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Testing alternative explanations of character shifts against ecological character displacement in brook sticklebacks (*Culaea inconstans*) that coexist with ninespine sticklebacks (*Pungitius pungitius*)

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Abstract Ecological character displacement (ECD) provides opportunities to test how resource competition generates diversifying selection that results in adaptive divergence. We quantify an association between phenotypic and ecological divergence between two similar small fishes, brook (*Culaea inconstans*) and ninespine (*Pungitius pungitius*) sticklebacks, in replicate northern Ontario lakes, Canada. The two species partition resources and habitat, where they coexist, and brooks that coexist with ninespines are more benthically specialized in body form and diet than brooks from local allopatric populations. Here we test various explanations for this pattern. Chance is unlikely to have been the primary cause because divergence is replicated in three separate populations. Preliminary comparisons indicate that resource availability and a variety of abiotic ecological conditions are generally similar between sympatric and allopatric sites, and so do not readily account for the divergence. Biased colonization or extinction is less likely to account for the divergence because character values in sympatry tend to exceed those in allopatry, as expected if they have repeatedly evolved under diversifying selection. Recent studies have also demonstrated that these two species compete, and that competitive effects are more severe for allopatric compared to sympatric brook forms, as predicted

if divergence reflects the ghost of competition past. Ongoing studies indicate heritable variation in this system. Our results suggest that even small amounts of character shifts can influence competition and hence relative fitness, further implicating a role for ECD in the evolution of biodiversity.

Keywords Adaptive divergence · Competition · Northern freshwater fish · Ninespine sticklebacks · Species interactions

Introduction

Studies of divergent populations within and among species can increase our understanding of how biodiversity evolves because the mechanisms that drive adaptive divergence below and above the species threshold are thought to be one and the same (Simpson 1953). Diversifying natural selection imposed by ecological differences between habitats and resources, and from resource competition is thought to be the fundamental mechanism driving adaptive divergence (reviewed in Schluter 2000). When reproductive isolation evolves as a consequence, then adaptive divergence can lead to ecological speciation. While many approaches are being employed to study the origins of species (see TREE 7/01), there is a renewed interest in studies of the classic mechanism of ecological character displacement (ECD) (Schluter 2000, 2003; Melville 2002; Rundle et al. 2003; Adams 2004; Bolnick 2004; Day and Young 2004) because it provides opportunities to test how competitive interactions promote diversifying selection (Lack 1947; Simpson 1953; Grant 1972; Schluter and McPhail 1992; Schluter 2000). Further examples of ECD are also required to evaluate when and how ECD contributes to biodiversity in other ways. For example, is ECD more common between specialists or generalists? How much phenotypic divergence in sympatry is required to significantly reduce competitive effects? Is ECD more common at lower or higher trophic levels?

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These questions can only be addressed with a larger sample of systems that have evolved under ECD. Until recently, the problem has been that well-documented cases of ECD were difficult to find (reviewed in Schluter 2000).

Ecological character displacement is a shift in resource-related traits between coexisting species that is due to frequency-dependent competition over shared resources (Brown and Wilson 1956; Grant 1972; Schluter and McPhail 1992). The process results in exaggerated trait differences between species where they coexist (sympatry) compared to where they are found in the absence of the competitor (allopatry). For example, ECD predicts that sympatric forms represent postdisplacement phenotypes that have diverged under competition-mediated selection, while allopatric forms represent predisplacement phenotypes uninfluenced by the effects of interspecific competition. The traditional evidence for ECD, of character divergence between species in sympatry compared to allopatry, is inadequate because a variety of explanations can also account for this pattern (Grant 1972; Schluter and McPhail 1992).

One important goal of any study of ECD is to rule out at least six alternative explanations for such patterns of divergence (summarized in Schluter and McPhail 1992; Schluter 2000). These are that the phenotypic divergence: (1) results from species interactions other than resource-based competition; (2) reflects chance events such as drift; (3) results from other ecological differences between sympatric and allopatric sites; (4) reflects biased colonization or extinction events; (5) has evolved indirectly as a result of traits unrelated to resource use; and (6) represents induced plastic developmental responses to different local environmental conditions rather than evolved responses. Few ECD studies have tested all of these alternatives (Robinson and Wilson 1994) and direct tests of resource competition are often the weakest link (Schluter 2000). For example, only four studies have experimentally tested whether competitive effects decline with increased divergence under natural conditions (Pacala and Roughgarden 1985; Pritchard and Schluter 2001; Pfennig and Murphy 2002, 2003; Gray and Robinson 2002).

We have observed that brook (*Culaea inconstans*) and ninespine (*Pungitius pungitius*) sticklebacks are ecologically divergent where they coexist in a set of small and isolated lakes. The general purpose of this paper is to evaluate this system as an example of ECD. In a prior field manipulative experiment, Gray and Robinson (2002) tested for the “ghost of competition past” (Connell 1980). We found that allopatric brooks (the putative predisplacement phenotype) suffered stronger competitive effects on short-term growth than sympatric brooks (the putative postdisplacement form) when reared with ninespines. We interpret this as strong evidence of competition between the two species (criterion 1 above). But how much do characters have to shift in order to reduce competition (and increase relative fitness)? A survey of empirical ECD studies in vertebrates

suggests that the average ratio of exaggerated character values between sympatric fish species is 1.7 (Schluter 2000; Table 6.1). Here we quantify and evaluate the extent of ecological and phenotypic divergence in this system, test alternatives two through four above, and consider evidence relating to the last two alternatives.

Divergence of fishes in postglacial lakes

Many fishes are undergoing rapid divergence and species formation in postglacial lakes (Schluter 1996; Bernatchez and Wilson 1998). The most common environmental axis of divergence occurs between the shallow inshore (littoral) and the deeper offshore (pelagic) habitats, which differ with respect to: prey resources (larger macrobenthic invertebrate prey vs smaller and softer free-swimming zooplankton, respectively), competition with other fish taxa, habitat structure, predation risk, and many abiotic factors (such as temperature, light availability, and oxygen levels). In fishes, traits related to feeding and swimming performance have repeatedly and consistently diverged along this axis (Robinson and Wilson 1994; Skúlason and Smith 1995), in ways that are generally consistent with functional expectations (e.g., Keast and Webb 1966; Webb 1984). For example, pelagic forms that consume pelagic zooplankton have more and longer gill rakers, terminally oriented mouths, larger eyes, a more streamlined body form, and accentuated traits related to caudal locomotion. Littoral forms that feed inshore on larger and generally harder macro-benthic prey have fewer and shorter gill rakers, larger and more robust subterminal mouths and jaws, deeper body forms, and accentuated traits related to paired fin locomotion (Jastrebski and Robinson 2004). These patterns are well replicated both below and above the species threshold suggesting that intraspecific divergence may sometimes lead to the formation of new species (Robinson and Schluter 2000).

Stickleback study system and hypotheses

Variation between species of brook and ninespine sticklebacks conforms to these patterns. Ninespines have slimmer body form, more and longer gill rakers than the more robust brook sticklebacks (ESM S1). These species are more distantly related than the recently diverged pairs of threespine sticklebacks (*Gasterosteus aculeatus*) of British Columbia (McLennan 1996) and ninespines have a more northerly distribution than brook stickleback (Scott and Crossman 1998). Both sticklebacks favor inshore littoral habitats where they forage as generalists on a variety of invertebrates (Wootton 1976; Scott and Crossman 1998), which could result in competition where prey resources were limited.

We have found brook and ninespine sticklebacks in a series of small postglacial kettle lakes located on a

240-km-long esker formed 10,000 years BP (Connelly 1972; Pielou 1991) that runs north–south across the Arctic–Great Lakes watershed divide near Kirkland Lake, Ontario, Canada. These small lakes are all oligotrophic, have narrow littoral margins, a sandy benthic substrate that drops steeply into deep water, and sit inside small surface catchments. No lakes are directly connected by aboveground streams suggesting that the lake communities are currently independent. They share similar depauperate aquatic flora (primarily *Chara* sp.) and fish assemblages (primarily various dace species in the family Cyprinidae). Potential predators are few, including stocked brook charr (*Salvelinus fontinalis*) and the naturally occurring common loon (*Gavia immer*). Both species coexist in several lakes (sympatry) while brooks are found alone in many additional lakes (allopatry).

We predicted that: (1) the two species coexist by partitioning resources such that brook sticklebacks use more benthic and ninespines more pelagic resources; (2) the sympatric brooks have a more specialized macrobenthic diet compared to the more generalist diet of allopatric brooks which include both pelagic and benthic prey; (3) the sympatric brook sticklebacks have a more benthic body form (larger head, subterminal mouth, and deeper body) and fewer and/or shorter gill rakers compared to allopatric brooks; (4) if selection has favored a benthically specialized phenotype, then divergence in sympatry would be replicated among sympatric sites. Alternatively, if divergence in sympatry arose by chance, then we expect little similarity in the morphology of brook sticklebacks among replicated sympatric populations; (5) the local ecological factors that influence resources will not vary among sympatric and allopatric sites; and (6) if brook sticklebacks have diverged under ECD, then character values in sympatric brooks will exceed those in allopatry.

Materials and methods

Fish collection

Sticklebacks were collected from 20 kettle lakes between June and August of 1999, 2000, and 2001. Ninespines were only found in three lakes (Garrison, Lallan, and Rozon) and always in the presence of brooks (sympatry). Three lakes inhabited only by brook sticklebacks (Armitage, Bea, and Dewhirst) were selected for further study because their lakes' surface area approximated that of the three sympatric lakes. Brook sticklebacks were sampled haphazardly with 15 standard "G" minnow traps set and checked twice daily over two consecutive days in each lake. Ninespines often schooled and were sampled from a canoe with a large dip net (mesh size = 0.3 cm). Forty fish per species per lake were euthanized using 200 ppm clove oil and preserved in formalin (Robinson 2000).

Morphological variation

All specimens were stained with alizarin red in a 1% KOH solution to accentuate body form and then preserved in 70% ethanol (Robinson 2000). A digital photograph of the dorsal and lateral view of each fish was then taken using a Nikon 950 camera with a telephoto lens.

The left opercular flap was removed from each individual and the gill rakers, including rudiments, on the descending (ventral) portion of the first gill arch were counted. The gill arch was then removed and the length of the third (often the longest) raker was measured using an ocular micrometer in a dissecting microscope. Species were compared in each sympatric lake with respect to log-transformed gill raker length and number using separate ANCOVAs with log-transformed standard body length as covariate.

Variation in gill raker morphology between sympatric and allopatric brook populations was compared in order to test for an effect of ninespines on brook sticklebacks. Gill raker number was unrelated to body length among brook sticklebacks ($r = 0.15$, $p = 0.75$). Gill raker length varied consistently with body length across all six brook populations (slopes were homogeneous; interaction between population and body length, $p = 0.09$). We used a nested ANOVA model with lake nested in the ninespine competition factor (sympatry vs allopatry) to test if mean raker length (adjusted for body length) and mean raker number of sympatric populations differed from allopatric populations (Zar 1999).

External body form was assessed using the landmark-based morphometric method of thin-plate splines (hereafter TPS). This approach quantifies the geometry of form and is a powerful method to detect subtle variation in morphology (Bookstein 1991; Parsons et al. 2003; Adams et al. 2004). An additional advantage of the TPS technique is that it implements the concept of D'Arcy Thompson Cartesian grid deformations (Thompson 1917) allowing visualization of how body form has been stretched, pulled, and shrunk between individuals.

The Cartesian coordinates of 16 homologous landmarks (ESM S2) were identified on the digital image of each specimen using tpsDig (Rohlf 2002), and adjusted by a generalized procrustes analysis using tpsRelw (referred to in the past as generalized-least squares analysis; Bookstein 1991; Adams et al. 2004). This procedure removes variation in the coordinate data among individuals that is unrelated to shape, such as location, orientation, and size. A consensus brook stickleback configuration was then estimated as the average body form across all individuals from the six populations. The adjusted coordinates of each individual were then compared against the consensus form in order to estimate its shape. The degree to which an individual departs from the consensus form was quantified in terms of partial warps that reflect the amount, direction, and location of bending energy required to deform the consensus form

into that specimen. Each individual's partial warp scores therefore collectively represent its shape independent of body size, and can be analyzed using conventional statistical procedures (Adams et al. 2004).

Variation in the external body form (quantified by 12 X and Y nonuniform, and one X and Y uniform partial warps estimated by the TPS analysis above) of sympatric and allopatric brook sticklebacks was summarized using principal components analysis (PCA) in order to identify independent components of multivariate shape variation. We use PCA instead of discriminate function analysis (DFA) because we wish to determine the relative amount of variation in body form that is related to the presence versus absence of ninespines, rather than optimizing the differences between these forms. PCA is a common approach used to analyze geometric morphometric data involving partial warps (Adams et al. 2004). For each principal component, we tested whether variation in the external body form of brooks was related to an effect of ninespines using a nested ANOVA (lakes nested in competition factor as above). Scores from principal components showing a ninespine competitor effect were then regressed against partial warp scores using TpsRegr to generate deformation grid plots in order to visually assess variation in external body form between sympatric and allopatric brook populations.

Diet and habitat use

Short-term prey use was assessed using stomach contents. The stomach contents of each fish from nine study populations (six brook and three ninespine) were removed and identified at least to order. Invertebrate taxa were placed in one of three functional prey categories: "Benthic" referred to taxa that specifically inhabit the littoral or benthic habitat; "Pelagic" referred to taxa that inhabit the water column and "Both" referred to generalist prey taxa found in both habitats (Peckarsky et al. 1990; Ruppert and Barnes 1994). The diet of each individual was then estimated as the proportional count of each prey category (e.g., no. of prey items in a prey category/total no. of items in all prey categories). Diet proportions were subsequently arcsine square root transformed for analyses (Zar 1999).

Mean diets between species in each sympatric lake were compared using separate ANOVAs of the proportion of benthic prey. We tested for diet differences between sympatric and allopatric brook populations using a nested ANOVA on each of the three prey categories (with lake nested in the ninespine effect as above). Bolnick et al. (2003) argue that individual rather than population level metrics provide more accurate estimates of dietary specialization, and so we also used the program IndSpec1 (Bolnick et al. 2002) to calculate and compare several indices of individual level dietary specialization.

Analysis of stomach contents provides only a snapshot of prey and habitat use for the 24 h prior to cap-

ture. Parasite taxa and load can indirectly reflect a longer-term signal of habitat and prey use when parasite life histories include particular prey species as intermediate hosts, these prey species are habitat specialists, and the parasite remains in the host consumer for a long period. We tested for longer-term differences in diet by comparing the infection frequencies by two prey-specific macro-parasites (Konovalov 1995; Robinson et al. 1996; Wilson et al. 1996; Bolnick et al. 2002). We predicted that ninespines consuming zooplankton prey throughout the year would have a higher frequency of infection by parasites that use pelagic plankton as intermediate hosts compared to coexisting brook sticklebacks that specialized on benthic prey. Secondly, if allopatric brooks are dietary generalists in the absence of competition from ninespines and so include more zooplankton in their diets, then we expected a higher frequency of infection by pelagic-type parasites in allopatric compared to sympatric brooks. We removed, identified, and counted the internal macro-parasites from the body cavities of brooks ($n = 165$ and 90) and ninespines ($n = 128$ and 90) from two of the sympatric lakes (Garrison and Rozon, respectively) and of brooks ($n = 40$ and 30) from each of two allopatric populations (Armitage and Bea, respectively). The two most common macro-parasites were *Schistocephalus solidus* and *Ligula intestinalis*. Both parasites use pelagic copepods as their first intermediate host and so reflect pelagic resource use by sticklebacks (Clarke 1954; Hopkins and Smyth 1951), however, only *S. solidus* was found in sticklebacks from the sympatric sites, while *L. intestinalis* was present only in fish from allopatric sites. We used a two-by-two contingency analysis of the frequency of *S. solidus* parasite infection between the two species in sympatry. To test for differences in parasite infection between sympatric and allopatric populations of brook populations, we used a multiple contrasts among proportions (arcsine transformed) based on the Scheffé's procedure (Zar 1999). The total frequency of *S. solidus* and *L. intestinalis* combined were compared between populations.

Ecological comparisons among lakes

To test if ecological factors were related to resource availability between sympatric and allopatric lakes, we compared various abiotic and biotic properties of the six focal lakes between August 15 and 18, 2001. Because allopatric lakes were chosen to be roughly similar in area to sympatric lakes, any statistical inference may be limited to kettle lakes of this size. Seven abiotic properties were assessed for each lake: maximum depth and fetch (ascertained from Ontario Ministry of Natural Resources records), pH, dissolved oxygen, conductivity, temperature, and secchi depth (measured three times in the pelagic habitat of each lake). A WTW Multiline Water Analyzer was used to measure pH, dissolved oxygen, and conductivity at a depth of 3.0 m, and temperature was taken at 5.0 m. Sympatric and allo-

patric lakes were compared using univariate one-factor ANOVAs without adjusting family-wise error rates (resulting in more liberal tests).

Resource availability was also estimated in each of these six focal lakes by taking samples of littoral zone benthos and pelagic zone zooplankton between 1100 and 1300 hours over the same 4-day period. A vertical plankton tow was taken at each of three pelagic locations in each lake using a 0.5 μm plankton net lowered to a standard depth of 5.0 m. Each subsequent 50-ml plankton sample was preserved with 100% ethanol. Benthic samples were also collected using a 2.4 l Eckman grab of the substrate between 0.5 and 1.0 m in the littoral habitat of each lake. The contents of each grab were sorted and invertebrates were stored in 95% ethanol.

Zooplankton and benthic invertebrates were identified at least to Order. Zooplankton richness and abundance were estimated from the mean of three 1.0 ml subsamples of each of the three plankton tows from each lake. Each of the benthic samples was completely examined in order to estimate macro-invertebrate richness and abundance. Total mean zooplankton abundance and total mean benthic invertebrate abundance were determined for each lake by averaging the total count for all taxa per sample, and for all samples per lake. The abundance of the four most common zooplankton taxa, and five most common macro-invertebrate benthic taxa were analyzed using univariate one-factor ANOVAs to test for differences between sympatric and allopatric lakes without adjusting family-wise error rates.

Results

Habitat use by brook sticklebacks varied among lakes. In the three sympatric lakes, brooks were found in the littoral habitat generally in vegetation or near the substrate, while ninespines tended to school in the higher water column of the littoral (but not the pelagic) habitat. In allopatric lakes, brooks were found in the littoral vegetation, at the substrate and also in the water column.

Phenotypic and diet variation between species in sympatry

Brooks had on an average 28% shorter and 23% fewer gill rakers than ninespines in each sympatric lake (Gill raker length: ANCOVAs Garrison: $F_{2,114} = 64.5$, $p < 0.0001$; Lallan: $F_{2,62} = 368$, $p < 0.0001$; Rozon: $F_{2,67} = 78.0$, $p < 0.0001$; Gill raker number: ANCOVAs Garrison $F_{2,114} = 127$, $p < 0.001$; Lallan $F_{2,62} = 242$, $p < 0.0001$; Rozon $F_{2,67} = 197$, $p < 0.0001$).

Brooks consistently consumed a larger proportion of benthic type prey and a smaller proportion of pelagic prey relative to ninespines at all sympatric sites, al-

though this was not significant in Lallan Lake (Benthic prey group: Garrison: ANOVA $F_{1,95} = 89.5$, $p < 0.0001$; Lallan: ANOVA $F_{1,51} = 1.9$, $p = 0.18$; Rozon: ANOVA $F_{1,64} = 8.2$, $p = 0.006$; Table 1). A combined probability test indicates distinct differences between the diets of sympatric brook and ninespine sticklebacks ($\chi^2 = 32.1$, d.f. = 6, $p < 0.001$). On average, the diets of brooks included 61% benthic type prey, whereas ninespines included only 24% benthic type prey and instead included a greater proportion of pelagic prey. Consistent with these differences in prey use, fewer sympatric brooks were infected with *S. solidus* than ninespines (percentage infection rates of brooks and ninespines for Garrison Lake: 0.02 and 16.4%, $\chi^2 = 20.4$, d.f. = 1, $p < 0.001$; Rozon Lake: 0.0 and 11.1%, $\chi^2 = 10.6$, d.f. = 1, $p < 0.01$). The associations between external body form (ESM S1), gill raker architecture, diet, and parasite load collectively suggest that sympatric brook sticklebacks have a benthically specialized phenotype, while ninespines are better suited for consuming pelagic prey from the water column.

Morphological variation among sympatric and allopatric brook populations

The morphology of sympatric brooks also appears more benthic than that of allopatric brook stickleback. Sympatric brooks had on average 8% shorter and 2% fewer gill rakers than allopatric brook sticklebacks (Fig. 1; raker length $F_{1,4} = 92.5$, $p < 0.0001$; raker number $F_{1,4} = 28.5$, $p < 0.0001$). The size-adjusted mean values for gill raker length in sympatric brook populations (Garrison = 0.72 mm, Lallan = 0.62 mm, Rozon = 0.71 mm) are generally less than the values obtained from allopatric populations except for Bea Lake (Armitage = 0.79 mm, Bea = 0.67 mm, Dewhurst = 0.74 mm). In other words, more than 25% of the difference in mean size-adjusted gill raker length between the two coexisting stickleback species above is due to the reduced gill raker lengths in sympatric compared to allopatric brook sticklebacks.

There was considerable variation in brook stickleback body form within and among populations, and variability in multivariate shape was expressed on many principal components (Table 2). For example, the first three components only accounted for 52% of the total variation in body form. Brook sticklebacks sympatric with ninespines also had somewhat more benthic body forms than allopatric brook sticklebacks, primarily expressed on PC4 and PC8 which together accounted for only 11.5% of the variation in external body form (Fig. 2). The evidence for a ninespine effect on variation in brook body form was stronger for PC8 ($F_{1,4} = 9.1$, $p = 0.042$) than for PC4 ($F_{1,4} = 5.0$, $p = 0.093$; for all other principal components $p \geq 0.19$). Deformation grids generated from these two components indicate that brooks sympatric with ninespines have on average slightly deeper bodies, blunter snouts, narrower pectoral

Table 1 A comparison of diet measures among three sympatric brook and ninespine stickleback populations, and three allopatric brook stickleback populations. (a) The mean proportion (\pm SE) of benthic, pelagic, or both (present in both habitats) functional prey categories were calculated for individual fish and then averaged for each prey category in each population. Proportions were arcsine square root transformed for analysis. Matching letters across a row denote significant pair-wise differences between means among brook populations (using Tukey's HSD). Populations without letters were not significantly different from any other in that prey category. (b) Individual diet specialization indices calculated using IndSpec1 (Bolnick et al. 2002) with discrete data (i.e., counts of individual prey types found in the stomach contents of each individual fish). WIC, within individual component; BIC, between individual component; TNW, total niche width; subscript "s" denotes Shannon-Weaver index (see Bolnick et al. 2002 for explanation of calculations), IS, individual specialization index for the population (average proportional similarity indices, PSi, for each population)

	Allopatric			Sympatric					
	Brooks			Brooks			Ninespines		
	Armitage <i>n</i> = 24	Bea <i>n</i> = 31	Dewhurst <i>n</i> = 17	Garrison <i>n</i> = 49	Lallan <i>n</i> = 32	Rozon <i>n</i> = 36	Garrison <i>n</i> = 48	Lallan <i>n</i> = 21	Rozon <i>n</i> = 30
(a) Mean proportion of prey by category									
Benthic ^a	0.554 a (0.089)	0.884 abc (0.046)	0.755 (0.079)	0.593 b (0.058)	0.559 c (0.086)	0.802 (0.045)	0.022 (0.021)	0.403 (0.069)	0.536 (0.025)
Pelagic ^b	0.329 d (0.042)	0.094 a (0.043)	0.061 b (0.041)	0.274 e (0.054)	0.409 abc (0.084)	0.007 cde (0.005)	0.975 (0.021)	0.552 (0.066)	0.351 (0.026)
Both ^c	0.116 (0.057)	0.018 a (0.009)	0.184 (0.069)	0.133 (0.039)	0.031 b (0.019)	0.190 ab (0.045)	0.003 (0.002)	0.045 (0.029)	0.112 (0.015)
(b) Individual specialization									
WICs	0.65	0.65	0.81	0.68	0.26	0.94	–	–	–
BICs	0.57	1.11	0.75	0.97	0.71	0.66	–	–	–
TNWs	1.22	1.77	1.57	1.65	0.97	1.60	–	–	–
WICs/TNWs	0.53	0.37	0.52	0.41	0.27	0.58	–	–	–
Psi	0.38	0.20	0.25	0.31	0.29	0.29	–	–	–
IS	0.41	0.34	0.35	0.32	0.35	0.47	–	–	–

^a Amphipoda, anisoptera, bivalvia, chaoboridae (larvae), chironomidae (larvae) ephemoptera, gastropoda, harpacticoid (Copepoda), hemiptera, hirudinea, insecta, isopoda, oligochaeta, ostracoda, plecoptera, trichoptera, zygoptera.

^b Bosminidae, calanoid (copepoda), daphnidae, rotifera. c: chdoridae, cyclopoid (copepoda) hydrachnidia.

fin insertions, and longer and thicker caudal peduncles (Fig. 3, Table 2). Except for pectoral fin insertion, this variation was consistent with our expectations of greater benthic specialization in sympatric brooks. Unlike the better-known threespine stickleback species pairs, the

body size of sympatric (benthically specialized) brook sticklebacks here was less than that of the more generalist allopatric brook sticklebacks (Fig. 1; mean stan-

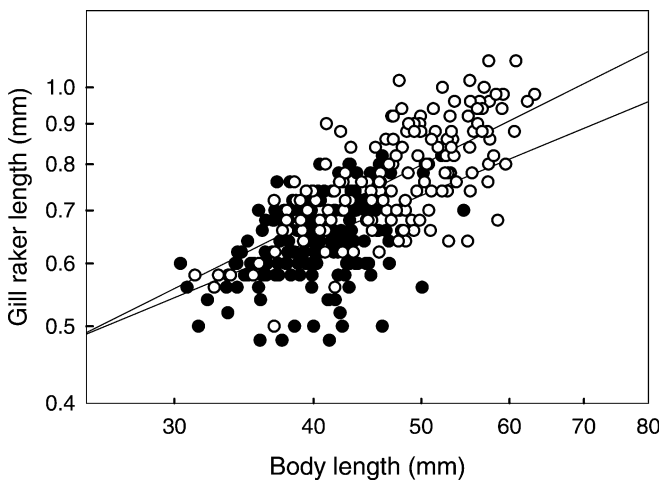


Fig. 1 Linear relationships between gill raker length and body length (both log transformed) in sympatric (closed circle) and allopatric (open circle) brook stickleback (*Culaea inconstans*) populations. Sympatric brook gill rakers were on average 8% shorter than allopatric brook gill rakers (ANOVA $F_{1,4} = 92.5, p < 0.0001$)

Table 2 Summary of the principal components analysis of the geometric-based body form of all brook sticklebacks. For each of the first ten components is shown the percent of total variation in body form, and a description of biological traits that vary on that component as interpreted from an analysis of deformation grid plots (e.g., as shown in Fig. 3)

Principal components	Percent variance	Trait variability
1	27	Body depth, head length, midbody length, caudal length
2	17	Head orientation, caudal length, belly length
3	9	Body depth, head length, caudal length, belly length
4	8	Body depth, midbody length, caudal length, pectoral width, pelvic position
5	7	Body depth, caudal length, belly length
6	6	Caudal length, pectoral and pelvic position
7	5	Snout shape, midbody length, pectoral and pelvic position
8	4	Head orientation, snout shape, pectoral width and position
9	3	Eye size, pectoral width
10	3	Head depth, caudal length, pectoral width

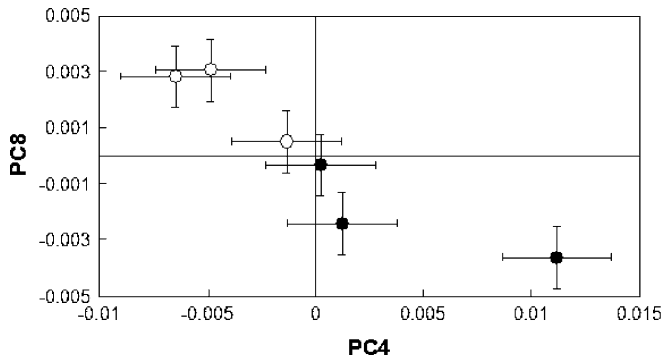


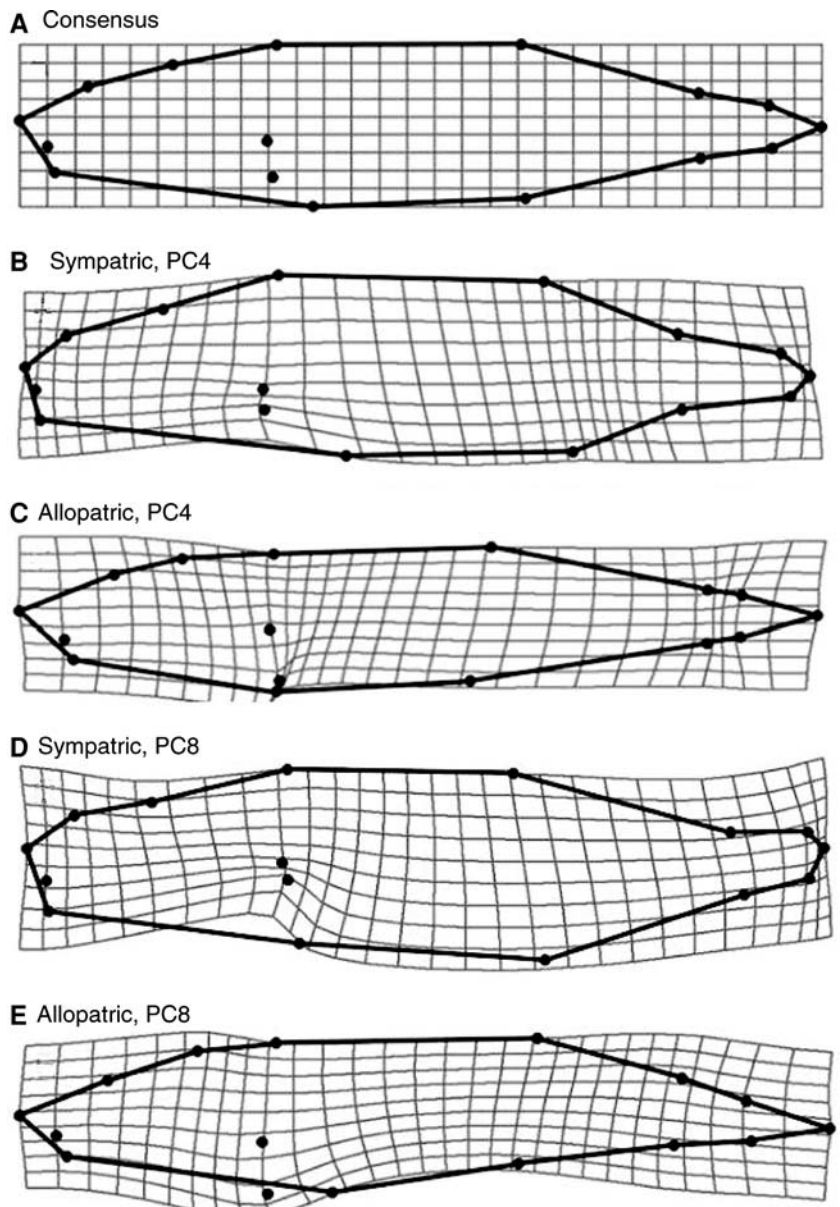
Fig. 2 The external body-shape values of each population (mean \pm SE) on axes 4 and 8 of a principal components analysis of variation in external body form (partial warps scores) of three sympatric (*closed circle*) and three allopatric (*open circle*) brook stickleback populations

standard length 40.7 mm (\pm 0.44SE) and 47.7 mm (\pm 0.46SE), respectively). Taken together, these results suggest greater character shifts in gill raker length than in body form for the brook stickleback here.

Variation in diet and parasite load among brook populations

The proportion of specialized benthic, pelagic, and generalist prey categories did not vary significantly between allopatric and sympatric brook populations (Table 1; Benthic prey: $F_{1,4} = 0.6$, $p > 0.25$; Pelagic prey: $F_{1,4} = 0.20$, $p > 0.25$; Generalist prey: $F_{1,4} = 0.034$, $p > 0.5$). Benthic prey on average comprised 69% of the diets of all brook sticklebacks, but this and other prey categories varied significantly among populations within

Fig. 3 Deformation grid plots showing variation in the external body form of individual fish from sympatric (**b, d**) and allopatric (**c, e**) brook stickleback populations. **a** A consensus grid form representing a brook stickleback body form averaged over all six populations, against which individuals are compared (see text for description). The head region is to the left. Individuals shown here were selected from the extremes of body-shape variation along PC4 (**b, c**) and PC8 (**d, e**) (see Fig. 2). Note the more robust and blunt headed body form of brooks sympatric with ninespines (**b, d**) reflecting a more benthic phenotype. **b** Rozon S-33, sympatric, PC4 score value = -0.022 . **c** Bea S-40, allopatric, PC4 score value = 0.026 . **d** Rozon S-19, sympatric, PC8 score value = -0.018 . **e** Bea S-34, allopatric, PC8 score value = 0.023



sympatric and allopatric lakes (Table 1; Benthic: $F_{4,187} = 3.94$, $p = 0.004$; Pelagic: $F_{4,183} = 8.02$, $p < 0.0001$; Generalist prey: $F_{4,183} = 4.4$, $p < 0.002$). There was also little evidence of increased benthic resource specialization by sympatric brooks using the individual based metrics (Table 1) of Bolnick et al. (2002).

Although no short-term variation in stomach contents were detected between sympatric and allopatric brook populations in these samples, the frequency of prey-specific parasite infection suggests that some allopatric brook sticklebacks include pelagic copepod resources in their diets while sympatric brooks generally do not. Pelagic source macro-parasites (*S. solidus* and *L. intestinalis* combined) were almost completely absent from brook sticklebacks in the two sympatric populations (Garrison = 1.8%, Rozon = 0%), but were present in fish from the two allopatric populations (Armitage = 5%, Bea = 30%; Scheffé's $S = 4.67$, $p < 0.05$).

Ecological comparisons of sympatric and allopatric lakes

The three sympatric and three allopatric lakes containing brook populations were qualitatively similar with respect to almost all of the ecological parameters measured with two exceptions. Allopatric lakes appear to have reduced levels of conductivity and pH (Table 3a), although neither result would have been statistically significant under a sequential Bonferroni adjustment for multiple comparisons. There was no evidence that either pelagic or benthic invertebrate abundance varied consistently between sympatric and allopatric lakes either for total abundance of all taxa pooled or for any individual taxa (Table 3b). Five benthic invertebrates accounted for 68.6% of the total number of individuals sampled. Four zooplankton taxa accounted for 99.6% of the total number of individuals sampled.

Discussion

In a previous manipulative field experiment, we found that the putative pre-displacement allopatric brook stickleback forms suffered greater competition from ninespines than the putative post-displacement sympatric brook forms, suggesting that ECD had occurred in this system (Gray and Robinson 2002). Our current results show that the ecological divergence of brook and ninespine sticklebacks in sympatry is associated with exaggerated phenotypic differences (ESM S1). The ratio of species means in sympatry for gill raker length and number are 1.28 and 1.23, respectively (ninespines:brooks). Of these species level differences in sympatry, one quarter of the difference in gill raker length and 9% of the difference in gill raker numbers resulted from reductions in sympatric compared to allopatric brook sticklebacks. The external body form of

Table 3 The mean (\pm SE) of: (a) physical parameters and (b) biotic parameters in three sympatric versus three allopatric lakes

	Allopatric	Sympatric	<i>p</i> value
(a) Physical parameters			
Conductivity (μ S)	21.7 (7.26)	127 (24.0)	0.01
Maximum depth (m)	10.0 (2.75)	19.9 (12.3)	0.50
Dissolved O ₂ (mg/l)	7.66 (0.13)	9.02 (1.40)	0.39
Maximum fetch (km)	0.62 (0.16)	0.39 (0.12)	0.31
pH	8.05 (0.18)	8.94 (0.26)	0.05
Temperature at 5.0 m ($^{\circ}$ C)	23.8 (0.38)	15.2 (3.88)	0.09
Secchi depth (m)	4.83 (0.36)	5.83 (2.33)	0.69
(b) Biotic parameters			
Benthic invertebrates (mean total abundance)	12.3 (4.25)	17.7 (4.90)	0.45
Amphipods	2.38 (1.74)	1.89 (2.01)	0.86
Chironomid larva	2.98 (1.69)	6.17 (1.95)	0.27
Snails (Gastropoda)	0.29 (0.29)	0.84 (0.33)	0.27
Hydrachnids	2.38 (1.05)	2.77 (1.22)	0.82
Trichopterans	0.54 (0.21)	0.83 (0.31)	0.51
Zooplankton (mean total abundance)	25.8 (6.33)	28.0 (7.30)	0.83
Bosminids	69.9 (45.7)	73.2 (5.28)	0.96
Calanoid copepods	104 (34.9)	36.3 (4.03)	0.26
Cyclopoid copepods	5.86 (4.05)	2.04 (4.67)	0.56
Daphnids	25.5 (25.4)	111 (29.3)	0.08

Physical parameters were measured in three locations per lake at the same time of day within a 4-day period. All repeated measures within lakes were averaged within lake before comparison. The five benthic and four pelagic zooplankton invertebrate taxa shown represent the most common taxa in samples across lakes. Invertebrate abundances were estimated per 2.4 l for benthic samples and per 1.0 ml for zooplankton. *p* values reflect two-sided *t* tests of the means of each variable between the three sympatric and three allopatric lakes.

brook sticklebacks also exhibited predictable character shifts between sympatry and allopatry but to a much smaller extent. This suggests that the obviously different body forms of the two species are mostly ancestral and may have preadapted them for the niche shifts observed here. We cannot similarly evaluate the nature of character shifts in ninespine sticklebacks because allopatric populations are absent from this system. Nonetheless, we can compare our results to those reported elsewhere. Schluter (2000, table 6.1) reviewed the empirical literature on ECD, and from his data we have calculated the mean species ratios in sympatry for vertebrates in general as 1.43 ($n = 25$ cases), 1.7 for fish (5 cases), and 1.6 for gill raker number in fish (3 cases). Our results are striking because they are lower than all of these values, and so demonstrate that subtle shifts in resource-related traits can be related to significant reductions in competitive effects and presumably to increased fitness.

We found strong evidence of dietary differentiation between stickleback species in sympatry, but no consistent evidence of differences in short-term diet between sympatric and allopatric brook populations (although variation in pelagic source parasite load suggested a greater use of pelagic resources by some allopatric brook sticklebacks). Thus, our evidence for the ecological shift toward benthic specialization in sympatric brook sticklebacks is indirect and qualitative, coming from three

complimentary sources: the relative absence of pelagic parasites, observations of habitat use, and predictable shifts in body form. More rigorous tests that integrate diet composition over longer time periods are required to better evaluate the degree to which sympatric brooks are ecologically specialized.

The adaptive nature of these character shifts is indirectly supported by replicated parallel patterns of divergence between the benthic and water column habitats at two levels. The character shifts detected between sympatric and allopatric brook populations parallel to a lesser degree the differences between the two species; sympatric brooks have shorter and fewer gill rakers and a more “benthic” body form than allopatric brooks, which mimics the pattern of phenotypic and ecological divergence between brook and ninespine sticklebacks. Additionally, divergence here parallels that found between threespine stickleback (*G. aculeatus*) species pairs in British Columbia (Schluter and McPhail 1992; McPhail 1994) with one exception. The more benthically specialized (sympatric) brooks here tend to be smaller than the more generalist (allopatric) brooks, whereas the benthic species of *G. aculeatus* is larger than the pelagic species in B.C. Nonetheless, a consistent signal of parallel divergence in body form and gill raker traits among different populations and fish taxa over this ecological gradient strongly suggests that aspects of phenotypic variation are functionally significant and under diversifying selection here as elsewhere (Schluter 1996).

Collectively, our results suggest that this stickleback system represents a recently evolved example of ECD. We now explicitly address alternative explanations for these character shifts (see also Pfennig and Murphy 2003). First, a critical and one of the least supported components of any ECD study, is to test for evidence of interspecific competition between focal species (Schluter 2000). These stickleback species compete under natural conditions because experimental manipulations in field enclosures have demonstrated that the negative effects of competition decline as expected with exaggerated divergence in sympatry (Gray and Robinson 2002).

Second, we can test the likelihood that chance accounts for this divergence. We provisionally conclude that this is unlikely because the patterns of divergence with respect to gill raker length and number, and body form are replicated among three sympatric populations of brook sticklebacks that are geographically isolated in separate kettle lakes. If these populations are evolutionarily independent, then selection is the only deterministic mechanism that is expected to result in similar patterns of divergence under similar ecological conditions (Nagel and Schluter 1998). Molecular techniques could be used to more rigorously test our assumption of evolutionary independence.

Third, we found little evidence that sympatric and allopatric sites varied ecologically with respect to resource availability, at least during the late summer season. There was only weak evidence that two abiotic

parameters consistently varied among lakes, and no evidence that prey resource abundance varied at all. So far, this suggests that the principal ecological factor differentiating sympatric and allopatric sites is the respective presence and absence of ninespine sticklebacks. Although not statistically significant, water temperature was on average 8°C cooler in sympatric compared to allopatric lakes. This is unlikely to influence resource availability, though, because temperature was measured at 5 m depth in the pelagic habitat, while the littoral habitat rarely exceeded 1.5 m. Subsequent tests have found no evidence of variation in littoral water temperature between sympatric and allopatric lakes in late summer ($t = 1.94$, d.f. = 4, $p = 0.17$). The cooler pelagic waters of sympatric lakes may nonetheless explain why ninespines, which prefer cooler waters (Wootton 1976; Scott and Crossman 1998), are found in the sympatric lakes. Similarly, pelagic water temperature may affect the distribution of the two macro-parasites (*L. intestinalis* and *S. solidus*) between sympatric and allopatric lakes because both use pelagic copepods as their first intermediate hosts (Hopkins and Smyth 1951; Clarke 1954).

There was no evidence of any consistent variation in resource abundance among lakes that could have accounted for the divergence among sticklebacks. Similar benthic and pelagic prey taxa were found in all lakes with the exception of several taxa that were inconsistently absent in some and present in other lakes. Obviously, these results are also preliminary, because a longer-term comparison of resource abundance throughout nonwinter seasons would provide a better test of the ecological similarity of these lakes than our current “snapshot”. We also have to assume that current patterns of resource availability reflect historic patterns. This seems reasonable, though, because these small lakes are relatively young (formed less than 10,000 years BP), are all highly oligotrophic (reflecting an early phase in natural eutrophication), and have generally similar physical and chemical properties.

A fourth explanation of the diversity among brook stickleback populations is that it reflects a sorting process due to biased colonization or extinction of different forms of ancestral brook sticklebacks and not an evolutionary shift driven by selection. This implies that there were historically at least two brook phenotypes, with only one now found with ninespines in sympatric lakes. ECD predicts that exaggerated trait values evolve under diversifying selection between coexisting species (Schluter 2000). We have some evidence that mean gill raker length and gill raker number of sympatric brooks generally lies below the range of values observed among allopatric brooks (Fig. 1). The same is true for various aspects of body form (Fig. 2). Evaluation of phenotypic variation among additional allopatric brook populations would be useful to confirm this finding. These preliminary results are not consistent with a role played by biased colonization in the diversity between brook and ninespine sticklebacks.

We have only indirect evidence that these character shifts are functionally related to resource use here. The divergence between stickleback species, and between sympatric and allopatric brook sticklebacks follows a common pattern of diversification between benthic and pelagic forms observed in many other fishes in postglacial lakes (Robinson and Wilson 1994; Robinson and Schluter 2000). Brook sticklebacks are more benthic in body form with fewer, shorter gill rakers than ninespines. These traits are believed to be related to swimming and foraging performance (e.g., Keast and Webb 1966; Ehlinger 1990; Schluter 1993). Hence, we expect that brooks will be more efficient at utilizing benthic resources, while ninespines will be better at foraging on pelagic prey. In principle, a similar prediction should hold between the more benthically specialized sympatric and generalist allopatric brook sticklebacks. Detailed foraging trials could test these predictions.

One final alternative explanation for trait divergence here is that it could represent plastic developmental responses induced by local conditions as opposed to evolved genetic changes. Preliminary results from brook sticklebacks reared under common laboratory conditions provide strong evidence of heritable variation in morphology and behavior among brook stickleback families and populations (Robinson and Peiman, unpubl. results). Those results are consistent with other studies that have shown heritable morphological variation in sticklebacks (e.g., McPhail 1994; Hatfield 1997), and will be presented elsewhere.

Neutralist and contingency views of evolution challenge biologists to provide evidence for the role of natural selection in the evolution of biodiversity. Many empirical tests reveal that natural selection regularly acts to create biodiversity (reviewed in Schluter 2000). The focus now is on whether selection is frequently diversifying between environments, and whether and how competition and other forms of species interactions induce and shape selection. This stickleback system provides a window into how species interactions drive natural selection and consequently diversification in lake fishes. The evidence for morphological divergence, habitat shifts, repeated patterns of divergence among ecologically similar lakes, along with our competition study (Gray and Robinson 2002) provide consistent evidence that ECD has occurred in this system. Relatively small character shifts were related to reduced competition in this compared to other systems, suggesting that ECD has had a more subtle evolutionary outcome here. Strong inferences about ECD requires an approach where alternative explanations for divergence are identified and tested along with predictions derived from the processes yielding ECD, such as competition over resources. The tractability and replication inherent in this system has made such an approach possible.

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