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Along the speciation continuum in sticklebacks

A. P. Hendry*†, D. I. Bolnick‡, D. Berner§ and C. L. Peichel‖

**Redpath Museum & Department of Biology, McGill University, 859 Sherbrooke St. W., Montréal, Québec, H3A 2K6 Canada, ‡Section of Integrative Biology, University of Texas at Austin, Austin, TX 78712, U.S.A., §Zoological Institute, University of Basel, Vesalgasse 1, 4051 Basel, Switzerland and ‖Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, WA 98109, U.S.A.*

Speciation can be viewed as a continuum, potentially divisible into several states: (1) continuous variation within panmictic populations, (2) partially discontinuous variation with minor reproductive isolation, (3) strongly discontinuous variation with strong but reversible reproductive isolation and (4) complete and irreversible reproductive isolation. Research on sticklebacks (Gasterosteidae) reveals factors that influence progress back and forth along this continuum, as well as transitions between the states. Most populations exist in state 1, even though some of these show evidence of disruptive selection and positive assortative mating. Transitions to state 2 seem to usually involve strong divergent selection coupled with at least a bit of geographic separation, such as parapatry (*e.g.* lake and stream pairs and mud and lava pairs) or allopatry (*e.g.* different lakes). Transitions to state 3 can occur when allopatric or parapatric populations that evolved under strong divergent selection come into secondary contact (most obviously the sympatric benthic and limnetic pairs), but might also occur between populations that remained in parapatry or allopatry. Transitions to state 4 might be decoupled from these selective processes, because the known situations of complete, or nearly complete, reproductive isolation (Japan Sea and Pacific Ocean pair and the recognized gasterosteid species) are always associated with chromosomal rearrangements and environment-independent genetic incompatibilities. Research on sticklebacks has thus revealed complex and shifting interactions between selection, adaptation, mutation and geography during the course of speciation.

Key words: adaptive radiation; chromosomal speciation; ecological speciation; *Gasterosteus aculeatus*; genetic incompatibilities; three-spined stickleback.

INTRODUCTION

It is often said that 'On the Origin of Species' was misnamed given that Darwin focused more explicitly on how natural selection drives change within lineages than on how one lineage becomes sundered into two or more. Reading between the lines, and through his notes and letters, however, it becomes clear that Darwin did have a well-formed view of speciation. In particular, he felt that speciation was often the result of adaptation to different environments (Schilthuizen, 2000). In addition, Darwin felt that speciation would be easier if the range of a species was divided by

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†Author to whom correspondence should be addressed. Tel.: +1 514 398 4086, ext. 00880; fax: +1 514 398 3185; email: andrew.hendry@mcgill.ca

barriers to movement (Sulloway, 1979). Thus, not only did Darwin start the study of speciation, he also raised two important questions that remain current today: the causes of speciation and its geographic context. These two themes will recur in the present paper, and so a brief overview is warranted.

Speciation involves the evolution of reproductive isolation among groups of individuals; isolation that is often coupled with genetic and phenotypic differences (Dobzhansky, 1937; Mayr, 1963; Schluter, 2000). In the present paper, group is used broadly to refer to sets of individuals that differ from other sets in some way, such as geographically, ecologically, morphologically, behaviourally or genetically. Different groups can thus run the gamut from resource polymorphisms within populations to different populations to different species. The potential causes of speciation can be categorized in a variety of ways (Schluter, 2000; Coyne & Orr, 2004; Gavrilets, 2004; Schluter, 2009); here is one. First, mutation coupled with genetic drift might cause groups in different places to gradually fix different alleles, which are then incompatible when brought together in hybrids. Second, convergent (similar or uniform) natural selection in different groups might fix different alleles (because different beneficial alleles arose by mutation in the different groups), which are then incompatible when brought together in hybrids. Third, divergent or disruptive natural selection might cause allele or trait divergence that leads to reproductive isolation (*i.e.* ecological speciation). Fourth, genomic rearrangements, such as polyploidy, inversions, or new sex chromosomes, can sometimes generate reproductive isolation between an individual (and its descendents) and the rest of a group. Of course, these different mechanisms can act in collaboration with each other, and with sexual selection.

The present paper will partly focus on ecological speciation, which has two basic parts. First, divergent or disruptive selection causes the adaptive divergence of groups in different environments, broadly construed to include different resources or habitats. This, of course, is the backbone of Darwin's great theory (Lack, 1947; Endler, 1986; Schluter, 2000, 2009; Rundle & Nosil, 2005; Grant & Grant, 2008). Second, the adaptive divergence leads to the evolution of reproductive isolation, which can be manifest in a variety of pre and post-mating barriers. For example, individuals moving between different environments might be maladapted for the new environment and should therefore show reduced fitness (Schluter, 2000; Hendry, 2004; Nosil *et al.*, 2005; Hereford, 2009; Thibert-Plante & Hendry, 2009). In addition, positive assortative mating might arise if the traits undergoing adaptive divergence also influence reproductive timing, reproductive location, or mate choice (Schluter, 2000; Coyne & Orr, 2004; Doebeli *et al.*, 2007). Even if these pre-mating barriers are breached, hybrid offspring might be maladapted for available environments and therefore also suffer low fitness (Schluter, 2000; Rundle & Whitlock, 2001; Rundle & Nosil, 2005). Many of the above barriers are expected to be ecologically dependent; that is, manifest owing to interactions between an individual's genes or traits and the environment. Alternatively, adaptive divergence might cause ecologically independent reproductive barriers; such intrinsic genetic incompatibilities that reduce the viability or fertility of hybrids (Ting *et al.,* 1998; Barbash *et al.*, 2003; Presgraves *et al.*, 2003; Brideau *et al.*, 2006; Phadnis & Orr, 2009; Tang & Presgraves, 2009).

Transitioning from causes to contexts, the geography of speciation has traditionally been categorized as either allopatric, parapatric or sympatric. The definitions of these terms have been highly variable (Mayr, 1963; Bush, 1994; Via, 2001; Coyne & Orr, 2004; Gavrilets, 2004; Butlin *et al.*, 2008; Fitzpatrick *et al.*, 2008), leading different evolutionary biologists to often talk past each other. The terms are probably best avoided in future work but are retained here owing to a specific focus on revisiting classic problems in speciation through the lens of stickleback research. The following definitions therefore largely match traditional interpretations, but are stated so as to make sense in the context of sticklebacks.

Allopatry is a context where populations are geographically isolated to the extent that dispersal ability does not allow cross-population interactions during breeding. Sympatry is the opposite extreme; geographic isolation is absent and dispersal ability allows frequent interactions during breeding (although dispersal behaviour might restrict such interactions). Parapatry is the broad range of intermediate possibilities, and it is typically evoked when groups interact along a relatively narrow spatial zone of contact. Allopatric speciation is uncontroversial, parapatric speciation slightly more so and sympatric speciation immensely more so (Bush, 1994; Via, 2001; Coyne & Orr, 2004; Bolnick & Fitzpatrick, 2007). The upshot of many decades of debate is that increased spatial overlap between groups should make their divergence less likely early in the course of speciation (Gavrilets, 2004; Bürger *et al.*, 2006; Bolnick & Fitzpatrick, 2007; Doebeli *et al.*, 2007), but perhaps more likely late in the course of speciation, such as when two formerly allopatric groups come into secondary contact (Lack, 1947; Servedio & Noor, 2003; Grant & Grant, 2008). Indeed, speciation in many taxa probably involves a variety of geographical contexts that change through time (Feder *et al.*, 2005; Rundle & Nosil, 2005; Butlin *et al.*, 2008; Fitzpatrick *et al.*, 2008; Grant & Grant, 2008). Another important generalization is that divergence with gene flow (parapatry or sympatry) is easiest when the same traits influence both adaptive divergence and reproductive isolation (Gavrilets, 2004; Bolnick & Fitzpatrick, 2007).

The preceding summary provides an entrée to some of the topics in speciation research that have persisted all the way from Darwin to the present. These topics are directly informed by research on sticklebacks. The rest of the present paper will thus consider the causes and contexts of speciation in sticklebacks.

SPECIATION AS A CONTINUUM OR A SERIES OF ‘STATES’

An important conceptual shift in speciation research has been the transition from an emphasis on speciation as an endpoint to speciation as a process. This shift is well motivated because it recognizes that speciation does not usually happen instantly (except under certain conditions), but rather usually involves the more-or-less gradual accumulation of genetic differences and reproductive isolation (Hendry *et al.*, 2000; Schluter, 2000; Wu, 2001; Drès & Mallet, 2002; Rundle & Nosil, 2005; Mallet, 2008; Butlin *et al.*, 2008; Berner *et al.*, 2009; Nosil *et al.*, 2009). In short, the point at which speciation is ‘complete’ is vague and varies with the species concept being applied and the stringency of its application. Must all hybridization cease? Must all loci be reciprocally monophyletic? It is thus most useful to consider different groups as falling at different places along a potential continuum from variation within panmictic populations all the way to complete and permanent reproductive isolation between distinct species showing genome-wide reciprocal monophyly. With this shift in focus, the interesting questions come more clearly into view: where do particular groups lie along this continuum, what got them there and why haven't they gone further?

To more easily evaluate these questions, several states along the potential speciation continuum can be defined (Hendry, 2009). State 1 is the presence of continuous (*e.g.* unimodal) variation within a group, without any reproductive isolation (*i.e.* panmixis). State 2 is the presence of partially discontinuous (*e.g.* bimodal) variation within or between groups, with some partial but relatively minor reproductive isolation. State 3 is the presence of strongly discontinuous variation, coupled with strong but potentially reversible reproductive isolation. State 4 is the presence of complete and irreversible reproductive isolation between groups. When considering these states, it is important to recognize that the borders between them are neither abrupt nor always obvious as they are part of a continuum. In addition, transitions between the states are not inevitable (groups can get stuck in one state), nor are they always unidirectional (groups can move back and forth between the states). Moreover, some groups might achieve one state (*e.g.* state 4) without necessarily having passed through states 2 and 3 (state 1 is always the starting point). In short, these states are not definitive but are instead a construct that allows a more convenient discussion of speciation as a process.

THE SPECIATION CONTINUUM IN STICKLEBACKS

Sticklebacks are a useful system for studying progress back and forth along the speciation continuum because different groups are positioned at different places along the continuum (McPhail, 1994; Foster *et al.*, 1998; McKinnon & Rundle, 2002; Boughman, 2007). State 1 (continuous variation without reproductive isolation) appears to be the case within most gasterosteid populations, whether they are found in lakes, streams or ocean. The present paper explores this state by reference to variation within lacustrine populations of three-spined stickleback *Gasterosteus aculeatus* L. State 2 (discontinuous variation with minor reproductive isolation) is often evident between *G. aculeatus* in physically separated (allopatric or parapatric) divergent environments. The present paper explores this state by reference to parapatric lake and stream and mud and lava pairs of *G. aculeatus*. State 3 (discontinuous variation with strong, but reversible, reproductive isolation) might be very common, but is only unambiguous where the groups are found in sympatry. The present paper explores this state by reference to the benthic and limnetic pairs found in a few small British Columbia (Canada) lakes and the anadromous and freshwater pairs found in the lower reaches of many streams. State 4 (complete and irreversible reproductive isolation) appears rare in sticklebacks, with the only known cases being the Japan Sea and Pacific Ocean *G. aculeatus* pair and the taxonomically recognized species: *G. aculeatus*, blackspotted stickleback *Gasterosteus wheatlandi* Putnam, nine-spined stickleback *Pungitius pungitius* (L.), brook stickleback *Culaea inconstans* (Kirtland), four-spined stickleback *Apeltes quadracus* (Mitchill) and 15-spined stickleback *Spinachia spinachia* (L.).

Several features of the stickleback, particularly *G. aculeatus*, system deserve special mention because they determine the evolutionary inferences that are possible. Highlights of these features are presented here, with the details discussed below and in several books (Wootton, 1976; Bell & Foster, 1994; Östlund-Nilsson *et al.*,

2007) and reviews (McPhail, 1994; Foster *et al*., 1998; McKinnon & Rundle, 2002; Boughman, 2007). First, different stickleback populations show remarkable levels of adaptation to different environments. Second, many of these adaptations arose independently in many different places, providing outstanding evolutionary replication (*i.e*. parallel or convergent evolution). Third, most extant freshwater populations were founded after the last glaciation (*c*. 10 000 B.P.), which means that the current phenotypic and genetic differences and reproductive barriers might reflect those that drove divergence in the first place, rather than just accumulating after the fact. Fourth, their relatively short generation time (1 to 2 years), small size (<10 g) and ease of rearing in the laboratory make sticklebacks well suited for both observational studies and manipulative experiments. Fifth, and for all of the above reasons, an outstanding set of genomic tools have been developed for stickleback research (Peichel *et al*., 2001; Kingsley *et al*., 2004; Kingsley & Peichel, 2007; Baird *et al*., 2008).

The following sections separately consider each state along the speciation continuum in sticklebacks. In each section, the nature of phenotypic and genetic variation is first described, and then any evidence for reproductive isolation is considered. Each section then closes with a discussion of how the current state was achieved, and why the system has not transitioned to another state. The paper then closes by considering how all of this work on sticklebacks can inform the general understanding of speciation.

STATE 1: CONTINUOUS VARIATION WITHOUT REPRODUCTIVE ISOLATION

Nearly all natural populations in all taxonomic groups exhibit appreciable levels of within-population genetic and phenotypic variation. Any given lake population of *G. aculeatus*, for example, includes individuals of different size, defensive and trophic morphology, colour and behaviour. This variation poses two distinct questions in the context of speciation. First, what determines the amount of within-population variation for any given trait? Second, in what ways might this variation promote or constrain progress towards speciation?

WHAT INFLUENCES WITHIN-POPULATION VARIATION?

Three major processes are considered here: sampling effects, sources of variation and selection. Genetic variation is usually reduced by sampling effects, such as genetic drift or founder effects, and by directional or stabilizing selection. Conversely, genetic variation is usually inflated by mutation, immigration and diversifying selection. In addition, plasticity might increase or decrease phenotypic variation in ways that are partially decoupled from genetic variation. Evidence for each of these processes in *G. aculeatus* is now considered.

Sampling effects

In some cases, genetic drift and founder effects are interrelated: that is, founder effects can create genetic bottlenecks that facilitate drift. In other cases, however, they can be somewhat distinct, that is, founder effects can have a deterministic component that is lacking in classic genetic drift. For example, certain genotypes might be more likely to colonize a new environment (Haag *et al*., 2005). Both genetic drift and founder effects are known to influence diversity at individual loci in freshwater *G. aculeatus*. Caldera & Bolnick (2008) showed that genetic diversity at six microsatellite loci within a catchment is lower for lake populations that are further from the ocean (the founding source). Due to a cascade at the base of the watershed, this effect is not due to continued immigration from the ocean. Instead, successive founder effects reduced genetic diversity as the fish colonized further upstream. In the same study, genetic diversity was positively correlated with lake size, suggesting that effective population size (genetic drift) influences single-locus diversity. Raeymaekers *et al*. (2008) found analogous patterns for riverine *G. aculeatus* in a European catchment.

Although the above results suggest that demographic history affects genetic diversity, only a small sample of loci was examined, and these were presumed-neutral microsatellites. Quantitative genetic variation, by contrast, might be much less sensitive to sampling effects, because such variation is based on numerous loci of which only a few might be affected by a bottleneck (Barton & Charlesworth, 1984; Dlugosch & Parker, 2008). Consequently, the sampling effects summarized above may not hold for quantitative traits of fitness consequence. Indeed, no correlation exists among the 13 lakes surveyed by Caldera & Bolnick (2008) between mean allelic diversity at microsatellites and the variance of morphological traits (body size, gape width, body depth, gill raker number and gill raker length; D. Bolnick, unpubl. data). The proposition that sampling effects influence adaptive variation within stickleback populations therefore remains unconfirmed.

Sources of variation

New genetic variation can arise within a population *via* mutation or recombination, or it can be imported *via* immigration. For sticklebacks, mutation and recombination rates have not been examined, nor have any studies formally considered the effects of immigration. Such effects are, however, indirectly supported by several observations. For example, marine populations repeatedly contribute genes to freshwater populations through ongoing immigration (Colosimo *et al*., 2005; Malhi *et al*., 2006; Kitano *et al*., 2008*a*), to a degree that presumably depends on the severity of hydrological barriers. In addition, dispersal among freshwater populations within a watershed is a potential source of variation. For example, some populations are strongly isolated from neighbours, whereas others exchange migrants at a high rate (Hendry *et al*., 2002; Hendry & Taylor, 2004; Moore *et al*., 2007; Berner *et al*., 2008, 2009; Bolnick *et al*., 2008; Caldera & Bolnick, 2008). In some cases, this dispersal appears to constrain adaptive divergence (Hendry *et al*., 2002; Hendry & Taylor, 2004; Moore *et al*., 2007; Berner *et al*., 2009) and generate maladaptive migration load (Bolnick *et al*., 2008; Moore & Hendry, 2009). But it is also possible that dispersal can sometimes promote adaptive evolution (Garant *et al*., 2007), which may be the case in some *G. aculeatus* populations (Kitano *et al*., 2008*a*).

Selection

Standard evolutionary theory predicts that stabilizing selection should predominate in natural populations, because phenotypes should converge towards fitness peaks that are reasonably stable equilibria (Endler, 1986; Estes & Arnold, 2007).

Following colonization of a new environment, the initially directional, and then stabilizing, selection should thus reduce genetic variation within populations. Under these simplified assumptions, adaptively important traits should exhibit little variation, whereas neutral traits might retain considerable variation (Blows & Hoffmann, 2005). As with many other organisms, however, *G. aculeatus* contradict this prediction; they show substantial within-population variation for fitness-related traits, including trophic morphology (Robinson, 2000; Nosil & Reimchen, 2005; Berner *et al.*, 2008; Bolnick *et al.*, 2008), life history (Cresko & Baker, 1996; Baker *et al.*, 2005) and defensive spines and lateral plates (Reimchen & Nosil, 2002; Kitano *et al.*, 2008*a*).

Possible solutions to the above contradiction can be illustrated by reference to trophic morphology in *G. aculeatus*. Within a given lake population, individuals vary in their propensity to use benthic prey, such as large insect larvae, *v.* limnetic prey, such as small copepods or cladocera (Fig. 1; Schluter & McPhail, 1992; Robinson, 2000; Araujo *et al.*, 2008; Berner *et al.*, 2008, 2009). For example, combined stomach content and stable isotope analyses reveal that some individuals primarily consume benthic prey, some primarily consume limnetic prey and some consume a relatively even mixture of both (Araujo *et al.*, 2008; Bolnick *et al.*, 2008; Snowberg & Bolnick, 2008; J. Paull, L. Snowberg & D. I. Bolnick, unpubl. data). This among-individual diet variation is not simply a consequence of spatial segregation, because within-location variation can also be high (Svanbäck & Bolnick, 2007; J. Paull, L. Snowberg & D. I. Bolnick, unpubl. data). This diet variation is at least partly phenotype-dependent: individuals with more and longer gill rakers, narrower gapes and shallower bodies consume more limnetic prey (Robinson, 2000, Araujo *et al.*, 2008; J. Paull, L. Snowberg & D. I. Bolnick, unpubl. data). This phenotypic variation is typically manifest as continuous and (often) unimodal trait distributions within lakes, as opposed to discrete *G. aculeatus* types. Laboratory foraging trials have confirmed that these variable traits (*e.g.* gill raker and gape width) differentially influence foraging efficiency on benthic *v.* limnetic prey, and therefore give rise to trade-offs that limit an individual's ability to optimally exploit both kinds of prey (Schluter, 1995; Robinson, 2000). Given that foraging is a key determinant of fitness, this coupled diet and morphological variation is probably not selectively neutral. How then is it maintained by (or despite) selection?

Adaptive variation can be enhanced *via* disruptive selection, which can arise owing to negative frequency-dependent interactions, such as intraspecific competition (Rosenzweig, 1978; Wilson & Turelli, 1986; Abrams *et al.*, 1993; Bürger & Schneider, 2006). In particular, individuals with the modal phenotype have disproportionately more competitors than do individuals with rare phenotypes, potentially leading to lower fitness in the former (Dieckmann & Doebeli, 1999; Doebeli & Dieckmann, 2003; Doebeli *et al.*, 2007). This hypothesis was tested in natural populations of *G. aculeatus* based on indirect proxies for fitness (gonad mass, body size and growth rate). First, Bolnick (2004*a*) showed that a major trophic trait, gill raker length, was subject to disruptive selection in natural lake populations. Moreover, experimental manipulations in large (>100 m^2) field enclosures showed that the disruptive selection was stronger in high-density than in low-density treatments, confirming the driving influence of competition. Second, Bolnick & Lau (2008) surveyed natural variation in 14 lake populations, showing that disruptive selection was largely absent in very large or very small lakes, but was more often present in

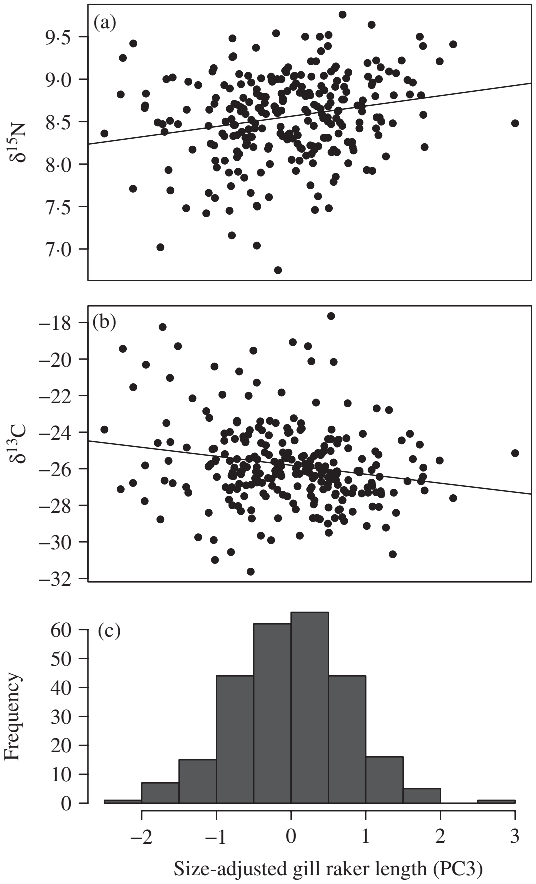


FIG. 1. State 1: within-population variation in (c) trophic morphology and (a), (b) corresponding variation in diet for *Gasterosteus aculeatus* (J. Paull, L. Snowberg, & D. I. Bolnick, unpubl. data). Within a single lake population (Robert's Lake, British Columbia, Canada), individuals vary in size-adjusted gill raker length [(c) third principal component axis]. This variation is normally distributed, highlighting the lack of speciation within this lake (the benthic and limnetic species pairs exhibit strongly bimodal gill raker length distributions). Nonetheless, variation in this trophic trait influences an individual's foraging behaviour, here measured by stable isotope signatures. Individuals with long gill rakers have a higher $\delta^{15}N$ ($P < 0{\cdot}001$), indicative of their feeding at a higher trophic position, consistent with feeding on limnetic copepods. Individuals with long gill rakers also exhibit lower $\delta^{13}C$ ($P < 0{\cdot}01$), again consistent with a more limnetic diet. Thus, morphological variation within a population coincides with diet variation among individuals.

intermediate-sized lakes. The authors suggested that intermediate-sized lakes have a relatively even mix of benthic and limnetic habitat types, thus favouring increased individual specialization and generating stronger disruptive selection. Supporting this idea, sites with greater variation in foraging opportunities appear to have greater variation and covariation in trophic traits (Berner *et al*., 2008; D. Berner & D. I. Bolnick, unpubl. data).

Although intraspecific competition thus seems to influence disruptive selection and trait variation in *G. aculeatus*, other potential contributing forces have yet to be examined in detail. For example, disruptive selection in *G. aculeatus* could be influenced by predation (Rundle *et al*., 2003), parasitism (MacColl, 2009), male–male competition and female mate preferences. Conversely, trait variation within a group can be constrained by interspecific interactions. For example, interspecific competition in sticklebacks drives ecological character displacement (Schluter, 2003; Gray & Robinson, 2002), and the resulting directional selection can reduce trait variation.

Phenotypic plasticity

Some studies have found that *G. aculeatus* feeding on different prey types develop different morphological traits that improve foraging (Day *et al*., 1994). Consequently, individuals exposed to different environmental conditions early in life may develop divergent phenotypes, contributing to trait variation within populations. This plasticity, however, is not always observed and is often small relative to genetic effects (Day & McPhail, 1996; Robinson, 2000; Wund *et al*., 2008). Furthermore, plasticity might reduce variation if individuals converge on a single adaptive phenotype during development, thereby masking underlying genetic variation.

Phenotypic variation might also be influenced by behavioural plasticity. Optimal foraging theory suggests that individuals will specialize on high-value prey when those prey are abundant, but will accept alternative prey when the preferred prey are scarce (Schoener, 1971). Genetic variation in foraging traits can mean that individuals have different first-ranked prey or different lower-ranked prey. In the latter case, increasing competition for shared high-value prey might cause individuals with different traits to increasingly include divergent lower-ranked prey (Robinson & Wilson, 1998). Indeed, increasing competition in *G. aculeatus* increases plastic diet variation and the correlation between diet and morphology (Svanbäck & Bolnick, 2005). By increasing this correlation, behavioural prey shifts increase the efficacy with which natural selection can drive morphological evolution. Svanbäck & Bolnick (2007) experimentally confirmed that intraspecific competition drives increased niche variation within *G. aculeatus* populations, especially through an increased diet-morphology correlation. As a result, disruptive selection arising from frequency-dependent competition (Bolnick, 2004*a*) should increase phenotypic variation.

WITHIN-POPULATION VARIATION AND THEN WHAT?

Within-population variation might play several roles in diversification. First, it should facilitate evolution within populations, and thereby increase divergence between allopatric or parapatic populations in different environments. This adaptive divergence might then lead to reproductive isolation. Second, within-population variation might contribute to fully sympatric speciation, particularly when two main conditions are satisfied (Gavrilets, 2004; Bolnick & Fitzpatrick, 2007). The remainder of this section will consider these two conditions in lacustrine *G. aculeatus* populations.

The first main condition for sympatric speciation is that a population should be subject to temporally consistent disruptive selection on ecological traits, thus reducing the fitness of intermediate phenotypes (Dieckmann & Doebeli, 1999; Bürger *et al*., 2006). This condition is at least sometimes satisfied in *G. aculeatus*, given that disruptive selection on trophic morphology has been documented in some lakes (Bolnick & Lau, 2008). This disruptive selection is likely to be temporally stable, given its association with factors that will persist through time, such as lake size and intraspecific competition (Bolnick, 2004*a*; Bolnick & Lau, 2008).

The second main condition is that the trait under disruptive selection should generate assortative mating. This might occur in several ways. First, the use of different resources might pleiotropically reduce encounter rates between individuals from different groups. For instance, individuals foraging on different prey types might come into breeding condition at different times, corresponding to different peak availabilities of their respective resources. No study has yet tested this hypothesis for sticklebacks. Alternatively, individuals with different foraging traits might breed in different microhabitats, *i.e.* the habitats where their preferred prey are most available. This spatial segregation seems possible in at least some *G. aculeatus* populations, because fishes captured at different sites within a lake often exhibit morphological and dietary differences (Baker *et al*., 2005; J. Paull, L. Snowberg & D. I. Bolnick, unpubl. data). Second, assortative mating might arise if individuals exhibit behavioural preferences for prospective mates that are adapted to similar resources (given a particular encounter rate). Such preferences might be possible if an individual's resource and habitat use is recognizable through cues influenced by ecological divergence. Possibilities for sticklebacks include body shape, colour, MHC genotype, excreted prey cues, behaviour and olfactory cues associated with diet (Ward *et al*., 2004). Supporting this possibility in *G. aculeatus*, Snowberg & Bolnick (2008) documented a positive association between the stable isotope signatures (reflecting past diet) of males and females in breeding pairs.

Given that *G. aculeatus* thus appear to be under the joint action of disruptive selection and assortative mating, both acting on the same ecological traits, standard theory might predict frequent sympatric speciation. But this prediction is not borne out; even in the lakes where disruptive selection and assortative mating have been documented, phenotypes remain unimodal (D. Bolnick, unpubl. data) and microsatellite genotypes are in Hardy–Weinberg equilibrium (Caldera & Bolnick, 2008). Although a few lakes do contain sympatric species pairs, their origin probably involved an allopatric phase (see below). Sympatric speciation in *G. aculeatus* thus appears to be 'all dressed up but with nowhere to go'.

One possibility for the rarity of sympatric speciation in *G. aculeatus* is that disruptive selection and assortative mating, although sometimes present, are too weak. Indeed, some theoretical models suggest that both forces need to be quite strong to drive noteworthy reproductive isolation in sympatry (Matessi *et al*., 2001; Bolnick, 2004*b*; Bürger & Schneider, 2006; Doebeli *et al*., 2007). Thus, some populations might exhibit enhanced (perhaps even bimodal) trait distributions, but never actually split into reproductively isolated groups. In effect, speciation might get permanently stuck in a state of trophic polymorphism within a population. The primary contribution of within-population variation to speciation in *G. aculeatus* thus appears to be the facilitation of adaptive divergence among parapatric or allopatric populations, which might then contribute to reproductive isolation.

STATE 2: DISCONTINUOUS VARIATION WITH MINOR REPRODUCTIVE ISOLATION

The previous section explained how genetic discontinuities do not easily emerge from the combined action of disruptive selection and assortative mating within stickleback populations. Discontinuities instead seem to arise from adaptive divergence among stickleback populations that inhabit ecologically disparate sites in allopatry or parapatry. This idea is now developed further and used to explain how adaptive divergence can lead to at least partial reproductive isolation. The main focus here is on parapatric *G. aculeatus* population pairs, where divergent selection and reproductive barriers are more easily examined than in allopatric populations, although these populations also can be very informative (Foster *et al.*, 1998; Vines & Schluter, 2006; Scotti & Foster, 2007; Shaw *et al.*, 2007).

One parapatric situation is represented by adjoining lake and stream habitats. These habitats differ in several important ecological features, including the prey resources that generate disruptive selection in lacustrine populations (as described above). In particular, lakes generally provide both benthic and limnetic foraging opportunities, whereas streams usually provide only benthic opportunities (Gross & Anderson, 1984; Berner *et al.*, 2008, 2009). This ecological difference generates divergent selection that drives genetically based adaptive divergence [Fig. 2(b), (c)]. Most obviously, lake *G. aculeatus* have shallower bodies, longer caudal peduncles and longer and more numerous gill rakers (Moodie, 1972*a*, *b*; Gross & Anderson, 1984; Reimchen *et al.*, 1985; Lavin & McPhail, 1993; Hendry *et al.*, 2002; Hendry & Taylor, 2004; Berner *et al.*, 2008, 2009; Sharpe *et al.*, 2008). Particularly useful features of this system are: (1) many different lake and stream population pairs have diverged independently since the last glaciation, (2) lake and stream phenotypic and genetic divergence varies considerably among the pairs and (3) this lake and stream divergence is sometimes maintained (and might have arisen) in close parapatry (Thompson *et al.*, 1997; Hendry & Taylor, 2004; Berner *et al.*, 2009). Furthermore, lake and stream transitions in resource use and morphology sometimes coincide closely with genetic discontinuities at neutral markers (Lavin & McPhail, 1993; Thompson *et al.*, 1997; Reusch *et al.*, 2001; Hendry *et al.*, 2002; Hendry & Taylor, 2004; Berner *et al.*, 2009). For example, clustering algorithms using microsatellite data often infer distinct lake and stream populations with a sometimes abrupt transition between them [Fig. 2(c)]. Microsatellite-based F_{ST} values between these clusters range from 0·05 to 0·23 for the different population pairs (Berner *et al.*, 2009). Substantial ecologically based reproductive isolation can thus arise across lake and stream transitions.

Another parapatric system is represented by the mud and lava habitats within some Icelandic lakes. In some lakes, phenotypic divergence between the habitats is evident in diet and trophic traits (Kristjánsson *et al.*, 2002*a*), but in other lakes it is more related to predator defence traits (Ólafsdóttir *et al.*, 2007*a*). This latter difference is thought to arise because the more complex lava habitat is thought to provide better protection from predatory Arctic charr *Salvelinus alpinus* (L.). The spatial distribution of the two habitat types has been examined in one lake, where they are separated by an open predation belt that lacks cover and should therefore restrict dispersal (Ólafsdóttir *et al.*, 2007*a*). Here is an example of the difficult application of the terms sympatry (they are in the same lake), parapatry (the two habitats are sometimes in close contact) and allopatry (dispersal may be very difficult). Some

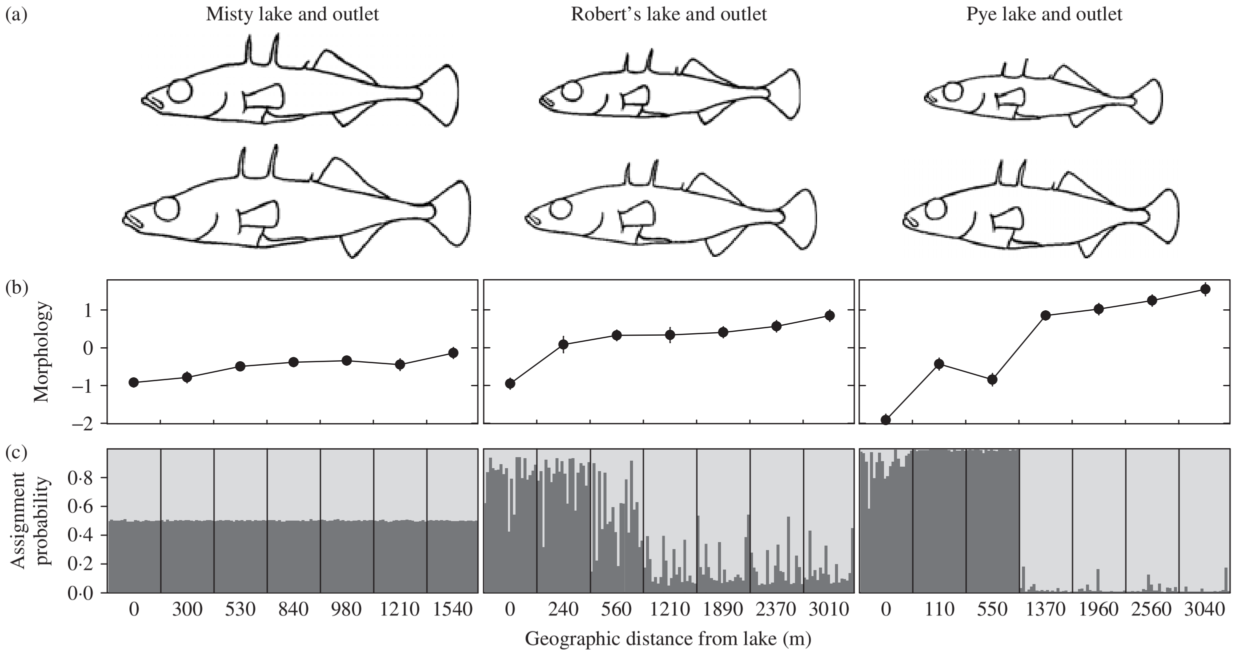


FIG. 2. State 2: morphological and neutral genetic divergence across three representative lake and outlet stream transitions (Misty Lake *v.* outlet, Robert's Lake *v.* outlet and Pye Lake *v.* outlet) for *Gasterosteus aculeatus*. Data are shown from seven clinal sample sites arranged by increasing distance from the lake site (distance = 0). (a) Average body shape for fish from the lake site (top) and the last stream site (bottom) in each system. The drawings are based on geometric-morphometric consensus shapes, scaled to preserve relative size differences observed in the wild. (b) Clinal shifts in morphology, here expressed as the size-standardized principal component of body shape and gill raker number (same scale for all systems). (c) Genetic clustering visualized by individual assignment probabilities to two predefined populations (dark grey, light grey), based on microsatellite data and the software STRUCTURE (Pritchard *et al.*, 2000). Note that the Misty Lake system displays only subtle (but significant) phenotypic change without any neutral genetic structure (as indicated by individual assignment probabilities close to 0·5 along the entire stream). The Robert's Lake system shows greater phenotypic change, and genetic markers suggest a hybrid zone. The Pye Lake system shows the greatest phenotypic change, and this is accompanied by substantial reproductive isolation across a very abrupt transition.

reproductive isolation is present in the one lake where this has been examined, although F_{ST} values are quite low (0·042–0·082) and hybridization can be extensive (Olafsdottir *et al.*, 2007*b*). The mud and lava *G. aculeatus* pairs thus show some ecologically based reproductive isolation, but its magnitude might be less than that seen among at least some of the lake and stream pairs.

REPRODUCTIVE BARRIERS

Different *G. aculeatus* populations are not known to show any intrinsic genetic incompatibilities (Bell & Foster, 1994; McKinnon & Rundle, 2002; Östlund-Nilsson *et al.*, 2007), although an exception is described later. The lack of such incompatibilities has also been confirmed for the one lake–stream pair (Misty Lake, British Columbia) where it has been examined (Lavin & McPhail, 1993; Hendry *et al.*, 2002; D. Berner, K. Räsänen & A. Hendry, unpubl. data). In general, then, reproductive barriers in the lake and stream and mud and lava systems would be due to the partial geographic separation and to ecological barriers, which are now considered. First, adaptation to a given habitat might result in maladaptation to alternative habitats, and hence selection against migrants (Hendry, 2004; Nosil *et al.*, 2005; Hereford, 2009). This barrier would seem likely for the lake and stream pairs where adaptive divergence is strong. To test for this possibility, transplant experiments were performed with the highly divergent lake and inlet stream populations in the Misty Lake watershed. Possible evidence of selection against migrants was observed, specifically a reduced growth rate in non-local individuals, but the performance differences were small (Hendry *et al.*, 2002; K. Räsänen & A. Hendry, unpubl. data). It is also possible that migrants between lakes and streams might show differences in survival as a result of adaptation to habitat-specific regimes of parasitism (M. Kalbe & C. Eizaguirre, pers. comm.) or predation. Additional work is necessary to establish the strength of this potential ecological barrier in the lake and stream system, and similar experiments have not been performed in the mud and lava system.

Another potential reproductive barrier is ecological selection against hybrids (Schluter, 1995; Rundle & Whitlock, 2001; Thibert-Plante & Hendry, 2009). In the lake and stream system, hybrids are generally morphologically intermediate between pure lake and stream fishes, at least in the one watershed (Misty Lake) where this has been examined (Lavin & McPhail, 1993; Hendry *et al.*, 2002). Hybrids might therefore be poorly adapted for either parental environment. No experiments have directly tested this hypothesis, but there is some indirect supporting evidence. In particular, lake and stream discontinuities in neutral genetic markers are sometimes displaced downstream relative to lake and stream discontinuities in morphology (Berner *et al.*, 2009), with a clear example seen for the Robert's Lake and outlet pair [*cf.* Fig. 2(b)]. This mismatch is what would be expected owing to ecological selection against hybrids in the F2 and later generations (Barton & Hewitt, 1985; Jiggins & Mallet, 2000). Similar data are not available for the mud and lava system.

Reproductive barriers might also arise if adaptation to contrasting habitats and resources leads to divergence in mate choice (Schluter, 2000). For the lake and stream system, mate choice experiments have been performed with laboratory-bred pure lake, pure stream and hybrid *G. aculeatus* from the Misty Lake catchment. Males of the two types differ in reproductive behaviour (Delcourt *et al.*, 2008), and so positive assortative mating would seem plausible. This has proven not to be the case, with mate choice instead being strongly asymmetric (K. Räsänen & A. Hendry, unpubl. data) and hybrids showing no apparent mating disadvantage (J. Raeymaekers, D. Berner, K. Räsänen & A. Hendry, unpubl. data). Asymmetric mate choice also appears to be present in limited data for another lake and stream pair (Moodie, 1982). Ecological divergence thus does not reliably generate positive assortative mating preferences in lake and stream *G. aculeatus* pairs, at least not when assayed in the laboratory. Laboratory mate choice experiments have also been performed with wild-caught mud and lava *G. aculeatus*, where positive assortative mating is present (Ólafsdóttir *et al*., 2006). The precise cues underlying this assortative mating are unknown, but they do not appear to involve differences in body size, as has proven the case in the other *G. aculeatus* systems (Nagel & Schluter, 1998; Rundle *et al*., 2000; McKinnon *et al*., 2004; Boughman *et al*., 2005).

Other ecologically based reproductive barriers seem likely. One is adaptive habitat choice. For example, Hendry *et al*. (2002) used a mark–recapture experiment to show that lake (but not inlet) *G. aculeatus* moved downstream when placed into the inlet stream. Presumably this means that lake *G. aculeatus* are not well suited for maintaining their position in a stream or that they are seeking different (possibly lake-like) conditions. Supporting the latter possibility, Bolnick *et al*. (2009) used a mark–recapture experiment to show that *G. aculeatus* often return to their respective source habitats (lake or stream) when released at the transition between these habitats. In addition, the few released stream fishes that dispersed into the lake were phenotypically more similar to lake fishes than were the many released stream fishes that returned to the stream, with the reverse being true for the released lake fishes (Bolnick *et al*., 2009). Habitat preference thus seems to be appropriately phenotype-dependent. Similar experiments have not been performed for the mud and lava system, but it is nevertheless clear that the two types generally stay in their respective habitats, despite being physically able to move between them. Finally, reproductive timing appears to differ between lake and stream populations in at least some locations (J.-S. Moore & A. Hendry, unpubl. data), although the reasons are not known.

To summarize, reproductive barriers between *G. aculeatus* populations displaying discontinuous variation across habitat transitions are variable, and they do not appear as strong as those in the benthic and limnetic pairs (see below). Some of these partial barriers have been identified, or at least appear likely, including natural selection against migrants, natural selection against hybrids, habitat choice and mate choice. Critically, however, not all of these barriers are present in all situations.

DISCONTINUOUS VARIATION, SOME REPRODUCTIVE ISOLATION AND THEN WHAT?

The results outlined above show that genetic and morphological discontinuities frequently arise between parapatric *G. aculeatus* populations adapted to ecologically contrasting environments. This finding suggests that the spatial context of diversification represents a pivotal determinant of the transition between states 1 and 2. In short, reasonable progress towards ecological speciation appears easier in parapatry than in sympatry, as is often predicted in theory (Endler, 1977; Doebeli & Dieckmann, 2003; Gavrilets, 2004). Tantalizingly, the apparently greater divergence between *G. aculeatus* in lake and stream habitats than between *G. aculeatus* in mud and lava habitats parallels the theoretical expectation that diversification should be easier in one dimension (linear) than in two dimensions (Gavrilets & Losos, 2009). But perhaps this is just coincidental, given the many other ways in which these two systems differ. Results from the lake and stream system further hint that substantial parapatric differentiation is facilitated by clear-cut spatial transitions between habitats (Berner *et al*., 2009). In other words, substantial progress towards ecological speciation is most likely in the presence of marked ecotones (and hence strong divergent selection), as is also predicted by theory (Gavrilets & Losos, 2009; Nosil *et al*., 2009; Thibert-Plante & Hendry, 2009).

Why, then, have parapatric *G. aculeatus* pairs apparently only rarely, if ever, achieved the strong reproductive isolation characteristic of state 3? The answer may lie in several additional modifying factors. One is that excessive dispersal between environments can constrain divergence, particularly for small populations that are adjacent to large populations (García-Ramos & Kirkpatrick, 1997; Richards *et al*., 1999; Nosil & Crespi, 2004). In the case of *G. aculeatus*, dispersal from the often-large lake populations might constrain adaptive divergence of the often small stream populations (Hendry *et al*., 2002; Hendry & Taylor, 2004; Moore *et al*., 2007). Another potential modifying factor is the number and specific types of traits targeted by divergent selection (Nosil *et al*., 2009). Perhaps mud and lava *G. aculeatus* cannot reach stronger isolation, because resource use, a key axis in many adaptive radiations (Robinson & Wilson, 1998; Skulason & Smith, 1995; Schluter, 2000), is not always strongly divergent (Kristjánsson *et al*., 2002*a*). In addition, progress beyond state 2 might sometimes require selection to reduce competition (leading to ecological character displacement) or to reduce maladaptive hybridization (leading to reproductive character displacement). Lake and stream and mud and lava transitions might not be favourable to strong selection of this sort because the zone of contact is often spatially restricted, which should make such selection weak (Servedio & Noor, 2003).

In summary, *G. aculeatus* provide evidence that strong divergent selection between habitats in parapatry (as opposed to sympatry) promote transitions from state 1 to 2 along the speciation continuum. Progress from state 2 to 3, however, may require increased secondary contact, a context addressed in the next section.

STATE 3. STRONG, BUT REVERSIBLE, REPRODUCTIVE ISOLATION

The previous section described situations in *G. aculeatus* where adaptive divergence has proceeded in parapatry and led to partial reproductive isolation. The present section considers situations where adaptive divergence has become coupled with very strong reproductive isolation that is nonetheless reversible. These situations are best examined by returning to instances of sympatry, because this context allows a more precise and clear examination of reproductive barriers.

One sympatric situation is represented by the benthic and limnetic *G. aculeatus* pairs coexisting in each of a few small lakes in south-western British Columbia. Each of these lakes is home to a pair of morphologically distinct types adapted to benthic *v*. limnetic foraging environments (McPhail, 1984, 1992, 1993, 1994; Schluter & McPhail, 1992; Gow *et al*., 2008). The limnetic type has a shallower body, a narrower gape, more and longer gill rakers and a larger eye, all traits that improve foraging success on the zooplankton found in limnetic zones. The two *G. aculeatus* types also differ in defensive armour, including spines, lateral plates and pelvic structures, seemingly because of differential exposure to predators. Importantly, the two types have diverged independently within each lake (Taylor & McPhail, 2000) and they are now strongly reproductively isolated. This isolation is evident in that they produce few ($<5\%$) hybrids in nature, form very distinct genetic clusters and show microsatellite-based F_{ST} values ranging from 0·209 to 0·336 (McPhail, 1984, 1992; Taylor & McPhail, 2000; Gow *et al.*, 2006, 2008).

The geographic context for *origins* of these sympatric benthic and limnetic pairs is not clear (McPhail, 1993; Taylor & McPhail, 2000; Gow *et al.*, 2008). One possibility is purely sympatric speciation within each lake following a single colonization by marine ancestors. Another possibility is a double invasion by the marine type into fresh water, corresponding to a two-stage allopatric–sympatric model of divergence (*sensu* Lack, 1947; Rundle & Nosil, 2005; Grant & Grant, 2008). The first invasion is most likely to have formed a generalized freshwater type that is more benthic than the ancestral marine type. The second invasion then probably generated character displacement causing the generalized freshwater type to become the benthic type and the invading marine type to become the limnetic type. Genetic data more clearly support the double-invasion hypothesis (Taylor & McPhail, 2000). Ambiguity remains, however, because the specific geologic scenario thought to allow a double invasion (multiple advances and retreats of glaciers) now seems unlikely (Gow *et al.*, 2008).

Another situation of interest is that of freshwater and anadromous *G. aculeatus* pairs in the lower reaches of many rivers and streams (McPhail, 1994; McKinnon *et al.*, 2004) and sometimes in lakes (Karve *et al.*, 2008). The two types show partially overlapping spatial distributions during breeding (Hagen, 1967; Jones *et al.*, 2006), and so are here considered sympatric (although some interpretations might consider them parapatric: ambiguity again). The two types are strongly morphologically differentiated in nature (Hagen, 1967; McPhail, 1994; McKinnon & Rundle, 2002), including in trophic morphology (the anadromous type is more limnetic) and defensive morphology. With respect to defence, the anadromous type has longer spines, better developed pelvic structures and numerous lateral plates. These differences probably reflect adaptation to the greater rate of piscivory in the ocean contrasted with the costs (mobility, growth and insect predation) of excessive armour in fresh water (Reimchen, 1980, 1983, 1992, 2000; Klepaker, 1993; Bell *et al.*, 1993, 2004; Marchinko & Schluter, 2007; Raeymaekers *et al.*, 2007; Barrett *et al.*, 2009; Marchinko, 2009). As seen in the benthic and limnetic pairs, the freshwater type appears to have evolved independently in many locations, where it now shows reduced hybridization and (especially) gene flow with the anadromous type (Hagen, 1967; McKinnon *et al.*, 2004; Raeymaekers *et al.*, 2005; Jones *et al.*, 2006; Mäkinen *et al.*, 2006).

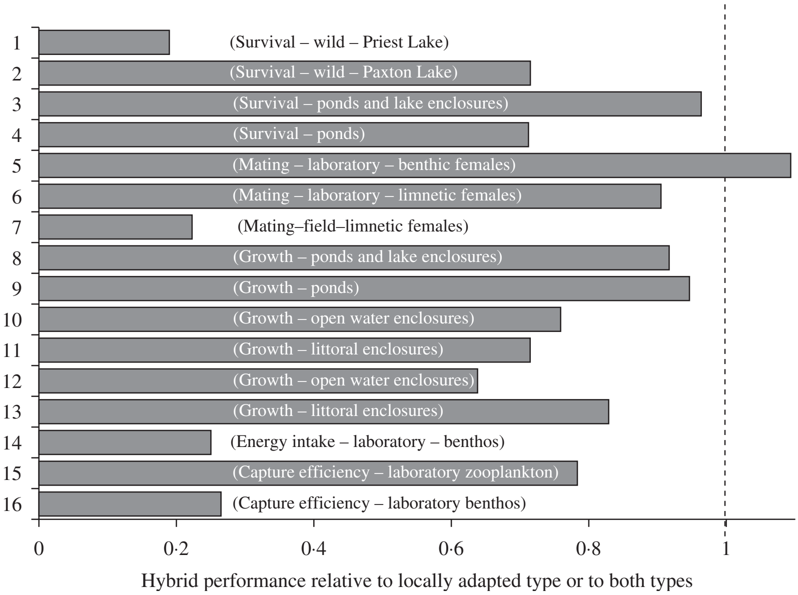
REPRODUCTIVE BARRIERS

As in the lake and stream and mud and lava systems, reproductive barriers are here probably ecological in origin. Selection against migrants (in the broad sense given that they are in the same lake) has been considered for the benthic and limnetic pairs by forcing each to feed on the other's main prey type (benthic or limnetic) in the laboratory or in experimental enclosures in their native lakes. The main finding of this work is that the locally adapted type usually feeds more efficiently and grows better than does the non-locally adapted type (Bentzen & McPhail, 1984; Schluter, 1993, 1995; Hatfield & Schluter, 1999). For the freshwater and anadromous pairs, laboratory or pond-enclosed experiments have shown that the marine type is disadvantaged in fresh water owing to reduced growth rates (Hagen, 1967; Marchinko & Schluter, 2007) and increased predation by insects (Marchinko, 2009). Moreover, pond experiments have shown that alleles for the marine armour type at the major-effect gene EDA are under negative selection after lateral plates are expressed during development (Barrett *et al.*, 2008, 2009). Perhaps the most direct evidence of selection against migrants, however, is that marine types colonizing fresh water very rapidly evolve reduced armour (Klepaker, 1993; Kristjánsson *et al.*, 2002*b*; Bell *et al.*, 2004). Selection against migrants in the opposite direction (fresh water to marine) has been less studied but seems likely given that freshwater *G. aculeatus* have low hatching rates in high salinity (McPhail, 1969; Marchinko & Schluter, 2007) and are not as good at prolonged swimming (Taylor & McPhail, 1986). In short, selection against migrants is probably a very important isolating barrier in the benthic and limnetic and freshwater and anadromous *G. aculeatus* pairs.

Selection against hybrids in the benthic and limnetic pairs has been studied in several different venues (Fig. 3), although nearly all of the studies are with a single pair (Paxton Lake). In artificial ponds, hybrids have lower survival than pure types under a variety of conditions (Vamosi *et al.*, 2000; Vamosi & Schluter, 2002). In experimental enclosures in their native lakes, hybrids grow slower (presumably indicating lower fitness) than the locally adapted pure type (benthic or limnetic) in each environment (Schluter, 1995; Hatfield & Schluter, 1999), and backcrosses sometimes grow at rates intermediate between hybrids and locally adapted pure types (Rundle, 2002). This last result is taken as particularly strong evidence that the reduced performance of hybrids has an ecological basis (Rundle & Whitlock, 2001; Rundle, 2002). In unmanipulated natural populations, genetic markers have been used to show that the frequency of hybrids decreases through the life cycle (Gow *et al.*, 2007). Selection against hybrids is therefore likely to be a very important part of reproductive isolation in the benthic and limnetic pairs. Similar studies have not been performed with freshwater and anadromous pairs, but selection against hybrids might be strong, because hybrids are often produced (Hagen, 1967; Jones *et al.*, 2006) but the two types remain genetically distinct in sympatry (Jones *et al.*, 2006).

Assortative mating has been examined in both systems. For particular benthic and limnetic pairs, laboratory studies have shown that each type prefers to mate with its own type (Ridgway & McPhail, 1984; Nagel & Schluter, 1998). Subsequent work showed that limnetic females would rather mate with limnetic males (and benthic females with benthic males) even if those males came from a different lake (Rundle *et al.*, 2000). This last result suggests that adaptation to benthic *v.* limnetic environments *per se* (*i.e.* regardless of the specific lake) generates the assortative mating. Further supporting this inference, the assortative mating was influenced by some of the same phenotypic traits (size, colour and mating behaviour) that diverge in parallel between benthic and limnetic environments (Boughman *et al.*, 2005). In follow-up work, Kozak *et al.* (2009) showed that it is the females rather than the males that care about mating with their own type, and Head *et al.* (2009) showed that assortative mating between species is not simply the result of male preferences within species. Finally, experiments with laboratory-reared fishes placed in lake enclosures revealed that limnetic females prefer limnetic males over hybrid males (Vamosi & Schluter, 1999). In the freshwater and anadromous system, mate choice experiments in the laboratory have sometimes revealed positive assortative mating by type within (Hay & McPhail, 1975) or between (McKinnon *et al*., 2004) different pairs. Another experiment, this time in experimental ponds with a different population pair, did not find assortative mating (Jones *et al*., 2008). One interpretation is that mating is positively assortative on average but that considerable variation is present among populations and experimental venues (J. McKinnon, pers. comm.). An important qualification at present is that most mate choice studies in both systems used wild-caught fish, and so the observed patterns do not necessarily reflect genetic differences in mate choice. They might instead reflect environmental factors such as sexual (Albert, 2005) or social (Kozak & Boughman, in press) imprinting, although the evidence so far supports the latter rather than the former.

A number of other reproductive barriers could be important in these systems. For instance, the different types prefer different breeding habitats, at least to some extent (Hagen, 1967; Vamosi & Schluter, 1999). Moreover, transplant experiments indicate that the freshwater and anadromous types move upstream and downstream, respectively, thus reducing their spatial overlap (Hagen, 1967). And, in the freshwater and anadromous system at least, the different types breed at somewhat different, although overlapping, times (Hagen, 1967; Jones *et al*., 2006; Karve *et al*., 2008). In short, multiple, strong reproductive barriers are present in the benthic and limnetic and freshwater and anadromous systems, with these barriers having an ecological and adaptive origin. And, most importantly, both systems pass the ultimate litmus test in that reproductive isolation is strong enough to often maintain their integrity in nature. (An exception is described below.)



DISCONTINUOUS VARIATION, STRONG REPRODUCTIVE ISOLATION AND THEN WHAT?

Reproductive isolation in the benthic and limnetic and freshwater and anadromous systems is so strong in sympatry that they are often considered separate species. What, then, has allowed ecological speciation to progress so far in these cases, as opposed to (for example) the mud and lava pairs and many of the lake and stream

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FIG. 3. State 3: results of tests for selection against hybrids in the *Gasterosteus aculeatus* benthic and limnetic pairs. Bars show the performance (based on a variety of metrics) of hybrids relative to one or both parental types (see below). Note that the different experiments used different measures of relative fitness (see below), and so are not directly comparable; the key point is that nearly all of them show a hybrid disadvantage (most bars fall short of the dashed line of equal fitness) that probably has an ecological basis. The text in the bars first indicates the fitness metric, then the study arena (*e.g.* wild populations, ponds, various types of field enclosures or the laboratory) and then additional key information. The last includes the specific lake for the two studies of wild hybrids (1 and 2), the specific pure type to which hybrid fitness is compared when this is critical (5, 6 and 7), and the type of laboratory foraging environment (14, 15 and 16). 1 and 2. Gow *et al.* (2006). The frequency of hybrids in wild adults sampled from a given lake (Priest or Paxton) divided by the frequency of hybrids in wild juveniles sampled from that lake. 3. Vamosi *et al.* (2000). The relative survival of hybrids is calculated as: 1 plus the frequency of hybrids at the start of the experiments minus the frequency of hybrids at the end of the experiments. The estimate is relative to both parental types (the Paxton Lake pair was used) and is averaged over four pond experiments and two lake enclosure experiments. 4. Vamosi & Schluter (2002). Frequency of young-of-the-year (YOY) hybrids at the end of the experiments divided by the frequency of YOY hybrids at the start of the experiment. The estimate is relative to both parental types (the Paxton Lake pair was used) and is averaged over a number of treatments (with and without predators and high or low density). 5 and 6. Hatfield & Schluter (1996). Frequency of hybrid males spawning with benthic (or limnetic) females divided by frequency of benthic (or limnetic) males spawning with benthic (or limnetic) females. 'No-choice' trials in laboratory aquaria were used with laboratory-reared individuals from the Paxton Lake pair. 7. Vamosi & Schluter (1999). Number of trials (Paxton Lake pair was used in Paxton Lake enclosures) where a hybrid male was preferred (had eggs in nest) by limnetic females divided by the number of trials where a limnetic male was preferred by limnetic females. Hybrid and limnetic males (both laboratory-reared) were introduced into the enclosures and induced to build nests. Wild-caught gravid limnetic females were then introduced and allowed to spawn. 8. Vamosi *et al.* (2000). Growth rate of hybrids relative to benthics (hybrids here grew better than limnetics). This value is averaged over four pond experiments with the Paxton Lake pair. Growth is calculated as the average size of surviving individuals of a given cross type at the end of the experiment divided by the average size of individuals of that cross type at the start of the experiment. 9. Vamosi & Schluter (2002). Growth rate of hybrids relative to benthics (hybrids here grew better than limnetics) for laboratory-reared fish (Paxton pair was used) in pond experiments. The estimate is an average of all of the treatment and replicate estimates for hybrids divided by the average of all of the treatment and replicate estimates for benthics. Growth is calculated as the average size of surviving individuals of a given cross type at the end of the experiment divided by the average size of individuals of that cross type at the start of the experiment. 10 and 11. Hatfield & Schluter (1999). The average growth rate of hybrids divided by the average growth rate of limnetics in open water enclosures or divided by the average growth rate of benthics in littoral enclosures. This experiment used second generation laboratory-reared fish of the Paxton Lake pair tested in Paxton Lake. 12 and 13. Schluter (1995). Average growth rate of hybrids divided by average growth rate of limnetics in open water enclosures or divided by average growth rate of benthics in littoral enclosures. This experiment used 10th generation laboratory-reared fish of the Paxton Lake pair tested in Paxton Lake. 14, 15 and 16. Schluter (1993). Experiments in laboratory tanks examining energy intake of hybrids relative to benthics when both were feeding on benthos (collected from the lake), and food capture efficiency of hybrids relative to limnetics when both were feeding on zooplankton (collected from the lake) and relative to benthic fish when both were feeding on benthos (collected from the lake). Tenth-generation laboratory-reared fish from the Paxton Lake pair were used.

pairs? One distinguishing feature is the geographic context, perhaps reproductive barriers become stronger through interspecific interactions following secondary contact. Supporting this idea, experiments in ponds have shown that competition between pure benthic or limnetic types and an intermediate type generates divergent selection on adaptive traits (Schluter, 2003). Moreover, mating probabilities in the laboratory are somewhat higher when the benthic and limnetic fish are from different lakes than when they are from the same lake (Rundle *et al.*, 2000). This possibility that 'familiarity breeds contempt' might be the result of classic reinforcement (indirect selection owing to low fitness of hybrid offspring) or direct selection in the sympatric pairs for limnetic males to avoid benthic females that might eat their eggs (Albert & Schluter, 2004). These interspecific interactions are probably weak in the lake and stream and mud and lava pairs, owing to the rarity with which individuals move between these habitats.

Despite the apparent strength and multitude of reproductive barriers in these sympatric pairs, those barriers are not unassailable, being instead sensitive to ecological conditions. A case in point is the Enos Lake benthic and limnetic pair, which historically showed strong adaptive divergence (Schluter & McPhail, 1992) and mating isolation (Boughman, 2001). Samples collected in 1999, however, suggested that this distinction and isolation had been lost (Kraak *et al.*, 2001). Subsequent work confirmed that the original species pair had collapsed into a hybrid swarm (Taylor *et al.*, 2006). Even very strong ecologically based reproductive barriers can thus fail if conditions change, here, seemingly, due to the introduction of crayfish *Pacifastacus leniusculus* (Taylor *et al.*, 2006).

Why have these sympatric pairs not proceeded to the point of irreversible reproductive isolation? One possibility is the limited time frame for their divergence, given that most freshwater populations were colonized from marine ancestors only after the last glaciation (Taylor & McPhail, 2000; Reusch *et al.*, 2001; Mäkinen *et al.*, 2006). Genetic incompatibilities (a possible route to complete isolation, see below) might take considerably longer to develop (Coyne & Orr, 2004; Bolnick & Near, 2005). This explanation is not entirely sufficient, however, given that genetic incompatibilities have not been documented across the range of *G. aculeatus*, including among populations separated for millions of years. A telling exception will be described below. Another possibility, then, is that the divergent selection is not strong enough to generate irreversible barriers through ecological speciation. Equivalently, assortative mating may not be strong enough, perhaps because trait divergence is not sufficient for females to reliably or profitably discriminate against heterospecifics. Finally, irreversible isolation in sticklebacks may require specific genetic changes, such as chromosomal rearrangements.

STATE 4. STRONG AND IRREVERSIBLE REPRODUCTIVE ISOLATION

In contrast to the systems described above, an apparently unique *G. aculeatus* pair has progressed substantially towards strong and irreversible reproductive isolation. This pair consists of two anadromous types: the ancestral Pacific Ocean type and the derived Japan Sea type. It is thought that the Japan Sea type evolved in allopatry because the Sea of Japan has been isolated from the Pacific Ocean several times within the past 2 million years (Higuchi & Goto, 1996; Kitano *et al.*, 2007). Unlike the Pacific Ocean type, which has also colonized freshwater habitats in Japan, the Japan Sea type is exclusively anadromous (Higuchi, 2003). The two types differ in a number of morphological traits, including body size, gill raker number, dorsal spine length and the size of lateral plates (Kitano *et al.*, 2007). Based on information from the above systems, these differences imply divergent selection and adaptation to different foraging and predation regimes. Ambiguity arises, however, because the two types are not currently found in different habitats, and the environment in which the Japan Sea type evolved is not known. Thus, it is more difficult to assess any adaptive significance of divergence than in the younger pairs discussed in the previous sections.

The two types also differ dramatically in male mating behaviour (Ishikawa & Mori, 2000; Ishikawa *et al.*, 2006; Kitano *et al.*, 2007, 2008*b*). First, Japan Sea males do not perform the traditional zig-zag dance so characteristic of the ancestral Pacific Ocean type; males instead slowly roll onto their sides as they approach females. Second, when Japan Sea males approach females, they open their mouths, extend their throats and erect their gill covers, behaviour not observed in the Pacific Ocean type. Third, when Japan Sea males get close to females, they perform much more aggressive dorsal pricking behaviour than seen in the Pacific Ocean type. The evolutionary reason, adaptive or otherwise, for these behavioural differences, as well as female responses to them, remains a mystery.

The Japan Sea type now occurs in sympatry with the Pacific Ocean type along the eastern coast of Hokkaido. In the one location where they have been studied in detail (Bekanbeushi River), $<1\%$ of adult fish are hybrids and the two types are strongly differentiated at microsatellite markers ($F_{ST} = 0{\cdot}15{-}0{\cdot}16$; $R_{st} = 0{\cdot}59{-}0{\cdot}60$; Kitano *et al.*, 2007; Kitano *et al.*, 2009). The probable reproductive barriers between these types are several. First, they show different, although partially overlapping, breeding habitats and seasons (Kume *et al.*, 2005; Kume, 2007). Second, they show behavioural isolation and hybrid male sterility (Kitano *et al.*, 2007; Kitano *et al.*, 2009). These latter two barriers are each asymmetric, but they act in opposite directions and thereby complement each other. On one hand, Pacific Ocean females have strong preferences for Pacific Ocean males, whereas Japan Sea females show no discrimination (Kitano *et al.*, 2007). On the other hand, the F1 males resulting from crosses between Japan Sea females and Pacific Ocean males are sterile, whereas those from the reciprocal cross are fertile (Kitano *et al.*, 2007). The Japan Sea type therefore appears unique among *G. aculeatus* in showing intrinsic genetic incompatibilities from other populations.

Given the unique evolutionary position of the Japan Sea type within *G. aculeatus*, it might prove especially useful for understanding how complete and irreversible reproductive isolation can arise. One key feature appears to be a chromosome fusion between the ancestral *G. aculeatus* Y chromosome and an autosome, creating an X_1X_2Y neo-sex chromosome system in the Japan Sea type (Peichel *et al.*, 2004; Ross & Peichel, 2008; Kitano *et al.*, 2009). Although this fusion does not contribute to hybrid sterility, the neo-sex chromosome does contain a locus that controls male dorsal pricking, an important component of behavioural isolation (Kitano *et al.*, 2009). These data suggest that chromosome rearrangements and genetic incompatibilities may play an important role in attaining complete and irreversible reproductive isolation in sticklebacks.

What about the six recognized species in the Gasterosteidae (Fig. 4; Wootton, 1976; Östlund-Nilsson *et al.*, 2007)? Like *G. aculeatus,* each of the other species shows apparently adaptive divergence between different environments. Some examples serve to illustrate this, although this listing is not complete. With the possible exception of *P. pungitius*, most of these species have not achieved the levels of diversity found within *G. aculeatus*. It is nevertheless true that each species shows apparently adaptive divergence between different environments. Like *G. aculeatus*, *P. pungitius* show pelvic reduction in sites without fish predators (Ziuganov & Zotin, 1995; Shapiro *et al.*, 2006), and they evolve different behaviours in different predation environments (Herczeg *et al.*, 2009). Similarly, *C. inconstans* differ in morphology between sites with different predator communities (Zimmerman, 2007), and they show different behaviour and foraging traits when in allopatry or sympatry with *P. pungitius* (Gray & Robinson, 2002; Gray *et al.*, 2005; Peiman & Robinson, 2007). These results suggest the possibility of progress towards ecological speciation within each of these species in ways that might parallel those discussed above for *G. aculeatus*. As one possible example, variation in pelvic structures influences mating isolation between *P. pungitius* populations (Ziuganov & Zotin, 1995).

Reproductive isolation can also be considered between the different species based on the regions where they occur in sympatry (Table I). Due to its broad distribution, different *P. pungitius* populations can be found in sympatry with each of the other species in at least some locations. Similarly, different *G. aculeatus* populations can be found in sympatry with each of the other gasterosteid species, except for *C. inconstans*. In several sites in eastern North America, up to four gasterosteid species can be found breeding in the same streams and tidepools, although they do show some differences in breeding habitats and seasons (Craig & FitzGerald, 1982; FitzGerald, 1983; Audet *et al.*, 1985; Delbeek & Williams, 1988; FitzGerald & Wootton, 1993; C. L. Peichel, unpubl. data). No evidence of natural hybrids between these species has ever been reported, and so they are presumably in a state of complete and irreversible reproductive isolation. Although the specific reproductive barriers might partly relate to the different breeding locations and times, they are even more obviously related to other factors.

One probable reproductive barrier between the gasterosteid species is their dramatic differences in reproductive behaviour and colour (Wootton, 1976, 1984; McLennan *et al.*, 1988; McLennan, 1993, 1996). Formal tests of behavioural isolation between species have been performed in two cases (Table I). First, *G. aculeatus* and *G. wheatlandi* males court females of either species indiscriminately, but females show strong preferences for conspecific males (Reisman, 1968; McInerney, 1969). Second, in regions of sympatry, *G. aculeatus* and *P. pungitius* males court females of both species, but females prefer conspecifics (Wilz, 1970). Thus, behavioural isolation between these species displays a similar pattern to that observed for some of the *G. aculeatus* pairs discussed above, in which female, rather than male, preferences are primarily responsible for behavioural isolation (Kitano *et al.*, 2007; Kozak *et al.*, 2009).

Probably even more important than behavioural differences are intrinsic hybrid incompatibilities. In most of the species combinations that have been tested, sperm from one species can fertilize eggs of the other species, but the resulting embryos die at early stages of development (Table I). The only exception is in crosses between *G. aculeatus* and *P. pungitius*, which yield viable F1s that are nevertheless sterile

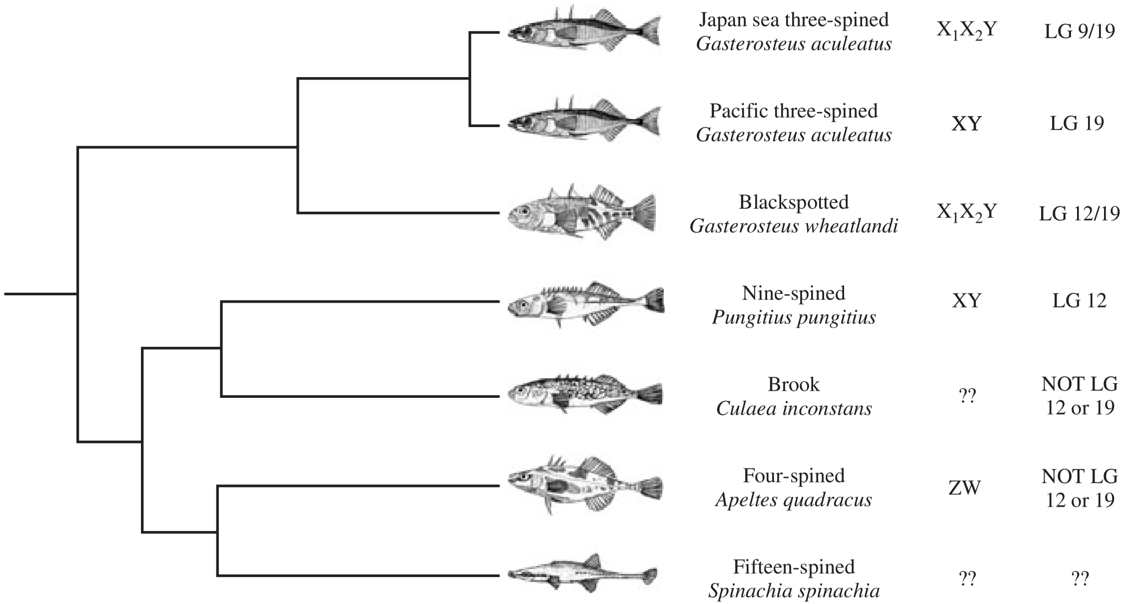

FIG. 4. State 4: a phylogeny of Gasterosteidae that illustrates how different species invariably have different sex determination systems. The phylogenetic relationships are based on Kawahara *et al*. (2009). The common and scientific names of each species are given, along with the type of sex determination system, and the *Gasterosteus aculeatus* linkage group to which the sex determination locus maps (Peichel *et al*., 2004; Ross & Peichel, 2008; Ross *et al*., 2009; Kitano *et al*., 2009). The sex determination system of *Culaea inconstans* is currently unknown, and no data are available for *Spinachia spinachia*. The drawings are from Wootton (1976).

TABLE I. Known isolating barriers among the six Gasterosteidae species. Each species is listed in the left column, with diploid chromosome number (2n) on the second line and known sex chromosome systems on the third line (Chen & Reisman, 1970; Ross *et al*., 2009). For each pair of species, the intersecting cell provides information about known isolating barriers with the other species. In each cell, the first line indicates whether the current ranges of the two species overlap (sympatric) or do not overlap (allopatric); the second line indicates whether behavioural isolation between the species has been identified and the third line indicates whether intrinsic isolating barriers have been identified

	Blackspotted *Gasterosteus wheatlandi*	Nine-spined *Pungitius pungitius*	Brook *Culaea inconstans*	Four-spined *Apeltes quadracus*	Fifteen-spined *Spinachia spinachia*
Gasterosteus aculeatus	Sympatric	Sympatric	Allopatric	Sympatric	Sympatric
$2n = 42$	Behavioural isolation[a,b]	Behavioural isolation[d]	N/T	N/T	N/T
$XY = LG19$	Hybrid inviability[c]	Hybrid sterility[e–i]	N/T	N/T	Hybrid inviability[g]
G. wheatlandi		Sympatric	Allopatric	Sympatric	Allopatric
$2n = 41, 42$		N/T	N/T	N/T	N/T
$X_1X_2Y = LG12/19$		Hybrid inviability[j]	N/T	N/T	N/T
P. pungitius			Sympatric	Sympatric	Sympatric
$2n = 42$			N/T	N/T	N/T
$XY = LG12$			Hybrid inviability[j]	N/T	Hybrid inviability[g]
C. inconstans				Allopatric	Allopatric
$2n = 46$				N/T	N/T
Unknown				N/T	N/T
A. quadracus					Allopatric
$2n = 46$					N/T
ZW					N/T

Table I. Continued

	Blackspotted *Gasterosteus wheatlandi*	Nine-spined *Pungitius pungitius*	Brook *Culaea inconstans*	Four-spined *Apeltes quadracus*	Fifteen-spined *Spinachia spinachia*
S. spinachia					
Unknown					
Unknown					

N/T, not tested.

[a] Reisman (1968).

[b] McInerney (1969).

[c] C. L. Peichel (unpubl. data): *G. aculeatus* female × *G. wheatlandi* male inviable; *G. wheatlandi* female × *G. aculeatus* male inviable.

[d] Wilz (1970).

[e] van Oordt (1925).

[f] Leiner (1940).

[g] Leiner (1957).

[h] Kobayashi (1962).

[i] Shapiro *et al*. (2006).

[j] M. D. Shapiro (pers. comm.): *G. wheatlandi* female × *P. pungitius* male inviable; *C. inconstans* female × *P. pungitius* male inviable.

(van Oordt, 1925; Leiner, 1940, 1957; Kobayashi, 1962; Shapiro *et al*., 2006). Interestingly, all five of the species that have been cytogenetically characterized have different karyotypes, resulting from differences both in chromosome number and chromosome morphology (Table I; Chen & Reisman, 1970; Ross *et al*., 2009). In addition, each species has a unique sex chromosome system, with independent XY systems in *G. aculeatus* and *P. pungitius* and a ZW system in *A. quadracus* (Fig. 4; Ross *et al*., 2009). Furthermore, *G. wheatlandi* have a X_1X_2Y sex chromosome system that results from a different Y chromosome–autosome fusion than that seen in the Japan Sea type (Ross *et al*., 2009; Kitano *et al*., 2009). These results suggest that the evolution of chromosome rearrangements and different sex chromosome systems may be required for complete and irreversible reproductive isolation in sticklebacks. As opposed to the Japan Sea and Pacific Ocean pair, however, the inability to cross these species precludes determining whether or not the chromosome rearrangements are directly linked to reproductive isolation.

HOW DID THEY GET THERE?

It seems likely that extended periods of allopatry have contributed to the evolution of the above-described reproductive isolation. It is not known, however, what selective regimes prevailed during the evolution of any of these species, whether particular morphologies or behaviours evolved as adaptations to particular environments, or whether reproductive isolation originally evolved as a result of adaptation. Also unknown is the extent of historical allopatry, parapatry and sympatry among the species. Thus, the specific roles of ecology and geography in the evolution of these species remain uncertain. The fact that irreversible isolation is always associated with chromosomal rearrangements, taken at face value, might suggest that ecology is not the driver of stickleback speciation, but, for the above reasons, this is hard to confirm.

Perhaps the identification of genes that underlie hybrid sterility and inviability in sticklebacks will yet uncover a role for adaptation, as has been the case in *Drosophila* sp. (Ting *et al*., 1998; Barbash *et al*., 2003; Presgraves *et al*., 2003; Brideau *et al*., 2006; Phadnis & Orr, 2009; Tang & Presgraves, 2009). Furthermore, theoretical work suggests that selection for linkage between genes with sexually antagonistic effects and the sex determination locus might drive the turnover of sex chromosome systems between closely related species (Charlesworth & Charlesworth, 1980; van Doorn & Kirkpatrick, 2007). For example, reproductive traits that are beneficial when expressed in males and detrimental when expressed in females might have selected for the spread of the Y-autosome fusion in the Japan Sea type. The male reproductive behaviours that map to the neo-sex chromosome in the Japan Sea type also contribute to reproductive isolation, suggesting that sex chromosome turnover and speciation may be directly linked. In this scenario, sex chromosome divergence between the gasterosteid species might result from selection, although any role for ecology will remain difficult to determine.

CONCLUSION

The above examination of variation in sticklebacks shows that speciation can be conceptualized as a continuum, as is the case in other taxa (Mallet, 2008; Hendry, 2009; Nosil *et al*., 2009). That is, different populations and species within a taxonomic group can exist all the way from continuous adaptive variation within populations to compete and irreversible reproductive barriers between species. At the same time, what might have seemed arbitrary states along this continuum might not be so arbitrary after all. That is, transitions between the states might sometimes require fundamental shifts driven by the action of some process or context not acting within a state. That is, speciation might be more of a continuum in condition than in process.

In sticklebacks, the transition from state 1 (continuous variation without reproductive isolation) to state 2 (discontinuous variation with minor reproductive isolation) seems to require not only adaptive divergence but also some degree of geographic separation (parapatry or allopatry). That is, the vast majority of lacustrine *G. aculeatus* show no signs of splitting into separate groups, even though a number of these populations experience disruptive selection and show some positive assortative mating. Couple these same processes to a bit of geographic separation, however, and the result can be discontinuous variation and some reproductive isolation, as seen in some lake and stream and mud and lava pairs. This is not to say that sympatric speciation never happens, but rather that it may require a set of conditions that are rarely if ever seen for sticklebacks.

One way to get from state 2 to 3 (strong, but reversible, reproductive isolation) appears to be a shift back to sympatry, where selection to avoid competition and interbreeding enhances adaptive divergence and reproductive barriers, as seen most clearly in the benthic and limnetic pairs. Shifts to state 3 might also be achievable without this strong secondary contact as long as divergent selection is very strong, as is apparently seen in some lake and stream pairs (Hendry & Taylor, 2004; Berner *et al*., 2009) and presumably also some allopatric populations (Foster *et al*., 1998; Vines & Schluter, 2006; Scotti & Foster, 2007; Shaw *et al*., 2007). The relative continuity of process from state 2 to 3 is also evident in the collapse of a benthic and limnetic pair into a hybrid swarm following a shift in ecological conditions (Taylor *et al*., 2006). Finally, achieving state 4 (strong and irreversible reproductive isolation) seems to require additional factors, such as chromosomal rearrangements, intrinsic genetic incompatibilities and extended periods of allopatry, all of which are less obviously linked to processes occurring along the rest of the speciation continuum.

Perhaps the greatest lesson that sticklebacks can reach is the value of plurality in the study of speciation. That is, speciation might often involve multiple and shifting geographic contexts and mechanistic drivers. This mosaic nature of speciation has also been suggested to characterize other taxa (Feder *et al*., 2005; Rundle & Nosil, 2005; Mallet, 2008; Nosil *et al*., 2009), and it might even be reasonably common in nature. The solution to Darwin's 'mystery of mysteries' might therefore be considerably more complicated that proponents of parsimony might desire. Nature is not parsimonious.

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