limnetic cross types (main effects; survival— $\chi^2_2 = 7.86$ ,  $p = 0.02$ ; growth— $F_{2,744} = 17.56$ ,  $p < 0.0001$ ). In all cases where significant differences were detected among cross types in ponds, the pattern was:  $F_1$  hybrid  $>$  pure =  $F_2$  hybrid. In aquaria, we detected growth but not survival differences among benthic cross types (figure 3a; survival— $\chi^2_2 = 2.88$ ;  $p < 0.23$ ; growth— $F_{2,432} = 50.75$ ;  $p < 0.0001$ ) and detected differences among limnetic cross types in both survival and growth (figure 3b; survival— $\chi^2_2 = 19.02$ ;  $p < 0.0001$ ; growth— $F_{2,258} = 50.75$ ;  $p < 0.0001$ ). Where differences were detected in aquaria, the pattern for benthic crosses was: pure  $>$   $F_1$  hybrid =  $F_2$  hybrid, while for limnetic crosses the pattern was:  $F_1$  hybrid =  $F_2$  hybrid  $>$  pure.

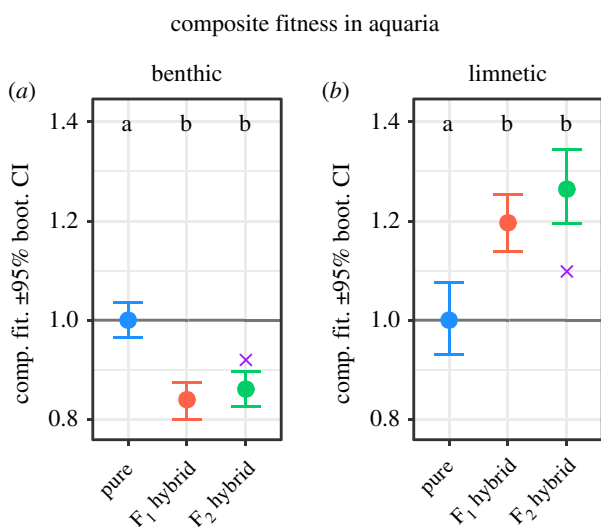
The remainder of our analyses report the composite fitness metric because differences among groups were similar for this metric as for survival and growth.

We detected significant heterosis—defined as a difference in fitness between pure crosses and  $F_1$  hybrids—in both ecotypes in ponds (electronic supplementary material, figure S6; pair-as-random models). Benthic  $F_1$  hybrids had 7% greater fitness than pure benthic crosses ( $p = 0.012$ ), and limnetic  $F_1$  hybrids had 27% greater fitness than pure limnetic crosses ( $p = 0.002$ ). This 3.9× greater heterosis in limnetic  $F_1$  hybrids compared to benthic  $F_1$  hybrids represents a significant difference in the magnitude of heterosis between ecotypes (electronic supplementary material, figure S7;  $p = 0.032$ ). In aquaria, we observed significant heterosis in limnetic crosses, with  $F_1$  hybrids having 19% greater fitness than pure crosses ( $p < 0.001$ ) whereas composite fitness was reduced by 16% in benthic  $F_1$  hybrids compared to pure crosses ( $p < 0.001$ ) (electronic supplementary material, figure S8). The magnitude of heterosis was significantly greater in ponds than in aquaria for benthic  $F_1$  hybrids ( $p = 0.024$ ), but did not differ between experiments for limnetic  $F_1$  hybrids ( $p = 0.68$ ) (electronic supplementary material, figure S9).

We detected significant hybrid breakdown—defined as a difference in fitness between  $F_2$  hybrids and the pure: $F_1$  hybrid midpoint—in both ecotypes in ponds (electronic supplementary material, figure S6; pair-as-random models). The composite fitness of benthic  $F_2$  hybrids was reduced by 5% below the pure: $F_1$  hybrid midpoint ( $p = 0.042$ ), and the composite fitness of limnetic  $F_2$  hybrids was reduced by 16% below the pure: $F_1$  hybrid midpoint ( $p = 0.016$ ). Similar to heterosis, the magnitude of breakdown was 3.1× greater among limnetic crosses than among benthic crosses, though this was not significant (electronic supplementary material, figure S7;  $p = 0.08$ ). In aquaria, we detected hybrid breakdown for benthic crosses ( $p = 0.001$ ) and found that the composite fitness of limnetic  $F_2$  hybrids was higher than expected under



**Figure 2.** Survival, growth and composite fitness of pure population crosses and their F<sub>1</sub> and F<sub>2</sub> hybrids in the experimental ponds (pair-as-random models) for both ecotypes (benthic: (a–c); limnetic: (d–f)). Survival and growth plots show estimated marginal means [51] ± 1 s.e. Points in (b,e) are partial residuals of final mass for individual fish. Composite fitness (c,f) was estimated by multiplying survival and growth and dividing by the ‘pure’ value, ± 95% bootstrap confidence intervals. Different letters indicate a significant difference at  $p = 0.05$ . Purple ‘x’ symbols indicate the pure:F<sub>1</sub> hybrid midpoint as a reference for the F<sub>2</sub> mean under additivity (uncertainty around the midpoint is not shown in the figure but is accounted for in the analysis). (Online version in colour.)



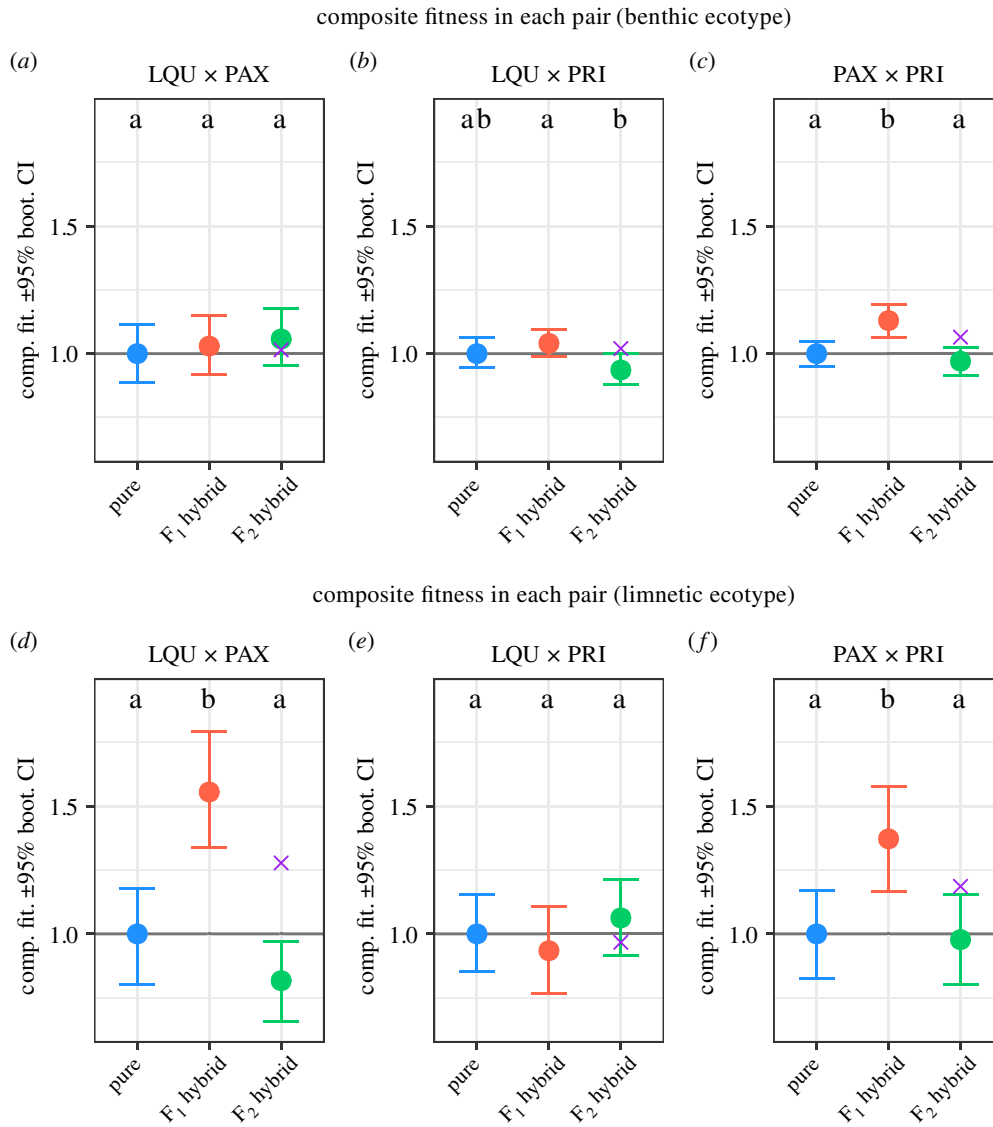
**Figure 3.** Composite fitness among (a) benthic and (b) limnetic cross types in aquaria. Points are composite fitness ± 95% bootstrap CI. See electronic supplementary material, figure S5 for underlying survival and growth data. Letters indicate significant differences at  $p = 0.05$ . Purple ‘x’ symbols indicate the expected F<sub>2</sub> mean under additivity (uncertainty around the midpoint is not shown in the figure but is accounted for in the analysis). (Online version in colour.)

additivity ( $p < 0.001$ ) (electronic supplementary material, figure S8). As a result, the magnitude of hybrid breakdown did not differ between ponds and aquaria for benthic crosses ( $p = 0.7$ ) and was significantly greater in ponds than in aquaria for limnetic crosses ( $p < 0.001$ ) (electronic supplementary material, figure S9).

We detected no evidence of post-zygotic isolation via composite fitness in ponds in the pair-as-random models. Specifically, for both the benthic and limnetic ecotype, the composite fitness of pure crosses and F<sub>2</sub> hybrids was statistically indistinguishable (benthic  $p = 0.68$ ; limnetic  $p = 0.63$ ).

### (c) Variation among lake pairs in ponds

Patterns differed among lake pairs (figure 4; see electronic supplementary material, figure S10 for underlying fitness components). In benthic crosses, we detected heterosis in one of the three lake pairs (Paxton × Priest [ $p < 0.001$ ]) and detected breakdown in two of the three lake pair crosses (Paxton × Priest [ $p = 0.002$ ] and Little Quarry × Priest [ $p = 0.014$ ]). In limnetic crosses, we detected heterosis in two of three lake pairs (Paxton × Priest [ $p = 0.01$ ] and Little Quarry × Paxton [ $p < 0.001$ ]) and breakdown in one of the three



**Figure 4.** The fitness consequences of hybridization differed among lake pairs (pair-as-fixed models) for both ecotypes (benthic: (a–c); limnetic: (d–f)). Points are composite fitness  $\pm$  95% bootstrap CI. Letters indicate significant differences at  $p = 0.05$ . Purple ‘x’ symbols indicate the expected  $F_2$  mean under additivity (uncertainty around the midpoint is not shown in the figure but is accounted for in the analysis). (Online version in colour.)

lake pairs (Little Quarry  $\times$  Paxton [ $p < 0.001$ ]; breakdown approached significance in the Paxton  $\times$  Priest cross [ $p = 0.054$ ]).

## 4. Discussion

In this study, we quantified the fitness consequences of hybridization after parallel phenotypic evolution in three-spine stickleback fish. In agreement with predictions from theory, we detected significant hybrid breakdown between benthic populations and between limnetic populations in experimental ponds. However, we also detected an equal magnitude of heterosis and thus the composite fitness of pure crosses and  $F_2$  hybrids were indistinguishable. The magnitudes of heterosis and hybrid breakdown were more than 3 $\times$  greater in limnetic crosses than in benthic crosses, contrasting the prediction that the ecotype most divergent from the ancestor should show the greatest amount of breakdown. Data from aquarium-raised fish indicate that heterosis is environment-independent (i.e. ‘intrinsic’) in limnetic crosses and that hybrid breakdown is environment-independent in benthic crosses. Although patterns differed among lake

pairs for crosses of both ecotypes (estimated via pair-as-fixed models), heterosis and hybrid breakdown were observed in both ecotypes via pair-as-random models. Below, we discuss the mechanisms that might underlie our findings and their implications for the process of speciation by parallel natural selection.

We detected hybrid breakdown that reduced  $F_2$  hybrid fitness below the pure:  $F_1$  midpoint, which suggests that incompatible alleles segregated in the  $F_2$  generation [18]. Hybrid breakdown was only detected in ponds in limnetic crosses, which thus suggests that hybrid incompatibilities [57,58] had environment-dependent effects on fitness [13]. In  $F_2$  benthic  $\times$  limnetic hybrids, previous studies [43,59] found evidence that mismatched trait combinations reduced individual fitness. By re-analysing the genetic data from one of these studies [43] and others, we previously found evidence that a genetic signature of selection against hybrid incompatibilities was detectable in pond-raised stickleback hybrids, but not those raised in aquaria [35]. Studies in yeast [60], anoles [61] and sunflowers [62] have similarly concluded that selection on hybrid incompatibilities can have an ecological basis. In line with emerging findings that recognize

ecology's role in shaping epistasis within populations [63], it is increasingly clear that ecology can mediate the fitness consequences of hybrid incompatibilities.

In spite of  $F_2$  hybrid breakdown, we detected no post-zygotic isolation in ponds because of counteracting heterosis. We detected heterosis in aquarium-raised limnetic  $F_1$  hybrids but not in benthic  $F_1$  hybrids, which is consistent with previous findings that features of the environment can underlie heterosis [64].  $F_1$  heterosis (and  $F_2$  breakdown) is regularly observed in plants [65–68], but is not as common in outbred wild animal species. Some models suggest that heterosis is a general property of hybrid systems [69], and it could be the case that ecological divergence overrides heterosis in most animal systems that have been subject to experimentation (e.g. in divergent crosses between [rather than within] benthic and limnetic ecotypes [8]). Studies of model systems often find evidence for heterosis during the early stages of divergence, followed by declines over longer time spans [70,71]. It is likely that heterosis is an ephemeral phenomenon in our system while hybrid breakdown is enduring.

Our findings are opposite to the theoretical prediction that crosses among derived populations should have more exaggerated fitness consequences than crosses among ancestor-like populations. Greater heterosis and breakdown among limnetic crosses might be explained by differences in the selective regimes experienced by the two ecotypes in the experimental ponds. Limnetic crosses survived at approximately half the rate of benthic crosses and so experienced greater opportunity for selection (i.e. greater variance in composite fitness). Food was unlikely to be limiting in ponds and we do not suspect starvation: all three experimental ponds lacked fish for at least 2 years prior to the experiment and pond-raised fish grew considerably larger than fish in aquaria (electronic supplementary material, figure S12). We suspect mortality was largely caused by frequently observed enemies such as piscivorous birds and/or predatory insects, though insects rarely attack stickleback in the size range that we introduced [72,73]. Several fish were found dead during the experiment with large wounds, and some recaptured fish had similar wounds which had healed. Worm-like parasites, visible through the skin, were not uncommon. Differences in growth among cross types, which generally showed similar qualitative patterns to differences in survival, might have resulted from particular genotypes being less able to capture prey. Our findings imply that the fitness consequences of hybridization might depart from theoretical expectations based on the ecology of the group being studied and the environment in which it is measured.

Although patterns of heterosis and hybrid breakdown varied among the three lake pairs for both benthic and limnetic crosses (figure 4), qualitative patterns were similar for the two ecotypes in the pair-as-random models (figure 2). This finding—variability among pairs, but consistency across pairs—is consistent with theory on the process of speciation by parallel natural selection. Under divergent evolution, populations are expected to fix different alleles during adaptation and hybrid fitness may be largely determined by selection against intermediate or mismatched hybrid phenotypes [8,74]. Although the same alleles might be favoured in different populations under parallel selection, stochasticity in the origin and fixation of *de novo* mutations or differences in the founding population's standing variation lead to

differences in the extent of genetic parallelism [15,20,23,75]. The fitness of hybrids between phenotypically similar parent populations is largely determined by the degree of genetic parallelism during adaptation [20]. Despite this variation among lake pairs, we found evidence for counteracting heterosis and hybrid breakdown among both benthic and limnetic cross types in the pair-as-random models, implying that broad patterns in the genetics of parallel phenotypic evolution are shared between benthic and limnetic populations. Thus, while we should expect stochasticity at the level of individual population pairs, predictable patterns might emerge if multiple such pairs are considered.

Our experiments make a number of assumptions. First, we assume that the ponds are reasonable stand-ins for natural lakes. Second, our design only captures natural selection and sexual selection could act differently [59]. Third, different populations of the same ecotype are phenotypically similar but not identical [37] and also have differences in mean fitness (electronic supplementary material, figure S11). Fourth, we cannot decouple 'lake pair' effects from 'pond' effects because all experiment data from each lake pair come from a single pond. While the magnitude of effects might differ among ponds if the same lake pair was considered in multiple ponds, we note that our conclusions derive from the rank-order of cross types. We suspect it is unlikely that the rank-order of cross types was affected by individual ponds—this would require there to be pond-specific niches that, for example, only  $F_1$  limnetic hybrids could access. Finally, our experiment did not consider ecological selection at the larval stage (i.e. <1 mo.), which can be extremely strong in fish [76,77].

In conclusion, our study did not detect post-zygotic barriers to gene flow between independently derived populations of either the benthic or limnetic ecotype in the threespine stickleback species pairs. Pre-mating isolation is weak between populations of the same ecotype [78] because mate choice is associated with body size [79–81] and shape [82]—preferences that have been strengthened by reinforcement [83]. Allopatric populations of the same ecotype would likely collapse if brought together in sympatry, and heterosis could hasten such a collapse [84]. Parallel evolution over longer timescales, where alternative alleles have substantial 'intrinsic' fitness effects, might be the primary route to speciation by parallel natural selection via the evolution of post-zygotic barriers to gene flow [16]. We conclude that there has been little progress toward speciation via parallel natural selection in this system.

**Data accessibility.** Data and analysis code are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.s7h44j187> [85].

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Both authors gave final approval for publication and agreed to be held accountable for the work performed therein.

**Conflict of interest declaration.** We declare we have no competing interests.

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