



## Origin and specificity of predatory fish cues detected by *Baetis* larvae (Ephemeroptera; Insecta)



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Some prey respond to predator cues by altering their behaviour, but the response may depend on predator diet and identity. There are few data on how predator feeding status and diet affect the behaviour of aquatic insects and on the specificity of the response of these prey to different predators. We present the results of four bioassays conducted in microcosms to evaluate the relationship between predator fish diet, predator identity and the predator avoidance behaviour of mayfly prey of the genus *Baetis* to elucidate the origin and specificity of the antipredator response. Results from the first bioassay indicated that the response of *Baetis* to predatory trout is independent of diet, which enabled us to rule out the following potential origins of the signal: alarm cues emitted by wounded *Baetis* conspecifics, salivary enzymes released by feeding fish and excretion products that may contain *Baetis* and conspecific odours. Thus, we focused further experiments on fish cutaneous mucus as the potential origin of cues that cause *Baetis* to reduce its activity. In two bioassays, we observed that North American *Baetis bicaudatus* did not distinguish between five freshwater fish species that co-inhabit the same drainage, despite differences in their relative risk of predation (four predatory salmonid species and one omnivorous sucker species). However, in another bioassay, we observed that European *Baetis rhodani* larvae did not respond to cutaneous mucus of novel predators (carp, seawater turbot or frog), indicating that *Baetis* do not show a general response to all mucus donor organisms. Our bioassays identified mucus as the potential origin of the cue eliciting antipredator behaviour in *Baetis*, providing much needed insight into the specificity of fish-associated chemical cues that cause some prey to respond. Experimental approaches similar to the one used in this study may increase our understanding of the role of chemical cues in aquatic ecosystems.

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Predators can affect prey populations and communities by increasing their mortality or by inducing changes in their behaviour, life history and morphology (Lima, 1998; Peckarsky, Kerans, Taylor, & McIntosh, 2008). In both scenarios, the strong selection pressure that predators impose on prey results in the evolution of a variety of behavioural responses that increase prey survival. However, predator avoidance behaviours are often costly to prey in terms of reduced foraging time, shifts to unfavourable food patches or changes in feeding periodicity (Alvarez & Peckarsky, 2005; Diehl et al., 2000; Peckarsky, 1996). Therefore, the ability of prey to assess predation threat accurately is crucial to minimizing costs of predator avoidance, and thus to balance predator evasion with other individual traits essential for foraging or reproductive success

(Åbjörnsson, Wagner, Axelsson, Bjerselius, & Olsén, 1997; Ferrari, Wisenden, & Chivers, 2010). Consequently, to maximize their fitness, prey must be able to identify predator cues and to respond adaptively to predation threats by assessing the level of risk associated with each threat posed by their predators (threat-sensitive predator avoidance hypothesis: Brown, Rive, Ferrari, & Chivers, 2006; Helfman, 1989; Ferrari, Trowell, Brown, & Chivers, 2005).

Predatory encounters escalate along a series of steps beginning with initial detection, attack, capture and prey ingestion by the predator (Brönmark & Hansson, 2000; Lima & Dill, 1990; Wisenden, 2000). Prey may respond to the presence of predators at any step in this predation sequence through perceived threat. For example, while some animals respond to visual cues and use predator appearance to distinguish between predatory and non-predatory species, predator-derived chemical cues released before or at the detection stage of the predation sequence provide prey the opportunity to reduce the probability of a predator encounter or to

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avoid attacks after detection (Brönmark & Hansson, 2000; Ferrari, Messier, & Chivers, 2008; Wisenden, 2000). Therefore, prey organisms may assess predation threat by detecting species-specific scents emitted by the predator itself (also known as 'predator odours' or 'kaironomes'; Brönmark & Hansson, 2000). Other prey organisms may assess predation risk by detecting chemical alarm cues released by nearby conspecifics or sympatric species whose tissues have been abraded during predator attack and capture (Chivers & Smith, 1998; Wisenden, 2000; Wisenden, Chivers, & Smith, 1997). In addition, chemical cues may be present in the excretion products of predators, and those cues may be related to predator diet (Chivers, Kiesecker, Anderson, Wildy, & Blaustein, 1996; Huryn & Chivers, 1999).

While the chemical ecology pertaining to predator–prey relationships in freshwater ecosystems has been well studied, there is ongoing debate about the origin of cues emanating from predatory fish that induce invertebrate prey to respond. Some studies have suggested that these cues originate from chemical compounds of the predator's diet (Brown, 2003; Huryn & Chivers, 1999), the cutaneous mucus of predatory fish (Forward & Rittschof, 1999; Mathuru et al., 2012; Rahman, Forward, & Rittschof, 2000), or certain bacterial populations associated with the cutaneous mucus or gut of predatory fish (Beklioglu, Telli, & Gozen, 2006; Ringelberg & Van Gool, 1998; Stabell, Ogebebo, & Primicerio, 2003). However, whether diet, the fish itself, or the fish mucus or associated bacteria are responsible for the production of predator cues has not been conclusively established for the zooplankton–fish system or for other bitrophic interactions involving fish predators (Beklioglu et al., 2006; Rahman et al., 2000; Van Donk, 2007). In fact, although several studies on stream-dwelling insects consider evolutionary aspects of chemical communication based on cost–benefit analyses from observed behaviours (e.g. Dodson et al., 1994; Martinez, 1986), there are relatively few studies on the origin of the chemical cue. Previous studies conducted in high-altitude headwater streams of western Colorado, U.S.A., have shown that mayfly larvae of the genus *Baetis* (Baetidae), which are highly mobile grazers (Alvarez & Peckarsky, 2005; McIntosh, Peckarsky, & Taylor, 2002), are also highly vulnerable to predation by salmonid fish (Peckarsky et al., 2008) and change their grazing behaviour most dramatically in the presence of salmonid fish (Alvarez & Peckarsky, 2005; Peckarsky, 1996), affecting foraging periodicity activity and becoming more nocturnal under predation risk (McIntosh & Peckarsky, 1996; McIntosh and Townsend, 1996). Moreover, *Baetis* has evolved numerous behaviours and life history strategies to reduce consumption by predatory fish, resulting in nonconsumptive effects on prey fitness (McPeck & Peckarsky, 1998). Studies of predator-naïve *Baetis* have also shown that the ability to recognize predatory trout is innate (Peckarsky & McIntosh, 1998) and suggest that the antipredator behaviour displayed by *Baetis* is mediated by chemical cues associated with fish, but of unknown origin (McIntosh & Peckarsky, 1996). Moreover, a feeding trial conducted to test whether *Baetis* responds to fish cues in proportion to predation risk indicated that risky behaviour of *Baetis* (drifting in the water column) decreased as predation risk increased (McIntosh & Peckarsky, 2004). Furthermore, McIntosh and Peckarsky (2004) observed that *Baetis* did not respond to a completely novel nonpredatory fish (goldfish) and, thus, are able to differentiate among chemical cues emanating from predatory and nonpredatory fish species.

The goals of this study were to (1) explore antipredator responses of aquatic insects (mayfly larvae of the genus *Baetis*) to chemical cues associated with a variety of predatory salmonid fish, (2) test whether the response of *Baetis* to salmonid fish is related to fish diet, (3) determine the origin of the chemical cues responsible for the antipredator behaviour observed in *Baetis* and (4) explore

whether antipredator behaviour of *Baetis* is a general response by *Baetis* to the scent of any potential predator (fish and nonfish).

## METHODS

We conducted three bioassays to study the origin and specificity of chemical cues eliciting antipredator responses of *Baetis bicaudatus* (Ephemeroptera; Baetidae) to fish cues and to test whether *Baetis* uses chemical cues to differentiate among freshwater fish species. Bioassays were conducted in a streamside system of 60 circular Plexiglas flow-through microcosms of 15 cm diameter (150 cm<sup>2</sup> area approximately; microcosms are described and illustrated in Alvarez & Peckarsky, 2005; Peckarsky & Cowan, 1991). The microcosms received natural stream water, gravity-fed from a nearby fishless stream, and were housed in an opaque white portable greenhouse (Weatherport) adjacent to the East River at the Rocky Mountain Biological Laboratory (RMBL), Gunnison County, Colorado (U.S.A.).

We conducted a fourth bioassay to test whether another species of *Baetis* (*Baetis rhodani*) was able to differentiate among chemical cues released by freshwater fish, saltwater fish and amphibians to test the hypothesis that *Baetis* responds to a general cue associated with cutaneous mucus of potential predators. This last bioassay was conducted in a closed system of 50 circular plastic microcosms 8 cm in diameter (~50 cm<sup>2</sup> area) provided with continuous aeration. Microcosms were supplied with stream water and housed in a laboratory of the Biology Faculty at the University of Vigo (Spain). Bioassays conducted on the European species of *Baetis* would not have been possible at RMBL because of strict conservation and environmental policies. Moreover, both *Baetis* species have been shown to respond similarly to fish predators, reducing their foraging activity during the day under the risk of predation and increasing their activity at night (see for example Malmqvist, 1988, on *B. rhodani*). Therefore, by using other predator and prey species, we also tested the generality of mechanisms of responses of *Baetis* to different fish species.

### *Effects of Fish Diet on the Antipredator Responses of Baetis*

The first microcosm experiment at RMBL tested one possible mechanism explaining antipredator responses of *B. bicaudatus* to identify the origin of chemical cues released by brook trout, *Salvelinus fontinalis*, which is the top predator in streams flowing through the RMBL. That is, we tested whether *Baetis* responds to chemical cues associated with fish feeding, and, if so, whether responses of *Baetis* to trout cues depended on variation in predator diet. Thus, a null response to this bioassay would enable us to rule out the following potential origins of the signal: alarm cues emitted by wounded *Baetis* conspecifics, salivary enzymes released by feeding fish and fish excretion products that may contain *Baetis* and conspecific odours.

This bioassay included four treatments: (1) *Baetis* exposed to the scent of brook trout fed conspecific *Baetis*; (2) *Baetis* exposed to the scent of brook trout fed texturized vegetable protein (TVP); (3) *Baetis* exposed to the scent of unfed brook trout; (4) control: *Baetis* not exposed to predator chemical cues.

Chemical cues from brook trout were added to the microcosms from one of three plastic bins (110 litres) located on a hillside outside the Weatherport so that cues could be gravity-fed to the microcosms. We placed two brook trout (mean fork length across treatments  $\pm$  SE = 178  $\pm$  2 mm) in each holding bin and added 50 *Baetis* daily (treatment 1), 50 TVP pellets daily (treatment 2), or no food for 3 days prior to starting the experiment (treatment 3). Fishless stream water was delivered by gravity to the holding tanks, and their outflows, including chemical cues from the three brook trout treatments, continually dripped into each microcosm via Tygon tubing at a rate of 3.0  $\pm$  0.2 ml/s (mean  $\pm$  SE). The same

source of fishless stream water was dripped into the control microcosms at a similar rate, by-passing the bins with brook trout. Treatments were allocated randomly to microcosms within the Weatherport, with 15 microcosms per treatment.

We added six *B. bicaudatus* to each microcosm (mean individual weight  $\pm$  SE =  $0.62 \pm 0.03$  mg) collected from a fishless tributary of the East River. To provide a food source for *Baetis*, we placed six  $4 \times 4$  cm tiles covered with benthic algae in each microcosm. In an attempt to standardize the starting amount of benthic algal biomass, tiles were acclimated in mesocosms with the same source of fishless stream water for 3 weeks prior to the experiment. Water temperature was recorded continuously using Onset Stowaway data loggers (Onset Computer, Pocasset, MA, U.S.A.) placed at four outflow locations of the system.

We estimated initial and final biomass of *Baetis* per microcosm by collecting and drying 15 sets of six individuals for 24 h at 60 °C and then weighing them to 0.01 mg accuracy. We calculated growth rates of individual *Baetis* over the 16-day experiment (29 June–15 July 2010) as the difference between the average dry mass at the end (mean  $\pm$  SE across treatments =  $1.10 \pm 0.04$  mg) and at the beginning ( $0.62 \pm 0.03$  mg) of the experiment.

We conducted behavioural observations of *Baetis* in each microcosm, recording day (0900 hours) and night (2100 hours) levels of dispersal behaviour (individuals drifting in the water column). Day versus night observations of drift allowed us to compare effects of treatment on diel periodicity of *Baetis*, which are more nocturnal when exposed to chemical cues associated with brook trout (McIntosh & Peckarsky, 1996). We also recorded the total number of individuals observed during daytime foraging on exposed surfaces of the tiles (tops and sides), which accumulate greater levels of algae and thus are the optimal surfaces for grazer foraging. We only recorded the number of *Baetis* exposed during the daytime observations for this first experiment, because daytime was the period when we expected differences among treatments to occur. However, in later bioassays we recorded the average number of *Baetis* drifting in the water column during both night and day observation periods to compare total activity among treatments.

To compare effects of treatment on changes in prey behaviour over time, we made observations once before initiating the drips of chemical cues (29–30 June 2010) and once each on days 6, 9, 13 and 15 after the drips were started. Night-time observations were made using headlamps with red light, which do not affect the behaviour of *Baetis* (Peckarsky & Cowan, 1991).

The experiment ran for 16 days, and we replaced all fish held in the bins after the first week (6 July). The replacement fish were treated the same as the first batch of fish before and after adding them into the holding bins. All trout were collected from the East River near RMBL using an electrofisher (Smith Root LR-24, Vancouver, WA, U.S.A.).

#### Specificity of Response by *Baetis* to Fish

We conducted three other microcosm experiments to test whether *Baetis* can differentiate among chemical cues associated with different fish species. Two experiments were conducted in the microcosm system at RMBL described above. One used a similar design with water from the fish holding tanks (each containing two fish) delivered into the microcosms and the other used only the fish cutaneous mucus. The third specificity bioassay was conducted in the microcosm system at the University of Vigo.

#### Specificity of response by *B. bicaudatus* to water-borne chemical cues from different species of fish

We compared responses of *B. bicaudatus* to the scent of four species of predatory salmonid and one omnivorous sucker species.

All species were familiar with each other, presently or historically cohabiting the same stream drainage. Wild brook trout, *S. fontinalis*, and brown trout, *Salmo trutta*, were collected in the East River near RMBL and the Slate River near Crested Butte, respectively, using an electrofisher (Smith Root LR-24). Rainbow trout, *Oncorhynchus mykiss*, and cutthroat trout, *Oncorhynchus clarkii*, were obtained from the Pitkin Hatchery (Pitkin, CO, U.S.A.), and longnose suckers, *Catostomus catostomus* (Cyprinidae) were collected from Emerald Lake (the source of the East River upstream of RMBL) by electrofishing.

We tested *B. bicaudatus* responses to three fish species and a fishless control in two separate trials. The first trial included four treatments ( $N = 15$ ) exposing *B. bicaudatus* to the scent from (1) two brook trout (mean fork length  $\pm$  SE =  $192 \pm 12$  mm), (2) two brown trout ( $197 \pm 37$  mm), (3) two longnose suckers ( $172 \pm 27$  mm) and (4) control (fishless) water. The second trial also included four treatments ( $N = 15$ ) exposing *Baetis* to the scent from (1) two brook trout (mean fork length  $\pm$  SE =  $197 \pm 43$  mm), (2) two rainbow trout ( $163 \pm 18$  mm), (3) two cutthroat trout ( $162 \pm 18$  mm) and (4) control (fishless) water. Similar to the first bioassay, we included six tiles colonized with benthic algae in the East River and six *Baetis* per microcosm (mean individual weight  $\pm$  SE =  $0.28 \pm 0.02$  mg) collected from the same fishless stream.

To confirm the effect of feeding status of fish on *Baetis* behaviour, all fish used in this bioassay were deprived of food for 3 days prior to exposing *Baetis* to their scent and were subsequently fed 50 *Baetis* collected from the East River on the second and third days of the experiment. The experiment ran for 3 days (first trial: 27–29 June; second trial: 10–12 July 2012) and, in each trial, we conducted day (0900 hours) and night (2100 hours) observations of the number of *Baetis* larvae foraging on exposed surfaces of the tiles the day after the drips were started, but before feeding the fish (days 1–2), and then after feeding the fish (days 2–3). Although salmonid fish pose the highest predation risk during the day, *Baetis* also reduce their nocturnal drift when brook trout odour is present (McIntosh, Peckarsky, & Taylor, 1999; McIntosh et al., 2002). Therefore, we used the number of *Baetis* larvae foraging on exposed surfaces of the tiles as the dependent variable, and added the total number of *Baetis* exposed (day + night) to test for the potential effect of fish scent on *Baetis* foraging activity. We excluded analyses of drift behaviour because changes in levels of drift activity of *Baetis* observed in controls between the first and second trials, which were conducted over a 2-week interval, compromised our ability to detect effects of treatments on *Baetis* foraging behaviour. In fact, previous studies revealed that mayflies can distinguish different concentrations of trout odour in natural streams and that the response is size specific, according to the relative risk of predation of large and small *Baetis* (McIntosh et al., 1999). Foraging behaviour (i.e. foraging on exposed tile surfaces) was a more robust response variable because it was unaffected by time.

#### Specificity of response by *B. bicaudatus* to cutaneous mucus from freshwater fish

We conducted a bioassay to test whether fish cutaneous mucus elicits the antipredator response of *Baetis* to predatory fish. We also tested whether *Baetis* can differentiate among chemical cues (specificity of response) associated with mucus from different fish species. We followed a similar design to that used in previous bioassays, but in this experiment we included six treatments ( $N = 10$ ) and used mucus instead of water delivered from the holding tanks containing fish. We exposed *B. bicaudatus* to cutaneous mucus from (1) wild brook trout (mean fork length  $\pm$  SE =  $183 \pm 7$  mm), (2) hatchery brook trout ( $104 \pm 2$  mm), (3) hatchery brown trout ( $202 \pm 23$  mm), (4) hatchery cutthroat trout



(mean fork length  $\pm$  SE = 272  $\pm$  11 mm), (5) wild longnose suckers (118  $\pm$  9 mm) and (6) control (fishless) water.

To collect the mucus, we held individuals of each fish species (12 fish per treatment) snout-down, gently dabbed the mouth 10 times with a cotton ball to eliminate excess of water and placed the fish in a plastic bag (following the procedure of Landeira-Dabarca, Sieiro, & Alvarez, 2013). Mucus was extracted by gently rubbing each fish for 20 s within the bag, carefully removing the fish, then storing bags containing mucus on ice for transport to the laboratory. In the laboratory (2–4 h after sample collection), mucus was removed from each bag with a micropipette and placed in a preweighed Eppendorf vial to estimate the amount of mucus obtained from each fish. A previous study conducted in a closed system, in which experienced *Baetis* (collected from salmonid streams) were exposed to different concentrations of fresh mucus solutions of Atlantic salmon, revealed that *Baetis* showed an antipredator response (reduced foraging on the tops and sides of tiles) when they were exposed to at least 0.3 g of fresh mucus/ml (1.2  $\pm$  0.2 mg of dry mucus/ml) (Landeira-Dabarca, Alvarez, & Peckarsky, 2014). Therefore, we based our calculations of cue concentration on this threshold, above which we predicted a change in foraging behaviour of *Baetis*. In fact, we observed that the magnitude of the behavioural response of *Baetis* remained constant even when exposed to higher concentrations of mucus solution. However, as this bioassay was conducted in an open system, we used twice the estimated threshold concentration to ensure that there were sufficient fish chemical cues to obtain a response. Thus, to prepare mucus solutions to add to each microcosm, we homogenized mucus from all individuals of each fish species and diluted it to a concentration of 0.6 g fresh mucus/ml of stream water.

We added mucus from the different fish species into the microcosms by continually releasing the mucus solutions from 50 ml Falcon tubes via silicone tubes of 7 mm diameter. We adjusted a valve that clamped each tube to create comparable drips among replicates and treatments (~one drop/3 s). Mucus solutions were dripped for 15–20 min in each microcosm, and thus each received approximately 300 drops (20 ml) of mucus solution. In this bioassay, we conducted behavioural observations of *Baetis* (number of individuals on exposed tile surfaces) 40 min after the drips stopped. Previous studies on *B. bicaudatus* revealed that changes in drift density occur within 5 min of the addition of trout odour, providing strong evidence of a rapid response (McIntosh et al., 1999). This experiment ran for 2 days (17–18 July 2013), and we conducted day (0900 hours) and night (2100 hours) observations before (baseline behaviour) and after adding the mucus cues.

#### *Specificity of response by B. rhodani to cutaneous mucus from multiple vertebrate donor species*

We conducted the third bioassay using the microcosm system in Spain to test whether *Baetis* displays a general response to the scent of any potential mucus donor or differentiates cutaneous mucus from predatory and nonpredatory fish species and predatory amphibians. We followed a similar design to that used in the bioassay 2 and included five treatments ( $N = 10$  per treatment). We exposed *B. rhodani* to cutaneous mucus of (1) predatory brown trout (mean fork length (FL)  $\pm$  SE = 182  $\pm$  6 mm), (2) omnivorous carp (165  $\pm$  13 mm FL), (3) novel predator seawater turbot (140  $\pm$  2 mm FL), (4) novel predator anuran frog (mean snout–vent length (SVL)  $\pm$  SE = 55  $\pm$  1 mm) and (5) control (fishless) stream water.

To obtain samples of cutaneous mucus, brown trout (*S. trutta*) were collected in the Carballedo Hatchery (northwestern Spain), carp (*Cyprinus carpio*) were collected by angling in a reservoir near the laboratory facilities at Vigo, and seawater turbot (*Psetta maxima*) were obtained from fish being reared at the ECIMAT (Marine Sciences Station of Toralla, University of Vigo, Spain). Mucus

samples from anuran frogs (*Lithobates iberica*) were obtained from individuals living in a small stream running through Castrelos Park (Vigo, Spain). Frogs were captured with hand-nets at night (2100–2300 hours) using headlamps.

To obtain enough fresh mucus to run the bioassay (~12 g), we took mucus samples from 12 fish per treatment and 18 frogs, following the same protocol described above, except that we freeze-dried the mucus in a speed-vacuum (Savant) for 5 h at maximum speed prior to its use to equalize the amount of mucus among treatments (fish species and frog). To prepare the mucus solutions to add to each microcosm, mucus from all individuals of each vertebrate species was homogenized and diluted to a concentration of 0.4 mg of dry mucus/ml of stream water (i.e. 3 $\times$  the threshold concentration estimated by Landeira-Dabarca et al., 2014). Each microcosm received 3 ml of mucus solution. As in the previous bioassays, we included six *Baetis* larvae per microcosm (mean individual weight  $\pm$  SE = 0.36  $\pm$  0.05 mg). Experiments ran 2 days (24–25 October 2013), and we conducted day (0900 hours) and night (2100 hours) observations of the number of *Baetis* foraging on exposed tile surfaces before (baseline behaviour) and after adding the mucus.

#### *Ethical Note*

The fish care and protocols used for experiments conducted at RMBL were approved by the RMBL Animal Welfare Committee. Protocols were evaluated for adherence to methodological guidelines proposed by professional societies and adherence to federal and state regulations. Moreover, all protocols complied with federal, state and local laws regarding the use of vertebrate animals in research.

#### *Data Analysis*

##### *Effects of fish diet on the antipredator responses of Baetis*

We conducted a one-way ANOVA to evaluate the effect of exposure to brook trout fed different diets on *Baetis* growth rates. Then, we used one-way ANCOVA to evaluate whether the rate of change over time (covariate) in the activity of *Baetis* depends on exposure to the scent of brook trout fed different diets (fixed factor) or whether drift avoidance behaviour of *Baetis* becomes fixed following exposure to trout odour. We used the average number of *Baetis* drifting in the water column at night and the number of *Baetis* foraging on exposed surfaces during the day as dependent variables in separate analyses. Those analyses determined whether the slopes of the relationship between the activity of *Baetis* and time during the experiment differed between individuals exposed to different fish treatments. If the slopes were homogeneous (i.e. interaction *Baetis* activity\*fish treatment:  $P > 0.05$ ), this term was omitted from further analysis. In this case, we subsequently conducted two separate one-way ANOVAs to evaluate the effect of fish diet on the average number of *Baetis* drifting in the water column (day + night activity) and the total number observed foraging on exposed tile surfaces during the day.

##### *Specificity of the response by Baetis to water-borne cues from different fish species*

As data from the bioassay using water-borne cues from different fish species did not meet the assumptions of normality, we conducted nonparametric tests to evaluate the effect of unfed fish scent from different fish species on the average number of *Baetis* observed foraging on exposed tile surfaces (day + night) in each microcosm.

Results from each trial were analysed separately. We first used a Mann–Whitney  $U$  test to repeat the test of the effect of predator

feeding status on *Baetis* total foraging activity. We then used a Kruskal–Wallis test to evaluate the effect of fish species on *Baetis* foraging activity. Pairwise comparisons of activity of *Baetis* between control microcosms and microcosms exposed to water-borne cues from different fish species were tested with Mann–Whitney *U* tests.

#### Specificity of the response by *Baetis* to cutaneous mucus

For similar reasons we used nonparametric tests to evaluate the effects of different mucus cues on the number of *Baetis* foraging on exposed tiles (either *B. bicaudatus* or *B. rhodani*). We first used separate one-way Kruskal–Wallis tests for comparison among treatments before adding any cue (baseline behaviour across treatments). Then, we conducted separate Scheirer–Ray–Hare extensions of the Kruskal–Wallis test to check for an interaction between treatment and period (before versus after cue exposure). If the interaction was significant, we used paired Wilcoxon signed-ranks tests to compare the number of *Baetis* foraging on exposed tile surfaces in each microcosm before and after cues were added.

## RESULTS

#### Effects of Fish Diet on the Antipredator Responses of *B. bicaudatus*

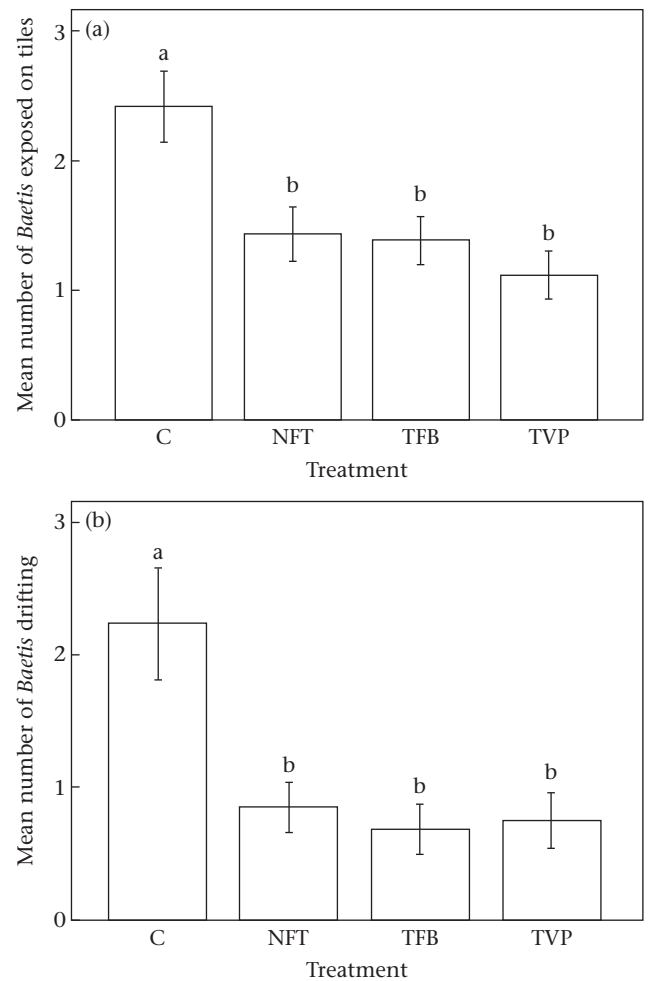
Mean size of *Baetis* was 75% larger at the end of the experiment compared to the beginning, but there were no differences in growth rates among treatments (mean individual growth rates across treatments  $\pm$  SE =  $0.030 \pm 0.002$  mg/day; ANOVA:  $F_{3,56} = 0.116$ ;  $P = 0.951$ ), indicating that growth rates of *Baetis* were not affected by fish cues.

The ANCOVA model revealed that the interaction (fish treatment \* time) was not significant for either the drift or foraging activity response variables; thus, the slopes of the relationship between the activity of *Baetis* and time was similar among fish treatments (ANCOVA: drifting:  $F_{3,292} = 0.047$ ;  $P = 0.986$ ; foraging:  $F_{3,292} = 2.258$ ;  $P = 0.098$ ), and that following exposure to fish odour, drift avoidance behaviour of *Baetis* became fixed. Therefore, to analyse the effect of fish feeding status and diet on *Baetis* activity, we used the number of *Baetis* drifting or foraging during the first observation period after cues were added for consistency with analyses of other bioassays. There were significant differences in the activity between *Baetis* exposed to fish cues of all diets and *Baetis* in control microcosms (ANOVA: drifting:  $F_{3,56} = 35.874$ ;  $P = 0.009$ ; foraging:  $F_{3,56} = 73.800$ ;  $P = 0.002$ ; Fig. 1). However, post hoc comparisons showed that the activity of *Baetis* did not depend on the feeding status of brook trout (fed versus unfed) or the composition of their diet (trout fed *Baetis* versus trout fed TVP) (Fig. 1). Therefore, the origin of *Baetis* responses to trout was not related to fish feeding status or diet. This bioassay allowed us to rule out alarm cues from conspecifics, salivary enzymes and fish excretion products associated with feeding as potential origins of the cues detected by *Baetis*. Thus, we focused further experiments on the fish cutaneous mucus as the potential origin of the cues.

#### Specificity of Response by *Baetis* to Different Fish Species

##### Response of *B. bicaudatus* to water-borne fish chemical cues from different fish species

Similar to the results obtained in the previous bioassay, feeding status of fish (fed versus unfed) did not affect the total number of *Baetis* foraging on exposed tile surfaces (Mann–Whitney *U* test: trial 1:  $U = 1564.5$ ,  $N_1 = 60$ ,  $N_2 = 60$ ,  $P = 0.213$ ; trial 2:  $U = 1698$ ,  $N_1 = 60$ ,  $N_2 = 60$ ,  $P = 0.588$ ). There were significant differences in the number of *Baetis* foraging on exposed tile surfaces among fish treatments (Kruskal–Wallis test: trial 1:  $H_3 = 12.749$ ,  $P = 0.005$ ;

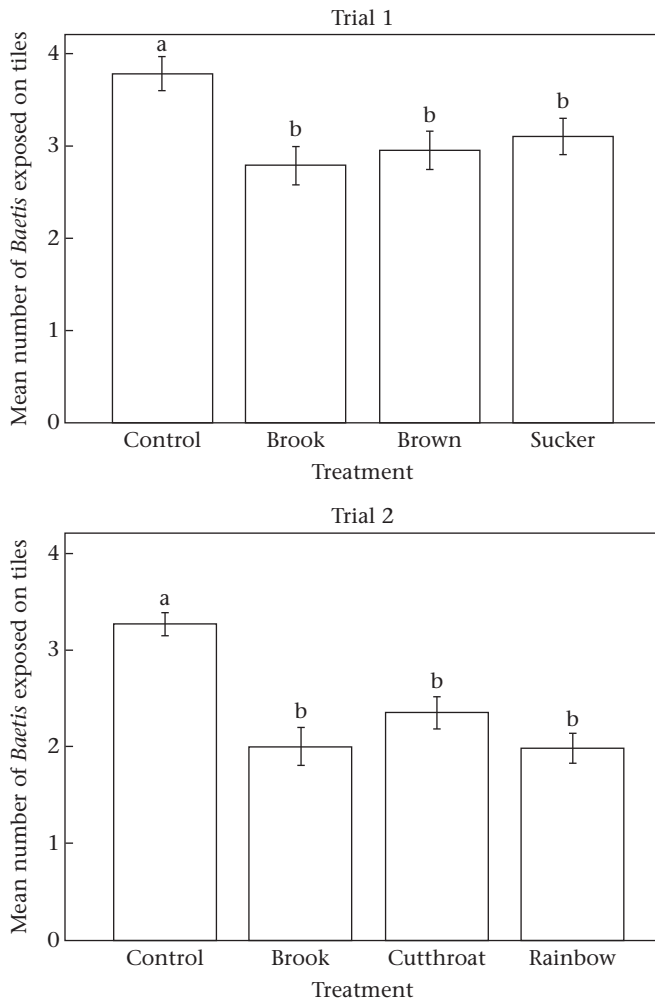


**Figure 1.** Effect of fish feeding status and diet on *Baetis bicaudatus* activity. Bars represent the mean number ( $\pm$ SE) of *Baetis* (a) foraging on exposed tile surfaces during the day and (b) drifting (day and night activity) after cues from different treatments were added. C: control no predator chemical cue; NFT: unfed fish; TFB: fish fed on *Baetis*; TVP: fish fed on texturized vegetable protein. Lower-case letters indicate significant differences between fish dietary treatments ( $P < 0.05$ ).

trial 2:  $H_3 = 33.695$ ,  $P < 0.001$ ; Fig. 2). However, the foraging activity of *Baetis* exposed to chemical cues from any fish species was significantly lower than that of *Baetis* in control microcosms without fish cues (Mann–Whitney *U* test: sucker:  $U = 296$ ,  $N_1 = 30$ ,  $N_2 = 30$ ,  $P = 0.021$ ; brown trout:  $U = 265.5$ ,  $N_1 = 30$ ,  $N_2 = 30$ ,  $P = 0.006$ ; cutthroat trout:  $U = 189$ ,  $N_1 = 30$ ,  $N_2 = 30$ ,  $P < 0.001$ ; rainbow trout:  $U = 108$ ,  $N_1 = 30$ ,  $N_2 = 30$ ,  $P < 0.001$ ; brook trout, trial 1:  $U = 321$ ,  $N_1 = 30$ ,  $N_2 = 30$ ,  $P = 0.001$ ; brook trout, trial 2:  $U = 143.5$ ,  $N_1 = 30$ ,  $N_2 = 30$ ,  $P < 0.001$ ; Fig. 2).

#### Response by *B. bicaudatus* to cutaneous mucus from freshwater fish

As expected, there were no differences in the levels of activity of *Baetis* before addition of the mucus cues (baseline behaviour) among treatments (Kruskal–Wallis test:  $H_3 = 3.350$ ;  $P = 0.646$ ). Furthermore, there was a significant interaction between treatment and period ( $H_1 = 25.905$ ;  $P < 0.0001$ ), indicating that although there were no differences in the activity of *Baetis* before adding the mucus cues to the microcosms, we observed differences between treatments after the addition of fish cues. There was a significant decrease in foraging activity of *Baetis* on exposed tile surfaces following exposure to cutaneous mucus of each fish species tested (Wilcoxon signed-ranks exact test: cutthroat trout:  $Z = -2.719$ ;



**Figure 2.** Effect of water-borne cues from different fish species on *Baetis bicaudatus* activity. Bars represent the mean number ( $\pm$ SE) of *Baetis* foraging on exposed tile surfaces. Trial 1: control, no predator chemical cue; brook trout; brown trout; and sucker. Trial 2: control, no predator chemical cue; brook trout; cutthroat trout; and rainbow trout. Lower-case letters indicate significant differences between fish treatments ( $P < 0.05$ ).

$N = 10$ ,  $P = 0.007$ ; hatchery brook trout:  $Z = -2.680$ ;  $N = 10$ ,  $P = 0.007$ ; wild brook trout:  $Z = -2.539$ ;  $N = 10$ ,  $P = 0.011$ ; brown trout:  $Z = -2.820$ ;  $N = 10$ ,  $P = 0.005$ ; suckers:  $Z = -2.259$ ;  $N = 10$ ,  $P = 0.024$ ; Fig. 3). However, in control microcosms, with no fish cues added, there were no differences in the foraging activity of *Baetis* between periods (baseline versus after:  $Z = -0.179$ ,  $N = 10$ ,  $P = 0.858$ ).

#### Response of *B. rhodani* to cutaneous mucus from multiple donor vertebrates

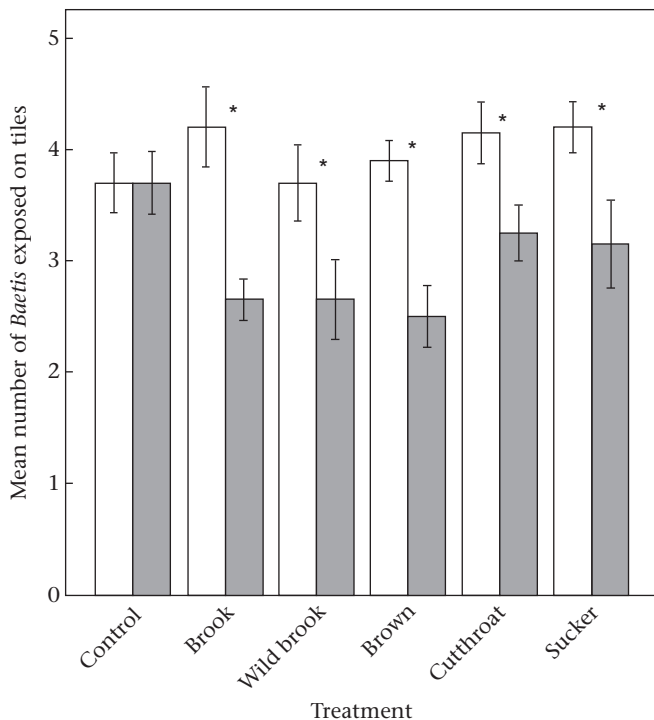
There were no significant differences between treatments in the activity of *Baetis* before the addition of cues (Kruskal–Wallis test:  $H_4 = 2.281$ ,  $P = 0.684$ ). However, as with the assay conducted on *B. bicaudatus* in RMBL (Colorado), there was a significant treatment\*period interaction ( $H_4 = 25.886$ ,  $P = 0.001$ ), indicating significant differences between treatments in the activity of *B. rhodani* after mucus cues were added. However, these differences were only significant when *B. rhodani* was exposed to the mucus of brown trout (Wilcoxon signed-ranks exact test:  $Z = -2.200$ ,  $N = 10$ ,  $P = 0.028$ ) but not when exposed to the mucus of the other three species tested (carp, turbot and frog) (Fig. 4). Therefore, the

foraging activity of *Baetis* exposed to carp, turbot and frog mucus was similar to that observed in the control microcosms (Fig. 4).

## DISCUSSION

Chemical signals play an important role in predator–prey interactions and are involved in many ecological and evolutionary processes (Burks & Lodge, 2002; Dodson et al., 1994). The general consensus of previous studies dealing with chemical alarm signalling in aquatic predator–prey systems is that chemical cues are detected by prey when predators chew or digest prey and thereby act as alarm substances. For example, studies on the effect of predatory fish on morphological traits of zooplankton prey (Stabell et al., 2003), on habitat choice and activity of fish prey (Pettersson, Andersson, & Nilsson, 2001) and even on day activity of mayfly insect prey (Douglas, Forrester, & Cooper, 1994; Hury & Chivers, 1999) have related the responses of prey to predator diet. Similarly, studies on salamanders (Chivers et al., 1996; Wilson & Lefcort, 1993) and crayfish (Covich, Crowl, Alexander, & Vaughn, 1994; Crowl & Covich, 1990; Turner, 2008) suggest that prey might not perceive chemical cues from nonforaging fish as an indication of imminent danger. In contrast to these studies, in our first and second bioassays, we observed that the antipredator response of *B. bicaudatus* from a fishless stream was independent of fish feeding status or fish diet. Our observations suggest that conspecific alarm substances, predator excretory products associated with feeding (such as urea and ammonia) and predator salivary enzymes do not elicit antipredator responses of *Baetis* to chemical cues from fish predators. Similarly, studies examining diurnal changes in behaviour of predator-naïve *Daphnia* have also demonstrated that chemical cues released by predatory fish are not related to predator diet (von Elert & Loose, 1996; von Elert & Pohnert, 2000; von Elert & Stibor, 2006). Specifically, in a laboratory experiment, Van Gool and Ringelberg (2002) showed that feeding on *Daphnia* was not necessary for detection of predatory fish cues, because *Daphnia* responded to chemical cues from newly hatched fish larvae, which utilize yolk sac reserves and have not yet begun feeding.

Many taxa respond to predators by altering their behaviour, including spatial avoidance, activity reduction and temporal changes in behavioural cycles (Dill, 1987; Lima & Dill, 1990; Sih, 1987). Because most predator-induced responses are commonly considered adaptive (e.g. reduced activity reduces predation risk; Lawler, 1989; Skelly, 1994; Woodward, 1983), it has been proposed that the magnitude of antipredator response should be related to the magnitude of risk posed by the predator (Botham, Kerfoot, Louca, & Krause, 2006; Lima & Dill, 1990; Sih, 1987). Indeed, there is substantial evidence that prey respond weakly or nonsignificantly to predators that pose little or no predation risk, whereas prey respond strongly to predators that pose a high risk (Kohler & McPeck, 1989; Peckarsky, 1996; Sih, 1987). In contrast to this general hypothesis, in our second and third bioassays we observed that *B. bicaudatus* did not distinguish between five familiar freshwater fish species that coexist in the same drainage, despite differences in the relative risk of predation posed by each species (four predatory salmonid species and one omnivorous sucker species). This result is similar to that found for *Daphnia*, where diel vertical migration, an antipredator behavioural response to visually hunting fish (Lampert, 1993), does not vary with cyprinid fish species (Burks & Lodge, 2002; von Elert & Loose, 1996). These results and those of other studies (e.g. von Elert & Loose, 1996; von Elert & Pohnert, 2000) showing generalized responses to predator cues have been informative in developing alternative hypothesis that a prey's magnitude of response to a predator may not always be related to the risk posed by the predator. In fact, as different antipredator traits are useful against different predators, and prey usually have



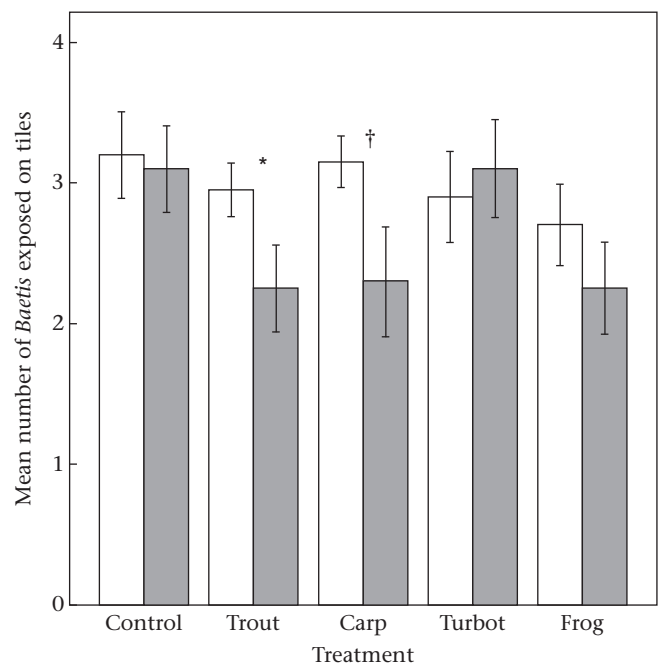
**Figure 3.** Effect of cutaneous mucus from different ‘familiar’ fish species on *Baetis bicaudatus* activity. Bars represent the mean number ( $\pm$ SE) of *Baetis* foraging on exposed tile surfaces across day and night observations. *Baetis* were exposed to control (fishless) water and mucus from hatchery brook trout, wild brook trout, wild brown trout, hatchery cutthroat trout and wild suckers. White bars indicate the mean number of *Baetis* foraging on exposed tile surfaces before adding the mucous cues; grey bars indicate the mean number of *Baetis* after adding the cues. An asterisk indicates a significant difference before and after adding the mucus cue for each fish species ( $^*P < 0.05$ ).

more than a single species of predator, prey may use a unique suite of responses against each predator species (Botham et al., 2006; Relyea, 2001a). For example, prey might reduce activity in the presence of a moderately risky predator, but use spatial avoidance against a highly risky predator (see for example Relyea, 2001b). In this case, the relationship between the magnitude of antipredator response and the predation risk for any one trait may not be scaled, thus highlighting the importance of comparing studies using similar traits and similar predator–prey systems. Moreover, several studies have shown that previous experience with a predator affects variation in the activity of prey. Specifically, experiments by Peckarsky and McIntosh (1998) showed that once *Baetis* are exposed to any concentration of trout odour (i.e. added artificially or collected from fish streams), drift avoidance behaviour during the day becomes fixed. Therefore, in order to make generalizations, the antipredator behaviour of naïve prey (with no previous contact with predators) should not be compared to the behaviour of experienced prey. Moreover, because the risk of predation depends on the size of the prey animal (Allan, Flecker, & McClintock, 1987; Brooks & Dodson, 1965), prey at a greater risk of predation should show more pronounced responses to predators. Thus, prey size should also be taken into account when comparing the response of prey to predators.

We focused our study on the hypothesis that the reduced foraging activity of *Baetis* exposed to fish predators is due to chemical cues emanating from the cutaneous mucus of fish. Using a similar experimental approach, this theory was previously suggested by other authors to explain the enhancement of light-induced swimming observed in brine shrimp (*Artemia*

*franciscana*) (Forward & Rittschof, 1999), the diel vertical migration of crab larva (*Rhithropanopeus harrisi*) (Forward & Rittschof, 2000) and the foraging-and-fright response behaviour of mud snails (*Ilyanassa obsoleta*) (Rahman et al., 2000) reacting to mummichog (*Fundulus heteroclitus*). In contrast, studies examining predator avoidance responses in *Daphnia* have hypothesized that predator cues are not produced by predatory fish, but by the activity of some other organism associated with fish predators (Loose, von Elert, & Dawidowicz, 1993; von Elert & Pohnert, 2000).

In our fourth bioassay, we observed that European *B. rhodani* larvae did not respond to cutaneous mucus of carp, seawater turbot or frogs, indicating that *Baetis* may not show a general response to all mucus donor organisms. Ringelberg, Flik, Aanen, and Van Gool (1997) suggested that diel vertical migration amplitude of *Daphnia* changes with predatory fish biomass and, consequently, with the concentration of fish chemical cues. Although we used fish of similar size to conduct all bioassays and we used similar amounts of mucus when dealing with different organisms, the proportion of the chemical components of mucus may differ among mucus donor species (Gomes & Dietrich, 1982); thus, the relationship between the relative fish biomass and the chemical signal strength may be different for different species. If this is the case, by using chemical cues from carp, seawater turbot or frogs, we may not have reached the minimal stimulus strength (threshold) necessary to elicit an antipredator response by *Baetis*. In line with this hypothesis, previous experiments have revealed that the response of *B. bicaudatus* to odour concentrations of brook trout (number of fish) is not linear and is probably best described by a threshold response, where only the odour from at least two fish (162–200 mm fork length) is sufficient to reduce nocturnal drift rate (McIntosh et al., 2002). Interestingly, the average amount of mucus obtained from one



**Figure 4.** Effect of cutaneous mucus from different donor vertebrates on *Baetis rhodani* activity. Bars represent the mean number ( $\pm$ SE) of *Baetis* foraging on exposed tile surfaces across day and night observations. *Baetis* were exposed to control (fishless) water and mucus from brown trout, carp, turbot and frog. White bars indicate the mean number of *Baetis* foraging on exposed tile surfaces before adding the mucous cues; grey bars indicate the mean number of *Baetis* after adding the cues. An asterisk indicates a significant difference before and after adding the mucus cue for each mucus donor predator species at different levels of significance (trout,  $^*P < 0.05$ ; carp,  $^\dagger P < 0.1$ ).



salmonid fish (trout or salmon) of this size (e.g. fresh mucus  $\pm$  SE =  $1.3 \pm 0.1$  g) is four times the threshold of 0.3 g, which corresponds to the average amount of mucus obtained from one small salmonid fish (mean fork length  $\pm$  SE =  $105.9 \pm 0.3$  cm; Landeira-Dabarca et al., 2014). Thus, although secretion of fish mucus occurs continuously (basal secretion) (Parrish & Kroen, 1988; Shephard, 1994), variation in the concentration of chemical cues from the predator may affect prey behaviour differently depending on the trait(s) examined.

As a second hypothesis to explain the sensitivity of antipredator responses of *Baetis* to different fish species, we also considered the historical coexistence of prey and potential predators. For example, studies conducted by Berendonk and O'Brien (1996) on the specificity of the response of different species of *Chaoborus* zooplankton to planktivorous fish showed that fish only elicited a response in those prey species with which they had coexisted. Similarly, although predator-naïve *B. bicaudatus* inhabit the same drainage as the sucker species tested in Colorado, *B. rhodani* has never coexisted with the carp, turbot or frog species tested. Therefore, given that some antipredator behaviour persists for many thousands of years even after isolation (Blumstein, 2006), evolutionary experience may play an important role in predator–prey recognition of *Baetis*.

McIntosh and Peckarsky (2004) showed that *B. bicaudatus* uses chemical cues produced by cutthroat and brook trout to adjust their nocturnal drift behaviour in proportion to the risk posed by these predators. In addition, *Baetis* showed no response to chemical cues from goldfish (*Carassius auratus*) obtained from a local pet shop, with which they had no prior evolutionary history (McIntosh & Peckarsky, 2004). Such threat-sensitive responses to predators require that prey are able to differentiate among chemicals associated with predators, which has been suggested by Mirza and Chivers (2001) to be an improbable mechanism. Exotic predators may also pose an additional problem, because prey may need to learn appropriate responses and adjust the intensity of their responses to novel predators accordingly (Ferrari, Gonzalo, Messier, & Chivers, 2007; Korpi & Wisenden, 2001; Laurila, 2000). Therefore, differences in the antipredator responses of Colorado and European *Baetis* species suggest the need for further investigation of the nature and the proportion of the metabolites released through the cutaneous mucus of different donor species (freshwater fish, salt-water fish and amphibians).

Our bioassays contribute to the limited information available regarding the origin and identity of the fish-associated chemical cues that cause mayfly prey to respond to fish predators (e.g. von Elert & Pohnert, 2000; Forward & Rittschof, 1999; Ringelberg & Van Gool, 1998). Given that different predators induced different antipredator responses and different prey showed different antipredator responses, comparison among studies does not constitute a rigorous test of a general hypothesis on the effect of predator diet on prey. Therefore, future studies on specific predator–prey systems, focusing on specific traits of prey, are needed to develop more general hypothesis and to identify not only the source but also the chemical composition of these chemical cues. This information is key to understanding the quantitative relationships between the antipredator responses of prey and predator biomass, cue composition and cue concentration. Therefore, approaches similar to that used in this study may contribute to our understanding of the role of chemical cues in aquatic ecosystems.

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