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Are populations of mayflies living in adjacent fish and fishless streams genetically differentiated?

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SUMMARY

1. Conspecific populations living in habitats with different risks of predation often show phenotypic variation in defensive traits. Traits of two species of mayflies (Baetidae: *Baetis bicaudatus* and *Baetis* sp. nov.) differ between populations living in fish and fishless streams in a high altitude drainage basin in western Colorado, U.S.A. We tested for genetic differentiation between mayfly populations in these two habitat types, assuming that lack of genetic differentiation would be consistent with the hypothesis that those traits are phenotypically plastic.

2. Previous work has shown that larvae of both species behave differently and undergo different developmental pathways in adjacent fish and fishless streams. These phenotypic differences in behaviour and development have been induced experimentally, suggesting that populations from fishless streams have the genetic capability to respond to fish.

3. During summer 2001 we collected *Baetis* larvae from several fish and fishless streams, and from fish and fishless sections of the same streams. We used allozymes and a fragment of the cytochrome oxidase subunit 1 mitochondrial gene to examine genetic variation of *Baetis* individuals within and among streams.

4. Results showed that genetic variation exists among populations of the same species of *Baetis* from different streams, but none of that variation was associated with the presence or absence of fish. These data confirm that populations of *Baetis* living in fish and fishless streams are not genetically distinct, and are consistent with the hypothesis that traits associated with environments of different risk are phenotypically plastic.

Keywords: allozymes, fish and fishless streams, genetic differentiation, mayflies, mitochondrial DNA

Introduction

Conspecific populations living in habitats with different risks of predation often show phenotypic variation in defensive traits (Flecker, 1992; McIntosh & Townsend, 1994; Ball & Baker, 1996; Dahl & Peckarsky, 2002). Natural selection for morphology, development or behaviours that reduces the risk of predation may result in fixed traits adapted to risky environments (Hairston & Walton, 1986; Reznick, Bryga & Endler, 1990; Wellborn, 1994). Alternatively, phenotypic variation may result from plastic responses of individuals genetically capable of responding to changes in predation risk (Crowl & Covich, 1990; Arnqvist & Johansson, 1998; DeWitt, 1998; Nijhout, 1999). Such phenotypic plasticity should be favoured when predation risk is variable but can be anticipated (DeWitt, Sih & Wilson, 1998). Thus, individuals that can change their behaviour, morphology or development in response to predation risk should have a fitness

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advantage in variable environments (Bradshaw, 1973; Newman, 1992).

Mayfly larvae of the genus *Baetis* (Baetidae) are highly mobile (Peckarsky, 1996), and often caught in drift nets (McIntosh, Peckarsky & Taylor, 2002). Thus, these mayflies are highly vulnerable to predation by drift-feeding fishes, such as salmonids (Elliott, 1973; Allan, 1981). Nonetheless, they can achieve high densities in trout streams (Allan, 1982) and, surprisingly, are sometimes more abundant in trout streams than in adjacent fishless tributaries (Peckarsky *et al*., 2001). Adult males form mating aggregations, often swarming at locations distant from streams (Peckarsky *et al*., 2002a). Females flying near these swarms mate with males on vegetation, and then fly predominantly upstream to oviposit (Flecker & Allan, 1988; Peckarsky, Taylor & Caudill, 2000). Thus, there is potential for extensive dispersal of adults of these mayflies, as corroborated by genetic analyses (Hughes *et al*., 2003).

Recent genetic analyses have also shown that there are two common species of *Baetis* that coexist in fish and fishless streams in the high altitude upper East River drainage basin in western Colorado near the Rocky Mountain Biological Laboratory (RMBL) (J. M. Hughes & M. Hillyer, unpublished data). The most abundant species *Baetis bicaudatus* Dodds, emerges in June and early July, lays eggs that hatch without delay, and overwinters as first instar larvae. Larval development begins in April or May and is completed as the winter snowmelt recedes. All larval stages of this species have two cerci and no middle caudal filament. A second species of *Baetis* emerges in August and September, lays eggs that overwinter and hatch in mid-summer the next year. Larvae undergo rapid development during late summer base-flow. Early instar larvae and adults of this species are hard to distinguish from *B. bicaudatus*, but later instar larvae develop a 2–20-segment middle caudal filament. Preliminary genetic analyses suggest that this is an undescribed cryptic species of *Baetis* (*Baetis* sp. nov., S. L. Ball, unpublished data). In previous publications these two species were called 'winter' (*bicaudatus*) and 'summer' (sp. nov.) *Baetis* (e.g. Peckarsky *et al*., 2000, 2001).

Differences in the phenology of these two species affect their relative vulnerability to predation by brook trout (*Salvelinus fontinalis* Mitchell). Most *B. bicaudatus* complete development during peak flow before water clears and predation pressure from trout becomes intense, although late-developing individuals are more vulnerable. In contrast, larvae of *Baetis* sp. nov. hatch into a hazardous environment when water level is low and trout are actively feeding (Peckarsky *et al*., 2001). Thus, they are vulnerable to trout predation throughout their larval development, but more so as they get larger (Allan, 1978).

Different populations of both *Baetis* species living in adjacent fish and fishless streams (<100 m apart) in the same catchment have significantly different phenotypes. Although foraging and drift behaviour are aperiodic in fishless streams, both species of mayflies are highly nocturnal in trout streams (McIntosh *et al*., 2002), possibly reducing the risk of predation while feeding and dispersing. We have induced nocturnal behaviour in *Baetis* individuals of both species collected from fishless streams by exposing them to chemical cues from brook trout in microcosms (Cowan & Peckarsky, 1994), mesocosms (McIntosh & Peckarsky, 1996), and whole streams (McIntosh, Peckarsky & Taylor, 2004).

Both species have an indeterminate number of instars, and developmental trajectories differ between of populations in fish and fishless streams (Peckarsky *et al*., 2001). Size and fecundity of mature *B. bicaudatus* decline in trout streams over one emergence period, but not in fishless streams, while size of emerging *Baetis* sp. nov. does not vary with date of emergence in either stream type. However, *Baetis* sp. nov. emerging from trout streams are significantly smaller than those emerging from fishless streams, and mean size is similar to the smallest *B. bicaudatus*. Observed size variation was associated with accelerated development, which reduced time of exposure and size-related vulnerability to predators in the more dangerous environment. We have also induced these developmental shifts in fishless *Baetis* of both species by exposing them to chemical cues from brook trout in mesocosms (Peckarsky & McIntosh, 1998) and whole streams (Peckarsky *et al*., 2002b).

Although previous induction experiments suggest that the traits of *Baetis* associated with fish and fishless streams are phenotypically plastic, we do not know whether populations living in adjacent fish and fishless streams are genetically differentiated. The goal of this study was to conduct genetic analyses of populations of *Baetis* of both species living in fish and fishless streams to test the null hypothesis that there

was no systematic genetic variation associated with variation in risk of predation among habitats.

Methods

In this study, we used allozyme and mitochondrial DNA variation to compare the genetic structure among populations of *Baetis* larvae and between populations from fish and fishless streams. Our sampling design enabled us to determine whether there was significant genetic variation among populations of *Baetis* from different locations, and whether that variation could be attributed to the presence or absence of fish. We estimated allozyme variation in both *B. bicaudatus* and *Baetis* sp. nov., and we took samples of *B. bicaudatus* sufficient to examine variation in a fragment of the mitochondrial cytochrome oxidase I gene.

During July and August 2001 we collected individuals of both species from 28 locations in fish and fishless streams flowing through the Upper East River Drainage Basin on the east side of Gothic Mountain near the RMBL and the Slate River Drainage Basin on the west side of Gothic Mountain, Colorado, U.S.A. (Fig. 1). Brook trout were the only species of fish present in some of the sites sampled, and other sites were completely fishless because of barriers to dispersal (e.g. waterfalls). While these species coexist in streams throughout the area, we obtained sufficient samples to compare genetic structure of *B. bicaudatus* populations from nine fish and nine fishless sites using allozymes and mtDNA. For *Baetis* sp. nov. we were able to compare genetic structure between 11 fish sites and six fishless sites using allozymes only.

We collected *Baetis* larvae from rocky riffles at all sites using a D-net, and sorted samples on site, placing *Baetis* in plastic bags on ice until they could be frozen in liquid nitrogen. We transferred the frozen specimens from liquid nitrogen to dry ice for transport to Australia, where we placed them in a −80° freezer until genetic analysis.

Allozyme analysis

We used cellulose acetate electrophoresis (Helena Laboratories, Titan III plates; see Schmidt, Hughes & Bunn, 1995 for detailed methods) and stains modified from Richardson, Baverstock & Adams (1986). We chose five of 25 enzymes that were initially screened for variation and could be reliably interpreted. Four of the enzymes were encoded by single loci (Glucose phosphate isomerase: PGI, IECC no.5.3.1.9, phosphglucomutase: PGM, IECC no.2.7.5.1, peptidase: PEPB and PEPC, IECC no. 3.4.11). The fifth, amino aspartate transferase (AAT, IECC no. 2.6.1.1), had two loci that were both variable. Thus, we determined allele frequencies at six different loci for both species at each site (Hughes *et al.*, 2003).

Mitochondrial DNA analysis

We modified several Chelex protocols to extract total genomic DNA from each *B. bicaudatus* individual (Singer-Sam, Tanguay & Riggs, 1989; Walsh, Metzger & Higuchi, 1991; Sweet *et al.*, 1996). We used the residual tissue from allozyme analysis so that we could obtain both types of data from the same individuals. We transferred this tissue to clean 1.5 mL Eppendorf tubes with 500 μL of 5% chelex solution (Bio-Rad, Hercules, CA, U.S.A.) and then ground them with a plastic micro-pestle. We added 7 μL of proteinase K (20 mg mL^{-1}) to each sample and then vortexed and incubated them overnight at 54 °C. The next day, samples were heated at 96 °C for 20 min, centrifuged at 16 000 *g* for 2 min at room temperature, and then subjected to polymerase chain reaction (PCR). We amplified approximately 710 bp fragment of the cytochrome oxidase subunit 1 gene (COI) using the primers LCO1490 and HCO2198 (Folmer *et al.*, 1994). We cleaned the DNA according to manufacturer's instructions and sequenced DNA fragments using an Applied Biosystems 377 automated sequencer (Foster City, CA, U.S.A.) (See Hughes *et al.*, 2003 for details of PCR.) Sample sizes for both species are given in Fig. 2.

Statistical analysis

Sequences were aligned in Bioedit (Hall, 1999). We used analysis of molecular variance (AMOVA in Arlequin, Schneider *et al.*, 1997) with 'fish status' (fish or fishless streams) and 'among streams within fish status' as levels in the hierarchy. For allozymes, *F*-statistics were calculated for each locus separately and for all loci combined. We also used allozyme data to conduct a cluster analysis using the unweighted pair group method in BIOSYS-1.7. We used this analysis to group samples from fish and fishless sites

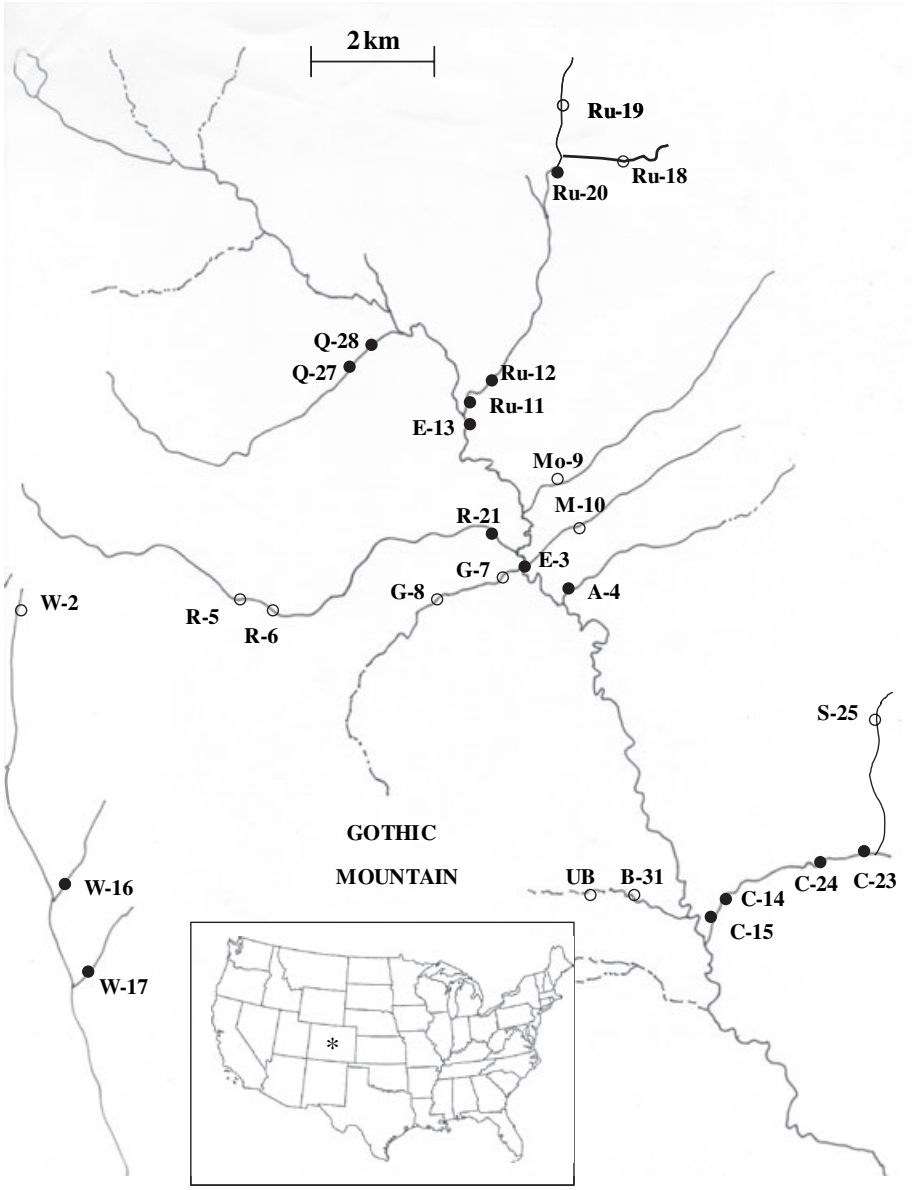

Fig. 1 Map of collection sites. Open symbols are fishless sites. Closed symbols are sites with brook trout. W, Washington Gulch Ck; B, Benthette Br; UB, Upper Benthette Br; C, Copper Ck; S, Sylvanite Ck; A, Avery Ck; G, Gothic Ck; E, East River; M, Marmot Ck; Mo, Mosca Ck; R, Rock Cr; Ru, Rustlers Gulch Ck; Q, Quigley Ck. Inset: Map of the U.S.A. indicating approximate location of study sites (*).

according to Nei's (1978) index of unbiased genetic similarity. For mtDNA data, we calculated *F*-statistics using haplotype frequencies alone and with both frequency and pair-wise sequence divergence included in the analysis. A significant F_{CT} value would indicate significant genetic differentiation between *Baetis* populations collected from fish and fishless streams. A significant F_{SC} and a non-significant F_{CT} would indicate more genetic differentiation among populations within 'fish' streams and within 'fishless' streams than between 'fish' and 'fishless' streams.

Results

Both the analyses of allozymes and mitochondrial DNA indicated that there was overall significant genetic variation among sites, but none of this variation in either species was accounted for by the fish/fishless

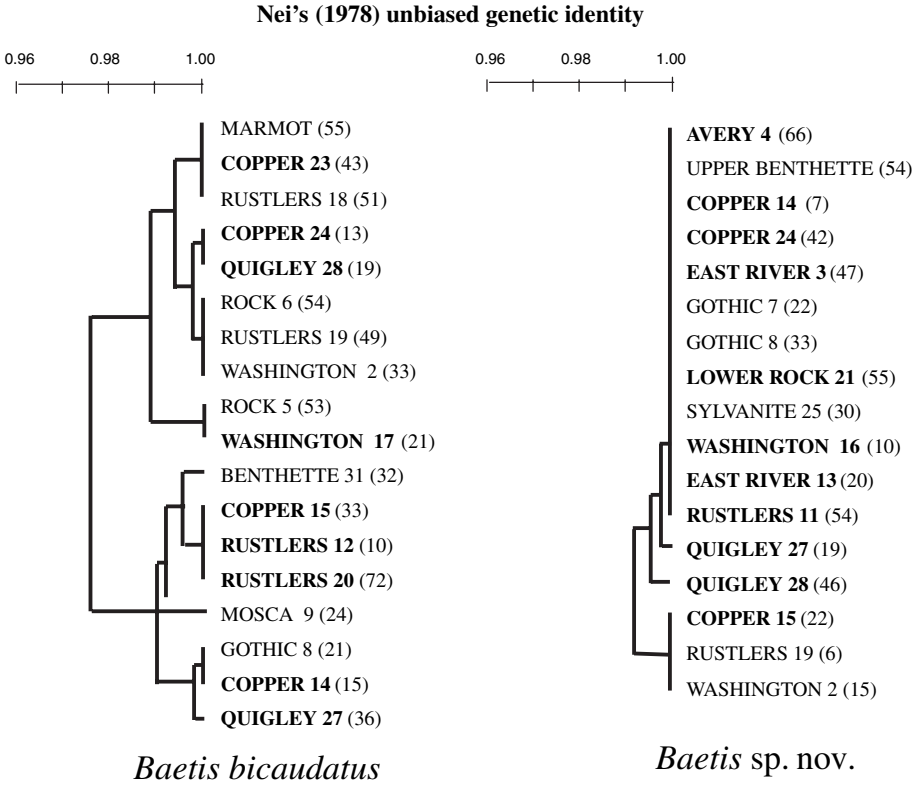

Fig. 2 Cluster analysis using an unweighted pair group method with sites arrayed according to Nei's (1978) genetic similarity for *B. bicaudatus* and *Baetis* sp. nov. Sites given in bold face type had brook trout, and others were fishless. Sample sizes for each site are given in parentheses.

status of streams. For *B. bicaudatus* the F_{ST} values for allozyme variation at all loci combined were small, but significant among sites and among sites within a category (F_{SC}) (Table 1). F_{CT} values were essentially zero between fish and fishless sites (Table 1), indicating that none of the genetic variation was between fish and fishless streams. For *Baetis* sp. nov. F_{ST} values for allozyme variation at all loci combined were even smaller, with no significant variation among sites, among sites within a category (F_{SC}), or between fish and fishless sites (F_{CT}) (Table 2). However, for some loci there was significant allozyme variation among sites (AAT-1, AAT-2, PGI), among sites within a category (AAT-2, PGI), and for one locus (PGI) between fish and fishless streams. Nonetheless, all F_{CT} values were very small, at least an order of magnitude less than F_{SC} values (Table 2), and when combined across loci F_{CT} all values were non-significant.

Table 1 Results of hierarchical analysis (Arlequin) for genetic variation among sites (F_{ST}), among sites within fish status (F_{SC}), and between fish and fishless sites (F_{CT}) for *B. bicaudatus*

	Locus						
Comparison	*AAT-1*	*AAT-2*	*PGI*	*PGM*	*PEP-B*	*PEP-C*	Average across loci
Fish status/total (F_{CT})	0.001	0.001	0.004	0.001	0.002	−0.005	0.002
Sites/fish status (F_{SC})	0.007*	0.012*	0.007*	0.005	0.065***	0.023*	0.035***
Sites/total (F_{ST})	0.008	0.013*	0.011*	0.067	0.067***	0.019**	0.036***

***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

Significant F_{SC} and non-significant F_{CT} values indicate more genetic differentiation among populations within fish streams and within fishless streams than between fish and fishless streams.

Table 2 Results of hierarchical analysis (Arlequin) for genetic variation among sites (F_{ST}), among sites within fish status (F_{SC}) and between fish and fishless sites (F_{CT}) for *Baetis* sp. nov

	Locus					
Comparison	*AAT-1*	*AAT-2*	*PGI*	*PGM*	*PEP-C*	Average across loci
Fish status/total (F_{CT})	−0.00168	−0.01497	0.00752*	−0.00013	−0.00097	0.00061
Sites/fish status (F_{SC})	0.02320	0.08809***	0.00852*	−0.00271	−0.00156	0.00614
Sites/total (F_{ST})	0.02156*	0.07443***	0.01598*	−0.00284	−0.00254	0.00675

***$P < 0.001$, *$P < 0.05$.
Averaged across all loci, non-significant F_{CT} and F_{SC} values indicate no genetic differentiation among sites, within fish or fishless streams, or between fish and fishless streams.

Table 3 Results of AMOVA based on 520 bp of mtDNA COI sequence data for *Baetis bicaudatus*, showing variation between fish and fishless streams, among populations within fish and fishless streams and among sites

	Phi_{CT}	F_{CT}
Fish/fishless	−0.0042	−0.0111
Sites within fish status	0.2061***	0.1460***
Sites within total	0.2027***	0.1365***

***$P < 0.001$. Results agree with allozyme analyses that genetic variation occurs among populations in different sites, but not between fish and fishless sites.

Similarly, the AMOVA for variation in the COI fragment for *B. bicaudatus* indicates highly significant differences among all sites and among sites within each of the fish status categories, but no significant difference between fish and fishless streams (Table 3). Both the phi_{CT} and the F_{CT} values were small and non-significant.

The cluster analysis using allozyme variation illustrates that overall genetic similarity among populations of both species was high, but populations of *Baetis* sp. nov. were more similar than *B. bicaudatus* populations (Fig. 2). Individuals from sites with and without fish were found in the same clusters and, as expected, the pattern of genetic similarity among sites was not related to the presence of fish.

Discussion

Analysis of genetic variation shows that populations of two species of *Baetis* in adjacent fish and fishless streams are not genetically differentiated. In contrast, others have observed clear genetic differences between populations of amphipods in adjacent fish and fishless ponds (McPeek & Wellborn, 1998). Nonetheless, we expected no genetic differentiation among fish and fishless *Baetis* populations, because traits associated with fish streams (nocturnal behaviour and accelerated development) could be induced in *Baetis* from fishless streams. Individuals of both *Baetis* species collected from fishless streams rapidly and consistently respond to trout chemical cues (Cowan & Peckarsky, 1994; McIntosh & Peckarsky, 1996; Peckarsky & McIntosh, 1998; McIntosh, Peckarsky & Taylor, 1999; Peckarsky *et al.*, 2002b). Although responses depended on the concentration of fish cues, they were not graded, but apparently 'switched on' at some threshold level of chemical cues. Furthermore, responses did not depend on fish density in natural streams (McIntosh *et al.*, 2002). These results suggest that individuals living in both fish and fishless streams are genetically programmed to respond phenotypically to the presence of fish.

Theoretical and empirical studies have shown that genetic adaptations need not be large-scale and thus, adjacent populations can differentiate despite the existence of gene flow (Antonovics, 1971; Endler, 1973; Nurnberger & Harrison, 1995; McPeek & Wellborn, 1998). Thus, local adaptation may uncouple dispersal from gene flow resulting in selection against colonisation by immigrants with less adaptive phenotypes (Bohonak & Jenkins, 2003). However, high levels of gene flow and low natural selection can prevent local adaptation (Antonovics, 1971; Slatkin, 1987), sometimes leading to ineffective antipredator behaviour caused by constraints of conflicting selection pressures (Storfer *et al.*, 1999). Alternatively, natural selection may result in the evolution of phenotypic plasticity if the risk of predation is variable but can be assessed (Gotthard & Nylin, 1995; DeWitt *et al.*, 1998). Furthermore, fixed risk-sensitive foraging behaviour and development could

be selected against if such traits have associated fitness costs, such as reduced fecundity.

Our genetic analyses showed very little genetic differentiation among populations of *Baetis* sp. nov. suggesting that populations of this species form a single, large panmictic population rather than a series of demographically-isolated populations. While this and a previous study (Hughes *et al*., 2003) showed significant structure and suggested restricted gene flow in *B. bicaudatus*, local differentiation sometimes exceeded genetic variation in more distant streams, and absolute genetic distances were small compared with other stream insects (Jackson & Resh, 1992; Hughes *et al*., 1999; Ketmaier *et al*., 2001; Kelley, Rundle & Bilton, 2002). However, small genetic differences among populations of these two *Baetis* species were typical of other mayflies (Sweeney, Funk & Vannote, 1986; Schmidt *et al*., 1995; Gibbs *et al*., 1998; Hughes *et al*., 2000; Smith & Collier, 2001). Interestingly, comparison of variation in allozymes to mitochondrial genes suggested that *B. bicaudatus* females may not disperse as far as males (Hughes *et al*., 2003). However, this pattern of sex-biased dispersal would still result in panmixia for nuclear genes.

Present and historic dispersal of ovipositing females (Peckarsky *et al*., 2000) should facilitate genetic mixing of populations derived from fish and fishless streams, countering fish-mediated selection on behaviour or development (Slatkin, 1987; Bohonak, 1999; Monaghan *et al*., 2002). The tendency of female *Baetis* to fly upstream to oviposit (Flecker & Allan, 1988; Peckarsky *et al*., 2000) will introduce new individuals into fishless streams from parents that emerged from fish streams. If highly-mobile *Baetis* larvae drift from fishless streams into fish streams, then selection pressure to adapt to a fishless environment could be low despite higher individual fitness of *Baetis* females emerging from fishless streams (larger and more fecund – Peckarsky *et al*., 2001). Consequently, extensive dispersal should facilitate genetic mixing of populations derived from fish and fishless streams, thereby preventing the evolution of fixed behaviour or developmental trajectories adapted to a single stream type.

Interestingly, we have never been able to induce risky behaviour or slowed development in larvae from trout streams by placing them in environments free of fish chemical cues (Cowan & Peckarsky, 1994; McIntosh & Peckarsky, 1996). Given that fishless streams are located upstream from fish streams, larvae are unlikely to drift from a fish stream to a fishless stream. Thus, we suspect that once fish cues are detected, the risk-sensitive behaviour observed in trout streams becomes fixed. Alternatively, switching developmental pathways during ontogeny probably involves hormonal and metabolic changes that may not be reversible (Nijhout, 1999).

Although both species of *Baetis* showed flexible patterns of behaviour and development adapting to increased risk of predation, the details of inducible traits differed between species, possibly because they develop under different environmental conditions. Larvae of *B. bicaudatus* begin growth and development during snowmelt, when water level is high and stream temperature and predator activity are low. As water levels recede, increasing water temperature and clarity are accompanied by intensified feeding by salmonids (Dunbrack & Dill, 1984; Metcalfe, Valdimarsson, & Fraser, 1997). We argue that seasonally increasing mortality risk should favour *B. bicaudatus* individuals that can adjust development to reduce the time of exposure to predation, despite incurring a loss of fecundity (Peckarsky *et al*., 2001).

In contrast, *Baetis* sp. nov. larvae complete their development entirely during a time of low flow and higher vulnerability to trout predation. Males and females of this species mature at invariant, but smaller and less vulnerable body sizes (Allan, 1978) in fish than fishless streams (Peckarsky *et al*., 2001). Thus, we suspect that selection pressure to avoid trout predation favours developmental flexibility enabling individuals of this *Baetis* species to mature at some minimum size in environments where predation risk is the highest. In safer (fishless) habitats larvae develop for longer periods thereby attaining higher fecundities.

We conclude that populations of two species of mayflies with highly mobile larvae and adult females that disperse by flying upstream to oviposit are not genetically differentiated between fish and fishless streams. Individuals from both environments are capable of responding to chemical cues associated with actively feeding trout, which trigger nocturnal feeding behaviour and accelerated development. This phenotypic plasticity enables larvae to adjust their behaviour and life history thus completing development quickly in risky environments and maximising survival, and extending the period of growth and thereby increasing fecundity in safer environments.

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