

# Differences in rheotactic responses contribute to divergent habitat use between parapatric lake and stream threespine stickleback

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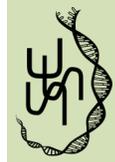
Migration among populations is widely thought to undermine adaptive divergence, assuming gene flow arises from random movement of individuals. If individuals instead differ in dispersal behavior, phenotype-dependent dispersal can reduce the effective rate of gene flow or even facilitate divergence. For example, parapatric populations of lake and stream stickleback tend to actively avoid dispersing into the adjoining habitat. However, the behavioral basis of this nonrandom dispersal was previously unknown. Here, we show that lake and stream stickleback exhibit divergent rheotactic responses (behavioral response to currents). During the breeding season, wild-caught inlet stream stickleback were better than lake fish at maintaining position in currents, faced upstream more, and spent more time in low-current areas. As a result, stream fish expended significantly less energy in currents than did lake fish. These divergent rheotactic responses likely contribute to divergent habitat use by lake and stream stickleback. Although rheotactic differences were absent in nonbreeding fish, divergent behavior of breeding-season fish may suffice for assortative mating by breeding location. The resulting reproductive isolation between lake and stream fish may explain the fine-scale evolutionary differentiation in parapatric stickleback populations.

**KEY WORDS:** Dispersal, *Gasterosteus aculeatus*, local adaptation, parapatry, rheotaxis, swimming.

Adaptive divergence is often explained as a balance between the diversifying effect of divergent selection and the homogenizing effect of random gene flow (Ehrlich and Raven 1969; Endler 1973; Slatkin 1987; Räsänen and Hendry 2008). However, there is increasing evidence that adaptive divergence can occur at surprisingly fine spatial scales compared to the movement range of individuals (Garant et al. 2004; Richardson et al. 2014). Such

fine-scale divergence might arise from exceptionally strong divergent selection over small distances, but may be most easily explained by invoking nonrandom dispersal and habitat use divergence (Garant et al. 2004; Bolnick et al. 2009).

Habitat use is a potentially important cause of reproductive isolation at fine spatial scales. The habitat use of individuals also determines the selection regime that they experience (Thorpe



1945; Jones and Probert 1980; Rice and Salt 1988). Thus, variation in habitat use may have profound evolutionary consequences. By altering both gene flow and selection, habitat use influences the maintenance of polymorphisms (Jones and Probert 1980; Garcia-Dorado 1986; De Meeus et al. 1993; Ravigné et al. 2004) adaptive divergence among populations (Holt and Barfield 2008; Ravigné et al. 2009; Bolnick and Otto 2013), and assortative mating (Maynard Smith 1966; Rice 1987; Beltman and Metz 2005; Snowberg and Bolnick 2012; Jiang et al. 2013; Taborsky et al. 2014). Note that we use habitat “use” rather than “preference” or “choice” to avoid anthropomorphizing or presupposing mechanisms.

Despite a wealth of evidence that the habitat use of individuals is often nonrandom with respect to phenotypes or genotypes (Jaenike and Holt 1991; Armsworth and Roughgarden 2005), nonrandom dispersal is still rarely incorporated into evolutionary thinking. Most empirical studies on divergent habitat use focus on the host preference of phytophagous insects (reviewed by Thompson and Pellmyr 1991; Dres and Mallet 2002; and Gripenberg et al. 2010; examples see Singer and Thomas 1996 and Via 1999). Furthermore, little is known about the proximate behavioral mechanism(s) of divergent habitat use (Nosil 2012), particularly in vertebrates (Jiggins et al. 2005).

Parapatric lake and stream populations of threespine stickleback (*Gasterosteus aculeatus*) provide an experimentally tractable example of divergent habitat use. Multiple lake/stream population pairs independently evolved divergent morphology (Hendry and Taylor 2004; Moore et al. 2007; Berner et al. 2008, 2009). This divergence is heritable and often parallel across replicate lake-stream pairs, suggesting that it is adaptive. However, gene flow (particularly from large lake populations into the smaller stream) constrains adaptive divergence (Hendry et al. 2002; Hendry and Taylor 2004; Moore and Hendry 2005; Garant et al. 2007), particularly because both natural and sexual selection against migrants are weak (Räsänen et al. 2012; Räsänen and Hendry 2014). Consequently, outlet streams (where water exits a lake) exhibit gradual phenotypic clines, often over a kilometer long (Hendry and Taylor 2004; Moore et al. 2007; Berner et al. 2008, 2009). In contrast, inlet streams (where water enters a lake) show abrupt phenotypic and genetic clines (Bolnick et al. 2009; Bolnick and Otto 2013). Strong lake-stream divergence can occur between sites just 10 m apart, without physical barriers to movement. This divergence is too abrupt to be plausibly explained by migration-selection balance alone, but could arise from divergent habitat use (Bolnick et al. 2009). A previous transplant experiment found that 90% of experimentally displaced fish returned to their original habitat, confirming that lake and stream stickleback differ in habitat use and dispersal behavior (Bolnick et al. 2009). This nonrandom movement was predicted to facilitate adaptive divergence relative

to the expectations for random movement (Bolnick et al. 2009; Bolnick and Otto 2013).

Water flow rates are an obvious difference between lake and stream environments. Thus, it is plausible that the previously demonstrated dispersal differences between lake and stream fish arise from divergent behavioral responses to water currents (“rheotactic responses”; Lyon 1904; Arnold 1974; Montgomery et al. 1997). The rheotactic response determines the direction of fish movement in currents (Montgomery et al. 1997; Pavlov et al. 2010). The rheotactic response is known to be important in the migration of salmonids (Kaya 1989; Kaya and Jeanes 1995). For example, sockeye salmon (*Oncorhynchus nerka*) juveniles usually live in lakes, but adults may spawn in inlet streams, outlet streams, or within lakes, depending on the population (Hartman et al. 1962; Hensleigh and Hendry 1998; Lohmann et al. 2008). Newly emerged fry in the inlet/outlet stream must migrate down-/upstream back to the lake, and this migration is known to be partly guided by genetically based rheotaxis (Hensleigh and Hendry 1998).

We therefore tested the hypothesis that lake and stream fish differ in rheotactic response, to evaluate a behavioral mechanism that might contribute toward the previously demonstrated lake-stream difference in habitat use (Bolnick et al. 2009). We first tested rheotactic responses of wild-caught lake and stream stickleback during their breeding season (Experiment I). Having found significant rheotactic differences, we then repeated the study on laboratory-reared, common-garden stickleback (Experiment II) to test for heritable divergence, and on wild-caught nonbreeding stickleback (Experiment III), to test for seasonal variation in rheotaxis.

## Methods

### STUDY SITE

We used unbaited minnow traps to sample threespine stickleback from four sites (see Appendix SA for geographical details): (1) the inlet stream of Blackwater Lake (“inlet stream fish”); (2) the south end of Blackwater Lake near the inlet (“inlet lake fish”); (3) the north end of Blackwater Lake near the outlet (“outlet lake fish”); and (4) the outlet stream of Blackwater Lake (“outlet stream fish”). We chose Blackwater Lake and stream because the inlet sites were the location of the previous experimental demonstration of divergent habitat use (Bolnick et al. 2009). The lake and stream habitats that fish were sampled from are the same habitats in which they spawn. Fish were collected with permits from the British Columbia Ministry of Forests, Lands and Natural Resources Operations (NA11-7031 and NA13-85103). Collection, transportation, and experimental procedures were approved by the University of Texas Institutional Animal Care and Use Committee (#AUP-2010-00059 and #AUP-2013-00027). Fish husbandry

at the Fred Hutchinson Cancer Research Center (FHCRC) was approved by the FHCRC Institutional Animal Care and Use Committee (#1575).

### CIRCULAR FLOW TANK DESIGN

We used a circular flow tank to quantify individuals' rheotactic responses (Fig. 1). This tank design allows fish to swim indefinitely into a current (upstream) or with a current (downstream). The flow tank was made of white smooth FRP plastic sheeting (tank height: 25 cm, water depth: 16 cm), equipped with two aquarium pumps that generated uni-directional circular flow (clockwise or counterclockwise, alternated across trials) with minimal turbulence. Flow was slowest at the innermost part of the tank and increased toward the outermost part of the tank (details below). Flow rates were within the natural range (0.135–0.513 m/s; see Appendix SA) observed in the Blackwater inlet stream.

### BEHAVIORAL ASSAY

Fish were tested individually in the flow tank, to avoid effects of social schooling. Each fish was tested once: it was first acclimated to the tank in still water for 15 min, then videotaped by an overhead webcam in two consecutive 5-min trials with still water or current. We randomized the order in which we assayed lake or stream fish, the direction of flow (clockwise or counterclockwise), and the order of flow treatments for each fish (still or current). Test fish were visually isolated from other fish and from investigators; observation only occurred through the webcam.

To avoid subjective bias when scoring behavior, one researcher named all videos with a random number, then a second researcher (blind to fish identity) tracked all fish movements using a single computer and constant zoom level (150%). Frames were extracted from each trial video at a rate of 3.4 frames/sec, and in each frame the anterior end (tip of the upper lip) and the posterior end (caudal peduncle) of the test individual were manually tracked using ImageJ analysis software (<http://rsb.info.nih.gov/ij/>) with MtrackJ plugin.

In each frame, the  $x$  and  $y$  coordinates of the focal fish's anterior end and posterior end were determined and then averaged to obtain the midpoint of the individual. We quantified four measures of rheotactic responses:

- (1) Net displacement: the arc distance (m) between the ending versus starting locations, including any full circuits of the tank upstream or downstream. Positive values indicate that the ending location is upstream to the starting location. Larger values of net displacement indicate more positive rheotactic responses.
- (2) Cumulative upstream movement: total upstream path length (m) that each individual swam during each 5-min trial. This differs from net displacement because the cumulative

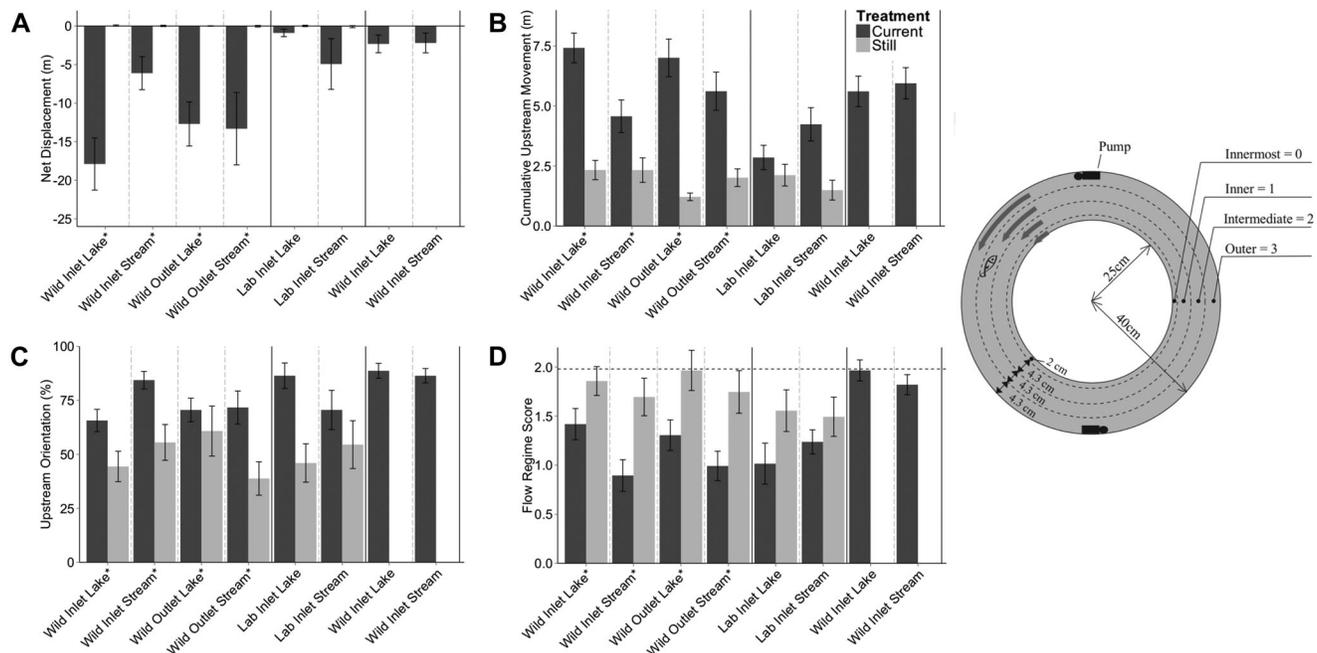
movement sums across multiple upstream swimming bursts that might not result in net displacement because of intervening downstream movements. Energy expenditure increases with cumulative movement, so fish with high cumulative movement exert substantial swimming effort. If this high effort results in little net displacement, fish exhibit poor energetic efficiency in the current.

- (3) Upstream orientation: the proportion of time each test individual faced upstream into currents ( $\pm 45^\circ$  relative to the tangent of circular flow at the midpoint of the fish). Facing upstream minimizes drag in currents. A higher proportion of time spent oriented upstream is indicative of a more positive rheotactic response. In still water, upstream orientation was (arbitrarily) calculated relative to the same tangents as in the current trial for that individual. A randomly oriented fish is expected to face upstream and downstream for equal proportions of time.
- (4) Flow regime: we defined four concentric circular zones within the test area, including a minimal flow rate innermost zone (0.01 m/s), a low flow rate inner zone (0.12 m/s), a medium flow rate middle zone (0.16 m/s), and a high flow rate outer zone (0.20 m/s; Fig. 1). In each video frame, the test individual was scored as being in the innermost, inner, middle, and outermost zone of the tank channel (scored as 0, 1, 2, 3), corresponding to increasing flow rates. These scores were averaged across all frames to obtain a single mean flow regime score per individual. Accounting for the relative surface areas of the four zones, a randomly moving fish is expected to have a flow score of 1.98, implying an inability to distinguish among, or lack of preference for, particular flow rates. Higher than expected scores imply a preference for high flow. Lower than expected scores imply active avoidance of high flow.

The total path length covered by a stickleback in current can be used to estimate its energetic expenditure. Boisclair and Tang (1993) described the energetic costs of swimming as a function of fish weight and swimming speed under different swimming patterns for various fish species. Our experiment resembled their "forced swimming" trials (fighting a constant unidirectional current). We calculated the upstream swimming speed (relative to the average current speed of the occupied tank zone) between each frame, for each fish. We then used this speed, and the focal individual's mass, to estimate (from Boisclair and Tang 1993) the transient energetic expenditure of the individual at each frame. We then calculated, for each individual, the average energetic expenditure over the 5-min trial.

### EXPERIMENT I: RHEOTACTIC RESPONSES OF WILD-CAUGHT BREEDING STICKLEBACK

We evaluated the rheotactic response of wild-caught stickleback from inlet and outlet Blackwater Lake and stream sites in June



**Figure 1.** Rheotactic responses of lake and stream stickleback from Experiments I, II, and III (breeding wild-caught; nonbreeding laboratory, and nonbreeding wild caught). Populations in breeding season are marked with asterisks. Rheotactic measures include: (A) net displacement (in meters) of all study populations in trials with flowing water (“current”; dark gray) and still-water trials (light gray). The height of each bar indicates the group mean and the error bars are the standard errors. Because all groups of individuals exhibited negative values in net displacement, a more positive rheotactic response is visualized as less net downstream displacement (shorter dark gray bars). (B) Cumulative upstream movement (in meters). (C) Upstream orientation frequency. The y-axis indicates the percentage of time an individual spent facing upstream during the 5-min trial period. (D) Use of flow regime within the circular flow tank. A random distribution of fish across flow regimes corresponds to a flow regime score of 1.98, which is indicated by a dashed line. The flow-rate regions of the circular tank are illustrated to the right of (D) in a schematic diagram showing counterclockwise currents. The tank was divided into the four labeled zones during the video quantification, as indicated by dashed concentric circles. For some metrics (e.g., A: net displacement), the treatment means for still-water treatments were very close to zero, so the light gray (still-water) bars are not visible. We omitted this still-water trial in Experiment III (nonbreeding wild inlet lake and stream fish, right side of each panel), so there are no light gray bars for the two farthest-right fish samples in each panel.

2011 (peak breeding season as in Bolnick et al. 2009). We captured 18 inlet lake fish, 18 inlet stream fish, 12 outlet lake fish, and 14 outlet stream fish. We tested the rheotactic responses of each fish 2–10 h after capture. See Appendix SA for animal care details.

We tested for significant pairwise differences among all four groups of stickleback (inlet stream, inlet lake, outlet lake, outlet stream), for each of the four rheotactic measures described above, both in still water and in currents. The data did not fit assumptions of parametric statistics, so we used Wilcoxon rank-sum tests to compare pairwise differences among the four groups of fish in currents, and pairwise differences among the four groups of fish in still water. We used Wilcoxon signed-rank tests to compare the swimming behavior of current- versus still-water trials within each group of fish. For each group of fish, we also tested whether upstream orientation and flow regime differed from null expectations (random orientation, random distribution across flow regimes; one-sample Wilcoxon signed-rank tests). Note that we combined males and females for behavioral analyses, as we found

no difference in any measures of rheotactic behavior between them (see Appendix SA for details). All analyses were done with R (Venables and Ripley 2002; R Core Team 2013; Legendre et al. 2014).

## EXPERIMENT II: RHEOTACTIC RESPONSES OF COMMON-GARDEN, NONBREEDING STICKLEBACK

To test whether the differences in rheotactic responses of lake and stream stickleback were heritable, we reared offspring of inlet lake stickleback, and offspring of inlet stream stickleback, in laboratory aquaria. We performed in vitro crosses between 14 pairs of wild-caught Blackwater inlet stream stickleback and between 34 pairs of inlet lake stickleback in early June 2010 (the same sample sites as in Experiment I). Eggs were hatched and reared to maturity at the University of Texas (see Appendix SA for details). Due to aquarium space limitations, as the fish grew we pooled families to generate one outbred population of lake fish (two fish from each of 11 surviving families) and one population

of stream fish (two fish from each of 30 surviving families). In November 2011, we sampled adult stickleback from each pooled population ( $N = 15$  each) for behavioral assays, as described above. All test individuals were naïve to water currents at the time of the behavioral assay. We tested for heritable differences between laboratory-reared inlet lake and stream fish, for each of the four behavior measures described above, both in still water and in currents, using the same statistical methods as in Experiment I. Fish were not in breeding condition (males were not brightly colored, females were not gravid) during rheotaxis trials.

### EXPERIMENT III: RHEOTACTIC RESPONSES OF WILD-CAUGHT, NONBREEDING STICKLEBACK

To assay seasonal variation in rheotaxis, we captured nonbreeding stickleback from the inlet stream and inlet lake sample sites in April 2013 before breeding began in early June. We marked stream individuals and lake individuals by clipping the tips of the first and second dorsal spine respectively, and brought them to the FHCRC for rheotactic behavioral assays (see Appendix SA for details). As described above, we tested randomly selected inlet stream and inlet lake stickleback for differences in rheotactic responses ( $N = 22$  per population). We omitted the still-water trial because prior assays had found no differences in still-water swimming behavior. We tested for differences between nonbreeding lake and stream fish in each of the four measures of rheotactic behavior in currents, using the same statistical methods as in Experiments I and II.

## Results

### EXPERIMENT I: RHEOTACTIC RESPONSES OF WILD-CAUGHT BREEDING STICKLEBACK

#### Behavioral assays

In still water, we found no differences in any measures of swimming behavior between any of the four groups of wild-caught stickleback (all  $P > 0.44$ , Fig. 1, all statistics in Tables SA1 and SA2). In addition, all four groups of fish exhibited random orientation (except for inlet stream fish,  $P = 0.048$ ) with no location preferences in still water (all  $P > 0.20$ ; Table SA2). Thus, any significant behavioral differences between lake and stream stickleback in currents represented flow-dependent differences in swimming behavior. Comparing the swimming behavior of each of the four groups in current versus still water (Table SA2), we found that in currents, all fish exhibited significantly more cumulative movement than in still water (all  $P < 0.001$ ). In currents, all four groups of fish disproportionately stayed within lower flow inner parts of the tank (all  $P < 0.003$ ) whereas still-water fish were distributed randomly across concentric zones of the tank (all  $P > 0.26$ ).

In currents, pairwise comparisons showed that wild-caught inlet stream fish and inlet lake fish differed in all four measures of

rheotactic responses (Table SA1). Stream fish were better at maintaining their positions in currents than lake fish. Both ecotypes exhibited negative (downstream) net displacement, but stream fish were displaced downstream less than their lake counterparts (averaging 6 m vs. 18 m downstream, respectively;  $P = 0.008$ , Fig. 1A). Despite maintaining their position better, stream fish actually swam significantly less distance than their lake counterparts (averaging 4.6 m vs. 7.4 m cumulative upstream movement, respectively;  $P = 0.005$ , Fig. 1B). Lake fish repeatedly swam or were forced downstream, and then compensated by swimming up-current. As a result, lake fish swam for longer total path lengths than did stream fish. There were no significant correlations between body weight and cumulative upstream movement in either inlet lake (Spearman's  $\rho = 0.001$ ,  $P = 1$ ) or inlet stream stickleback ( $\rho = 0.21$ ,  $P = 0.43$ ). Consistent with their greater swimming efficiency, stream fish faced into the current more often than lake fish (84% vs. 65% of the time;  $P = 0.007$ , Fig. 1C). Both ecotypes spent significantly more time facing upstream, compared with null expectations of random orientation (both  $P < 0.001$ ). Finally, inlet stream fish spent significantly more time in the slower current part of the tank, compared to their lake counterparts ( $P = 0.019$ , Fig. 1D). Both ecotypes disproportionately stayed within lower flow inner parts of the tank compared with null expectations (both  $P < 0.003$ ). In contrast to these striking differences between inlet stream and lake fish, outlet lake and outlet stream stickleback did not differ in any measure of rheotaxis behavior (all  $P > 0.16$ ).

#### Energetic expenditure

The dramatic difference in cumulative displacement, shown above, should lead to divergence in energetic costs of swimming in current. Using calculations from Boisclair and Tang (1993), we estimated that inlet lake fish swimming in a current incurred twice the energetic expenses of inlet stream fish (averaging 0.14 vs. 0.074 mg O<sub>2</sub> per hour, respectively; Wilcoxon rank-sum test,  $P < 0.05$ ). These estimates were comparable to prior studies of stickleback movement costs (e.g., 0.16–0.80 mg O<sub>2</sub> per hour; Dalziel et al. 2011; Grøtan et al. 2012). Although the magnitude of these expenses was a simplified estimate that ignores many other factors, the twofold difference between ecotypes makes it clear that lake fish were less-efficient swimmers in current.

### EXPERIMENT II: RHEOTACTIC RESPONSES OF COMMON-GARDEN, NONBREEDING STICKLEBACK

We did not find significant differences in rheotactic responses between laboratory-reared common garden inlet lake and stream fish, for any behavioral measure, in current or still water (all  $P > 0.16$ , Fig. 1, Table SA1). However, both ecotypes behaved differently in current versus still water (Table SA2). Compared with the results of Experiment I, both laboratory-reared lake and stream stickleback displayed more positive rheotactic responses

(less net displacement downstream and less cumulative upstream distance) compared to wild-caught breeding inlet lake fish (all  $P < 0.002$ , Fig. 1A, B). Both laboratory-reared ecotypes displayed rheotactic responses comparable to those of wild-caught breeding inlet stream fish for all four measures (all  $P > 0.05$ , Fig. 1), except that laboratory-reared lake fish displayed less net displacement downstream compared to the wild stream fish ( $P = 0.01$ ; Fig. 1A).

### EXPERIMENT III: RHEOTACTIC RESPONSES OF WILD-CAUGHT, NONBREEDING STICKLEBACK

We did not find significant differences in rheotactic responses between wild-caught inlet lake and stream fish prior to the breeding season, for any behavioral measure in currents (all  $P > 0.34$ , Fig. 1, Table SA1). In fact, the rheotactic responses of both groups were comparable to those of breeding inlet stream fish in Experiment I for all measures (all  $P > 0.05$ ; except for flow regime,  $P < 0.001$ ), but were significantly different from breeding inlet lake fish for all measures (all  $P < 0.04$ ). Thus, the rheotactic difference that we observed between lake and stream fish during the breeding season apparently resulted from a reduction of rheotaxis by breeding lake fish, relative to breeding stream fish and nonbreeding lake and stream fish.

## Discussion

Previous studies have shown that parapatric lake and stream stickleback have divergent dispersal behaviors that can facilitate adaptive divergence (Bolnick et al. 2009). Here, we tested for population divergence in rheotaxis response, one of several possible behavioral mechanisms that could generate divergent dispersal behaviors and thus divergent habitat use. We found strong differences in rheotactic responses between wild-caught breeding inlet ecotypes. These behavioral differences would facilitate the upstream movements of stream fish and downstream movements of lake fish, returning them to their respective habitats. These results are consistent with the previous experiment showing that displaced stream and lake fish disproportionately return to their respective habitats during the breeding season (Bolnick et al. 2009). Rheotaxis differences can thus reduce the effective rate of dispersal between these adjoining habitats and thereby promote adaptive divergence, regardless of whether the rheotactic behaviors are heritable.

During the breeding season, wild-caught inlet lake and stream fish exhibited distinctive rheotactic behaviors in currents. Inlet stream fish used slow-current areas more than their lake counterparts did. This pattern suggests that either stream fish more strongly prefer slow flow areas, or are better at maneuvering into narrow boundary layers with minimal flow. This microflow preference may reflect either biomechanical adaptations for

locomotion in variable flow regimes, or more prior experience seeking low-flow microhabitats. Inlet stream fish also more often faced toward currents and were more efficient at staying in place (less net displacement, with less effort) compared to their lake counterparts. These behavioral differences, regardless of their mechanistic basis, would tend to reduce migration between parapatric lake and stream populations. Any lake fish that immigrated into the stream would likely be displaced down-current back into the lake. Conversely, stream fish that entered the lake would be more likely to move back up-current into the stream. Any lake fish that did immigrate into and settle in the stream would probably be selected against due to their twofold higher energy expenditure when swimming in a current, compared to stream fish (McCormick et al. 1998; Mohammed et al. 2012). Importantly, the differences documented here are specific to performance in flowing water; lake and stream fish did not differ in swimming performance in still water.

In the outlet, roles must be reversed to reduce migration between lake and outlet stream fish; lake fish must swim upstream to return to the lake and stream fish must move downstream to re-enter the stream (but not so far as to be swept into the next lake). During the breeding season, outlet ecotypes did not differ in any measures of rheotactic response, although the presence of current triggered significant rheotactic responses in all four measures in both ecotypes. Outlet lake fish faced upstream for significantly longer periods of time in currents compared with null expectations. The lack of divergent rheotactic response is consistent with smaller differences in flow rates between the lake the outlet stream than between the lake and the inlet stream, as well as less morphological and genetic divergence between lake fish and outlet stream fish in other systems (Hendry et al. 2002; Moore et al. 2007).

Naïve common-garden inlet lake and stream fish that were never exposed to current did not differ in any measures of rheotactic response in current or still water. There are three possible explanations for the lack of differences. First, divergent rheotactic response may not be heritable. Second, all laboratory-reared test individuals were raised in still water with no prior exposure to currents, and the full development of rheotactic behavior may require prior exposure to currents, especially in inlet stream fish. Third, it is possible that divergent rheotaxis is expressed only during the breeding season, whereas our heritability assay used nonbreeding laboratory-reared fish. We cannot rule out any of these at present, but we find some additional support for seasonal rheotaxis. Specifically, we found no differences in rheotaxis behavior between the ecotypes in nonbreeding wild fish (Experiment III), suggesting that the differences found in Experiment 1 may be particular to stickleback during the breeding season.

Little is known about seasonal variation in rheotactic responses, although Schmitz (1992) examined the annual variation

in rheotaxis in a population of Arctic char (*Salvelinus alpinus*) that has been landlocked for about 6000 years. This population showed seasonal changes in rheotaxis (including rheotaxis reversal) which were directionally consistent with smolting, and even coupled with physiological changes in seawater adaptability (Schmitz 1992). Similarly, freshwater threespine stickleback populations are derived from marine ancestors, including anadromous populations that seasonally swim upstream into rivers from the ocean to breed in freshwater (McPhail 1994; Taylor and McPhail 2000; McKinnon and Rundle 2002; Hendry et al. 2009). We speculate that stickleback may exhibit elevated rheotactic behavior during breeding, a trait inherited from marine ancestors that may have subsequently been an exaptation that facilitated lake/stream divergence.

In conclusion, we identified an important behavioral mechanism (rheotaxis) that differs between populations, and therefore may contribute to biased dispersal and divergent habitat use between parapatric populations. Divergent rheotaxis specifically in breeding season may exaggerate its effect on assortative mating due to spatial segregation of the ecotypes, thereby reducing the effective rate of gene flow between the parapatric populations. If divergent rheotactic behavior were heritable, the resulting divergent habitat use would contribute to genetic divergence in rheotactic behavior, and associated neurological and mechanical traits, between the ecotypes. However, even if divergent rheotactic behavior was entirely plastic or learned, divergent habitat use would still reduce gene flow overall, thereby facilitating adaptive divergence for other heritable traits. Thus, evolutionary differentiation at a fine spatial scale with respect to the movement range of individuals can occur via variation in behavior that result in biased gene flow, and the prerequisite of strong divergent selection regime is circumvented (Bolnick and Otto 2013). Given the increasing evidence that gene flow is not random (Edelaar and Bolnick 2012), the role of gene flow in adaptive divergence and speciation may be far more complex than previously thought. This study helps to identify a proximate mechanism likely to underlie an example of nonrandom gene flow among diverging populations. Future studies on how multiple mechanisms (i.e., behavioral, neurological, morphological, physiological) interact and jointly influence gene flow may deepen our understanding of adaptive divergence in the presence of gene flow at fine spatial scales.

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#### DATA ARCHIVING

Data necessary to replicate results of the study are archived at Dryad, doi:10.5061/dryad.k3836.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

### Appendix SA. Study site.

**Table SA1.** Wilcoxon rank-sum test statistics and *P* values (in brackets) of pairwise comparisons between populations for four measures of rheotactic response (net displacement, cumulative upstream movement, upstream orientation, and flow regime), both in current trials and in still-water trials.

**Table SA2.** Within each population, test statistics and *P* values (in brackets) for comparing each of the four measures of rheotactic response (net displacement, cumulative upstream movement, upstream orientation, and flow regime) between current versus still water trials or relative to a random expectation.