Animal Behaviour 158 (2019) 35–45

Contents lists available at ScienceDirect

Animal Behaviour

journal homepage: www.elsevier.com/locate/anbehav

Mayflies avoid sweets: fish skin mucus amino sugars stimulate predator avoidance behaviour of *Baetis* larvae

Andrea Landeira-Dabarca [a, b, *], Maruxa Álvarez [a], Barbara Peckarsky [c, d]

[a] Departamento de Ecología y Biología Animal, Universidad de Vigo, Vigo, Spain
[b] Department of Ecosystem Biology & SoWa, University of South Bohemia, České Budějovice, Czech Republic
[c] Rocky Mountain Biological Laboratory, Crested Butte, CO, USA
[d] Departments of Integrative Biology and Entomology, University of Wisconsin, Madison, WI, USA

ARTICLE INFO

Article history:
Received 6 March 2019
Initial acceptance 19 June 2019
Final acceptance 27 August 2019

MS. number: 19-00170R

Keywords:
bacteria
Baetidae
chemical cues
fish skin mucus
glycosaminoglycans
hexosamine
kairomones
predator avoidance behaviour
Salmonidae

Nonconsumptive effects of predators can have knock-on effects on prey fitness, life history and population dynamics. However, the origin of cues stimulating predator avoidance behaviour and the mechanisms underlying prey responses need further investigation. Previous studies revealed that nonconsumptive effects of predatory fish on *Baetis* mayfly larvae are mediated by water-borne chemical cues released from fish mucus. However, there are conflicting results regarding the nature of these cues and the specific role of the activity of fish mucus-dwelling bacteria in stimulating predator avoidance by prey. To address those conflicting results, we investigated whether bacteria dwelling in fish mucus and/or chemical components present in fish mucus are responsible for the predator avoidance response by *Baetis* mayflies to salmonids. Results of five bioassays conducted in microcosms revealed that to stimulate *Baetis* predator avoidance behaviour: (1) bacteria do not need to be present in salmonid mucus; (2) the saccharide fraction of the fish mucus glycosaminoglycan component, but neither the protein fraction nor the whole molecule, functions as a kairomone; (3) specific active components of the saccharide fraction are primarily amino sugars in the form of hexosamines; and (4) there is a minimum dose of mucus and specifically of hexosamine needed. Our study provides the first experimental evidence that mayfly larvae recognize fish predators via the amino sugars naturally present in fish skin mucus. These sugars are released into the water by microbially mediated breakdown of glycosaminoglycans. Further research on the responses of different invertebrate prey species to similar predator cues are needed to understand the evolutionary history of this kairomone recognition behaviour.
© 2019 The Association for the Study of Animal Behaviour. Published by Elsevier Ltd. All rights reserved.

Increasing evidence shows that predators trigger nonconsumptive effects in prey, inducing changes in their behaviour, life history and morphology (e.g. Mitchell, Bairos-Novak, & Ferrari, 2017; Peckarsky, Kerans, Taylor, & McIntosh, 2008; Weissburg, Smee, & Ferner, 2014). The strong selection pressure that predators impose on prey results in the evolution of a variety of predator avoidance mechanisms that increase prey survival, although sometimes at a cost (e.g. Polačik & Janáč, 2017; Rosier & Langkilde, 2011). However, to respond to the threat from a potential predator, prey must first recognize predator cues (e.g. Brönmark & Hansson, 2000; Burks & Lodge, 2002; Mitchell et al., 2017; Paterson et al., 2013).

Extensive research on predator avoidance mechanisms has been carried out in freshwater ecosystems, as many organisms rely on chemical cues present in water to detect predators (e.g. Brönmark & Hansson, 2000; Burks & Lodge, 2002; Kats & Dill, 1998; Loose, Von Elert, & Dawidowicz, 1993; Scrimgeour, Culp, & Cash, 1994). Some of these chemical cues, also known as kairomones (Brönmark & Hansson, 2000), have been identified and chemically characterized (e.g. Akkas, Kepenek, Beklioglu, & Severcan, 2010; Bucciarelli & Kats, 2015; Forward & Rittschof, 1999, 2000; Rittschof & Cohen, 2004; Von Elert & Pohnert, 2000). However, the nature of cues involving interactions between predatory fish and aquatic insect larvae is still underexplored.

A recent study demonstrated that the chemical cue detected by mayfly larvae in the water has its origin in the skin mucus of fish (Álvarez, Landeira-Dabarca, & Peckarsky, 2014). Fish skin mucus is a complex biological matrix composed primarily of chains of mucopolysaccharides, of which glycosaminoglycans (GAG) are the most

* Correspondence: A. Landeira-Dabarca, Department of Ecology and Animal Biology, University of Vigo, 36310, Spain.
E-mail addresses: andrealandab@gmail.com, realand@uvigo.es (A. Landeira-Dabarca).

https://doi.org/10.1016/j.anbehav.2019.10.003
0003-3472/© 2019 The Association for the Study of Animal Behaviour. Published by Elsevier Ltd. All rights reserved.

abundant molecules (Reverter, Tapissier-Bontemps, Lecchini, Banaigs, & Sasal, 2018; Shephard, 1994). From an evolutionary perspective, predator cues released from the fish skin mucus are disadvantageous to the predator; thus natural selection may act against their production (e.g. Ringelberg & Van Gool, 1998). However, skin mucus has multiple functions in teleost fish, acting as a mechanical and chemical dynamic barrier (e.g. Reverter et al., 2018; Shephard, 1994). Part of its multifunctionality is attributed to the diverse microbial community inhabiting skin mucus, with bacteria being the major component (Hawkers, 1974, Sar & Rosenberg, 1989). Therefore, if the benefits provided by mucus counterbalance the negative effects of warning prey of the risk of potential predators (Ringelberg & Van Gool, 1998), then both the chemical components of skin mucus of fish and the mucus-dwelling bacterial communities could be good candidates for predatory fish cues.

Chemical components of fish skin mucus, such as disaccharide subunits of GAGs, have been shown to have a central role in mediating predatory fish recognition by *Daphnia*, brine shrimp, crab larvae, crustacean larvae and snails (Akkas et al., 2010; Cohen & Forward, 2003; Forward & Rittschof, 1999, 2000; Rahman, Forward, & Rittschof, 2000; Rittschof & Cohen, 2004). Nevertheless, a debate exists about the origin of the kairomone (Ringelberg & Van Gool, 1998), its mechanistic release (Akkas et al., 2010; Beklioglu, Telli, & Gozen, 2006) and the specific compounds stimulating predator avoidance responses. For example, some studies on predator avoidance by *Daphnia* argued that the hydroxyl group of the disaccharide subunits of GAGs is the key component of the kairomone released by fish (Von Elert & Loose, 1996; Von Elert & Pohnert, 2000), while others provide evidence that the predator cue is the acetylamino group of the disaccharides (Akkas et al., 2010; Forward & Rittschof, 2000). In addition, other studies have attributed the nature of the fish kairomone to the activity of the bacteria residing in the skin mucus (e.g. Akkas et al., 2010; Beklioglu et al., 2006; Forward & Rittschof, 1999; Rahman et al., 2000; Ringelberg & Van Gool, 1998).

The objectives of this study are: (1) to characterize the chemical components of fish skin mucus that could potentially act as kairomones emitted by fish; (2) to explore whether mucus-dwelling bacteria and/or specific chemical compounds of fish skin mucus act as kairomones in fish–*Baetis* predator–prey interactions; (3) to explore the existence of a threshold dose of mucus or specific kairomone compound needed to elicit a predator avoidance response in mayfly larvae.

METHODS

We implemented five bioassays to test whether natural chemical components and/or bacteria residing in fish skin mucus (Fig. 1) could act as kairomones mediating the interactions between salmonid fish (predator) and *Baetis* mayfly larvae (prey).

Test Organisms

For the first three bioassays we used European *Baetis rhodani* which has been shown to respond to the presence of water-borne chemical cues (Malmqvist, 1988; Tikkanen, Muotka, & Huhta, 1996; Winkelmann, Petzoldt, Koop, Matthaei, & Benndorf, 2008) released by Atlantic salmon, *Salmo salar*, which is one of the major predators of macroinvertebrates (specifically mayflies) in streams of northwestern Spain (e.g. Chapman, 1966; Álvarez et al., 2014). For the last two bioassays we used the well-studied North American mayfly species, *Baetis bicaudatus*, which has also been shown to respond to water-borne chemical cues (McIntosh & Peckarsky, 1999; McIntosh, Peckarsky, & Taylor, 2002; Peckarsky et al., 2008) emitted by brook trout, *Salvelinus fontinalis*. Brook trout are natural predators of mayflies that have coexisted with this mayfly species in streams of the East River drainage, in Gunnison County, Colorado (U.S.A.) for well over 150 years.

Mechanisms of Predator Avoidance

Previous studies showed that a chemical cue associated with the skin mucus of predatory fish (salmonids) and released into stream water changes the foraging behaviour and refuge use of the two *Baetis* mayfly species tested (e.g. Álvarez et al., 2014). Specifically, when exposed to waterborne fish kairomones, mayflies reduce drift in the water column, which functions to search for new food patches, thus decreasing the time spent foraging on exposed substrate surfaces where algal food resources are more abundant. In response to these kairomones, *Baetis* mayfly larvae also become more nocturnal, thereby reducing foraging time over a 24 h cycle (e.g. Flecker, 1992; McIntosh, Peckarsky, & Taylor, 1999). Therefore, responses by *Baetis* larvae to perceived risk of predation result in a potentially costly reduction in foraging behaviour.

Chemical Characterization of Salmonid Mucus

The most common GAGs present in freshwater fish mucus are chondroitin sulphate (CS) and hyaluronic acid (HA) (Rittschof & Cohen, 2004, Silbert, Bernfield, & Kokenyesi, 1997, Van de Winkel, Toin, van Kupevelt, Janssen, & Lock, 1986; Fig. 1). GAGs are composed of chains of repeated subunits of hexosamines [(N) acetyl-galactosamine (in CS-GAG) or (N) acetyl-glucosamine (in HA-GAG)] and uronic acid, such as glucuronic acid (in both GAGs). These long chains of polysaccharides are bound to a core linear protein (Bansil & Turner, 2018).

We characterized the chemical composition of the skin mucus of 10 hatchery-reared Atlantic salmon (mean fork length 16.58 ± 0.3 cm) from Carballedo hatchery (Spain). To collect the mucus, we held each fish snout down and gently dabbed its mouth 10 times with cotton to eliminate excess water before placing it in a plastic bag (following Landeira-Dabarca, Sieiro, & Álvarez, 2013). We extracted mucus by gently rubbing each fish for 20 s within the bag, removing the fish, and then storing the bags containing mucus on ice for transport to the laboratory. Then, we removed fresh mucus from each bag with a micropipette and placed it in a preweighed vial to estimate the amount of mucus obtained from each fish. To estimate the quantity of mucus for each fish, we lyophilized fresh mucus by freeze drying each mucus sample in a speed-vacuum (Savant) for 5 h at maximum speed, and calculated mucus dry weight (mg; vial weight − vial weight with lyophilized mucus corrected by the vial weight loss by the lyophilization process) and mucus water content (%; fresh mucus weight − mucus dry weight × 100).

We conducted metachromatic activity assays to quantify different fractions of proteins, GAGs, sulphated sugars, hexosamine and uronic acid. The protein concentration of mucus was determined using the Bradford assay (Bradford, 1976) measured at 596 nm in a spectrophotometer using bovine serum albumin (BSA) as the standard. Given that GAGs have a terminal sulphated group, quantifying sulphated sugars provides a good estimate of the amount of GAGs, such as CS, present in fresh mucus (Forward & Rittschof, 2000). To quantify sulphated sugars, we used the dimethyl methylene blue method (Farndale, Buttle, & Barrett, 1986) measured at 525 nm in a spectrophotometer using CS as a standard. For hexosamine quantification, we used the Morgan–Elson method modified by Reissig, Strominger, and Leloir (1955) based on dimethylaminobenzaldehyde (DMAB) reaction measured at 585 nm using (N) acetyl-galactosamine as a standard. To estimate the amount of uronic acid we used the phenyl-phenol method (Blumenkrantz & Asboe-Hansen, 1973; Filisetti-Cozzi & Carpita,

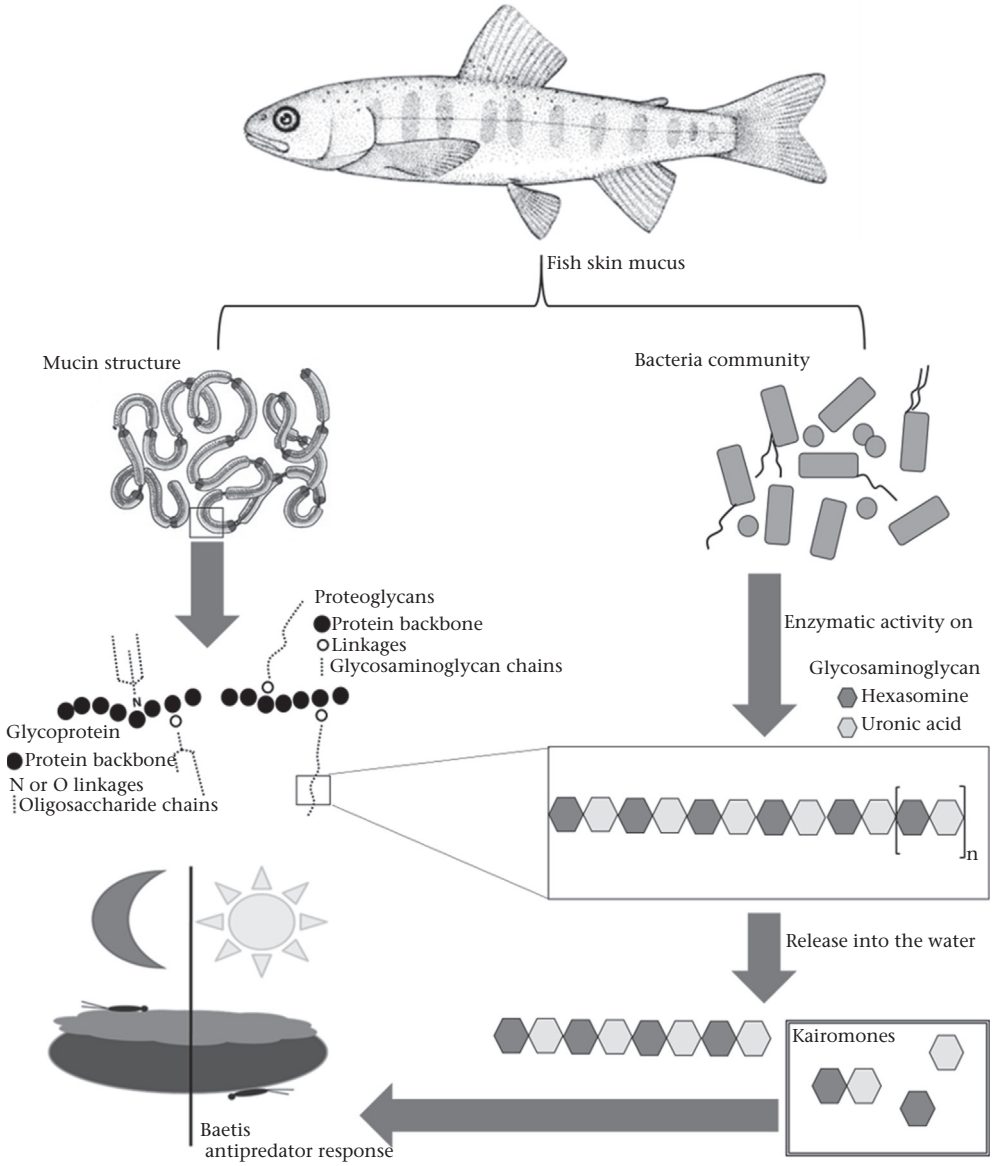

Figure 1. Schematic illustration of the origin and nature of the cue that causes *Baetis* larvae to exhibit risk-sensitive foraging behaviour: fish skin mucus has a mucin structure (based on Ichikawa & Ishihara, 2011), which is composed of polysaccharide chain structures (based on Rittschof & Cohen, 2004), and a mucus-dwelling microbial community (mutualistic interaction). As a result of bacterial enzymatic activity on O-linkages, breakdown products of glycosaminoglycans (GAGs) are released, warning of predator presence and stimulating *Baetis* larvae to reduce risky foraging behaviour.

1991), based on the hydroxy-biphenyl colorimetric reaction, with readings at 520 nm and galacturonic acid as a standard.

Bioassay 1: Fish Mucus Dose

To estimate the dose of salmonid fish mucus needed to stimulate *Baetis* predator avoidance responses, we used a closed system of 20 circular aerated plastic tanks (microcosms) of 8 cm diameter (volume = 0.9 litres) that were supplied with stream water and a natural day–night cycle. The system was in a laboratory at the University of Vigo (Spain), and had been used for previous experiments (Álvarez et al., 2014). We chose a range of mucus doses based on the results of the chemical characterization of Atlantic salmon mucus, which also included an assessment of mucus concentration. Based on these results, we exposed *B. rhodani* to 0, 0.1, 0.3 and 0.6 g of fresh mucus in 1 ml of phosphate buffer solution (PBS) as a stock solution, which resulted in an estimated kairomone concentration of 0, 0.11, 0.33 and 0.66 mg/ml of mucus present in the volume of the microcosm ($N = 5$). For the control treatment (0) we used 1 ml of fishless stream water. While there is a general lack of information about the levels of kairomones needed to stimulate prey responses in nature, these cue concentrations are within the range of levels of mucus-derived kairomones used in other studies (Cohen & Forward, 2003; Rahman et al., 2000).

We held six hatchery-reared Atlantic salmon in a 28-litre tank (59 × 30 cm and 16 cm deep), deprived them of food for 3 days, and collected mucus samples from individuals 2 h before conducting the behavioural observations. To provide a food source for *Baetis* and to homogenize the starting algal biomass, we incubated unglazed tiles in a mesocosm using the same source of stream water to allow benthic algae to colonize for 6 days. The tiles had ridges on their undersides which also provided a refuge for mayfly larvae. In

each microcosm we placed eight *Baetis* and one algae-colonized 4 cm^2 tile.

We ran this bioassay over 2 days in 2013 and performed one night-time (2100 h, 13 January) and then one daytime (0900 h, 14 January) instantaneous scan observation, recording the number of *Baetis* foraging on exposed tile surfaces as an estimate of risky foraging behaviour, to determine the dose of mucus necessary to stimulate predator avoidance behaviour. In all bioassays, we made night-time observations using headlamps with red light, which does not affect the behaviour of *Baetis* (Peckarsky & Cowan, 1995, A. Landeira-Dabarca, M. Álvarez & B. Peckarsky, personal observations).

Bioassay 2: Effects of Fish Mucus Bacteria

In this bioassay, we exposed experienced *B. rhodani* larvae (collected from a stream containing Atlantic salmon) to one of four treatments ($N = 5$): (1) water-borne cues from live Atlantic salmon; (2) fresh salmon mucus to test the effect of fish mucus containing its resident bacteria; (3) UV-sterilized salmon mucus to test the effect of mucus matrix components in the absence of bacteria ; and (4) control fishless stream water. We obtained water-borne salmon cues by holding, for 48 h, two salmon (mean fork length ±1 SE = 16.45 ± 0.37 cm) deprived of food for 3 days in a 28-litre tank (59 × 30 cm and 16 cm deep) filled with water, from which we added aliquots of 1 ml to replicates of this treatment. We collected mucus samples as described above from hatchery-reared salmon (mean fork length ±1 SE = 17.51 ± 0.31 cm).

In mucus treatments (2) and (3), we added 0.3 g of fresh mucus from the stock solution to 1 ml of stream water (estimated kairomone concentration in each microcosm of 0.33 mg/ml), which was the threshold determined by the dose response curve (see Results). In treatment (3) we eliminated bacteria by exposing salmon mucus to ultraviolet (UV) radiation overnight. We applied UVC rays for 16 h at 200 nm and 6.20 eV at a distance of 60 cm (LAF Telstar V-100). To check the effectiveness of the UV treatment, at the end of the bioassay we incubated 0.1 g samples of mucus from treatments (2) and (3) on PCA agar (plate count agar, Cultimed) for 6 days at 22 °C. After incubation, we counted the colony-forming units (CFU) of mucus-dwelling bacteria.

We conducted this bioassay in the same microcosms system used in Bioassay 1. In each microcosm we placed eight *Baetis* larvae (mean individual weight ±1 SE = 1.17 ± 0.30 mg) collected from Zamanes River (Vigo, Spain), and one algae-colonized 4 cm^2 tile.

We ran this bioassay over 3 days and conducted four instantaneous scan observations to record the number of *Baetis* foraging on exposed tile surfaces: two before adding the cue (baseline behaviour: night-time 2100 h on 16 January and daytime 0900 h on17 January 2013) and two after adding the cues (night-time 2100 h on 17 January and daytime 0900 h on18 January 2013). We added 1 ml of the cue treatment solution 1 h before each observation.

Bioassay 3: Role of Fish Mucus Components

We used the same microcosm system as in Bioassays 1 and 2 to test the potential effects of specific chemical components of Atlantic salmon skin mucus on *Baetis* predator avoidance behaviour. In each microcosm, we included eight predator-experienced *B. rhodani* larvae collected from the Zamanes River (individual weight ±1 SE = 0.80 ± 0.02 mg) and exposed them to one of seven treatments ($N = 5$) designed to systematically rule out potential kairomones: (1) Atlantic salmon water-borne cues; (2) salmon water-borne cues treated with proteinase to eliminate the proteins; (3) fresh mucus from Atlantic salmon; (4) lyophilized (freeze-dried) salmon mucus (to standardize the dose of mucus and to distinguish whether the kairomone is a volatile substance); (5) lyophilized salmon mucus treated with proteinase; (6) chondroitin sulphate glycosaminoglycan (CS), which is the major saccharide fraction of the mucus; and (7) control fishless stream water. We added 1 ml of treatment solution into each microcosm.

The rationale for treatment comparisons was as follows: treatments 1 versus 2 and treatments 4 versus 5 distinguish whether the kairomone released into the water by the live fish or fish mucus is protein or saccharide in nature, treatments 3 versus 4 determine whether the kairomone is a volatile or nonvolatile substance (Von Elert & Pohnert, 2000) and treatment 6 tests whether CS is the component acting as a kairomone mediating the interactions between fish and *Baetis* larvae.

We prepared water-borne treatments (1 and 2) by placing two salmon deprived of food for 3 days in a 28-litre tank for 48 h. We prepared treatments using proteinase (2 and 5) by incubating (37 °C for 1 h) the water-borne fish cues (2) or the mucus (5) with a nonspecific protein degradation enzyme (proteinase K [Sigma]; PK; following Mathuru et al., 2012) in a concentration of 0.1 mg/ml. We confirmed the effectiveness of the proteinase on those mucus samples using a polyacrylamide gel (SDS-PAGE) stained with Coomassie Blue. We collected samples of fresh mucus (3) from hatchery-reared salmon (mean fork length ±1 SE = 17.6 ± 0.5 cm) 2 h before conducting the behavioural observations, adding stock solution doses of 0.3 g of fresh mucus to 1 ml of stream water (following Álvarez et al., 2014). We prepared lyophilized mucus (4 and 5) by freeze-drying the mucus samples in a speed-vacuum (Savant) for 5 h at maximum speed. We diluted commercial CS (C4384 Sigma; for treatment 6) in PBS pH 7.2 to 6 mg/ml concentration, based on the results of the mucus chemical characterization. Each microcosm received 1 ml of cue, representing an estimated kairomone concentration of 13 μg/ml of dry mucus and CS calibrated by the volume of the microcosm.

We ran this bioassay over 3 days in 2013, during which we conducted two instantaneous scan observations before adding the cue (baseline behaviour: night-time 2100 h on 21 March and daytime 0900 h on 22 March) and two instantaneous scan observations after adding the cues (night-time 2100 h on 22 March and daytime 0900 h on 23 March), recording the number of *Baetis* foraging on exposed tile surfaces. Cues were added to this closed system once on 22 March 1 h before the night-time observation (2000 h), by administrating 1 ml of treatment solution.

Bioassay 4: Role of Fish Mucopolysaccharides

In this bioassay we tested the response of North American *B. bicaudatus* to specific sugars present in the mucopolysaccharide matrix of brook trout skin mucus: the complete GAG molecule (i.e. CS) and/or its monosaccharide breakdown products (i.e. hexosamine and uronic acid; Fig. 1).

We collected naïve *B. bicaudatus* larvae from a fishless stream and exposed them to one of the following treatments ($N = 12$): (1) fresh brook trout mucus; (2) commercial CS; (3) commercial hexosamine, i.e. (N) acetyl-glucosamine; (4) commercial uronic acid, i.e. glucuronic acid; and (5) control fishless stream water.

We implemented this bioassay in a streamside system of 60 circular Plexiglas flow-through microcosms (flow = 12.3 cm/s) of 15 cm diameter where many previous studies of *Baetis* responses to salmonid cues have been conducted (e.g. McIntosh & Peckarsky, 2004; Álvarez et al., 2014). The microcosms received natural stream water gravity-fed from a nearby fishless stream and were housed in an opaque white portable greenhouse (Hansen WeatherPort, Delta, CO, U.S.A.) adjacent to the East River at the Rocky Mountain Biological Laboratory (RMBL), Gunnison County, Colorado (U.S.A.). We used the similar food source and refuge tiles as in

previous bioassays. In each microcosm we placed six *Baetis* larvae (individual dry weight ±1 SE = 1.02 ± 0.03 mg) and six algae-colonized 6.25 cm^2 tiles.

For treatment 1 we collected mucus from 10 hatchery-reared brook trout (mean fork length ±1 SE = 9.93 ± 0.27 cm) following the same procedure as in previous bioassays. We used stock solution of 0.3 g of fresh mucus in 1 ml of fishless stream water as the dose of cue for this treatment (threshold estimated in Bioassay 1). The CS used in treatment 2 was added as a stock solution at a concentration of 6 mg/ml (concentration of CS present in salmonid skin mucus determined in mucus chemical characterization). Because CS is composed of repeated units of hexosamine and uronic acid (approximately 50–50%), for treatments 3 and 4 we prepared solutions of hexosamine ((*N*) acetyl-glucosamine A8625 Sigma previously detected in salmonid skin mucus; Landeira-Dabarca, Álvarez, & Molist, 2014) and uronic acid (glucuronic acid G5269 Sigma) at a stock concentration of 3 mg/ml.

Cues were added to the microcosms by continually releasing drops of the solutions from 50 ml Falcon tubes via Tygon tubes of 7 mm diameter. We adjusted a valve that clamped each tube to create comparable drips between replicates and treatments at 1 drop per 3 s. Solutions were dripped for 15–20 min into each microcosm; thus each microcosm received approximately 300 drops (20 ml) of solution, representing an estimated kairomone concentration of 3.25 μg of fresh mucus, 0.065 μg CS and 0.0325 μg of hexosamine or uronic acid calibrated by the volume of flow through the microcosms.

We ran this bioassay over 3 days, during which we conducted four instantaneous scan observations to record the number of *Baetis* foraging on exposed tile surfaces, two before adding the cue (baseline behaviour: night-time 2100 h on 1 July and daytime 0900 h on 2 July 2013) and two after adding the cues (night-time 2100 h on 2 July and daytime 0900 h on 3 July 2013). Cues were added 1 h before each observation in this flow-through system.

Bioassay 5: Hexosamine Dose

In the previous bioassay we observed that *Baetis* showed the strongest response to the monosaccharide hexosamine (see Results). Thus, we designed a dose–response bioassay to estimate the threshold dose of hexosamine needed to produce a significant behavioural change in *Baetis* larvae. We collected naïve *B. bicaudatus* larvae from a fishless stream and exposed them in microcosms ($N = 10$) to different stock concentrations of (N) acetyl-glucosamine by using dilutions in control fishless stream water: (1) 0 mg/ml; (2) 0.03 mg/ml; (3) 0.075 mg/ml; (4) 0.15 mg/ml; (5) 0.225 mg/ml; and (6) 0.3 mg/ml of N-acetyl-glucosamine.

We implemented this bioassay at the RMBL, in the same system of streamside microcosms and following the same design (six *Baetis* larvae and six benthic algae-colonized tiles) as Bioassay 4.

We ran this bioassay over 2 days in 2013, during which we conducted one night (2100 h on 27 July) and one day (0900 h on 28 July) instantaneous scan behavioural observation of the number of *Baetis* foraging on exposed substrate surfaces. Cues were added into the microcosms by continually releasing drops of the solutions from 50 ml Falcon tubes via Tygon tubes of 7 mm diameter. Drips were adjusted as in Bioassay 4, representing an estimated kairomone concentration of 0, 0.32, 0.81, 1.62, 2.43 and 3.25 ng of hexosamine calibrated by the volume of flow through the microcosms. These cue concentrations are within the range of levels of other aquatic infochemicals (hormones, pheromones and kairomones) that are found in nature (Guardiola, Cuesta, & Esteban, 2016; Bucciarelli & Kats, 2015). We conducted behavioural observations of *Baetis* 40 min after the drips stopped (Álvarez et al., 2014).

For all bioassays water temperature was recorded continuously using Onset Stowaway data loggers (Onset Computer, Pocasset, MA, U.S.A.).

Statistical Analysis

To test the effect of doses in Bioassays 1 and 5 we conducted an ANOVA and Tukey test for post hoc comparisons between treatments on the response variable: average number of *Baetis* observed on exposed substrate surfaces over a 24 h cycle (day and night). We conducted a one-way ANOVA to test the effectiveness of the UV treatment (Bioassay 2) on bacterial density associated with salmonid mucus.

We tested the difference between the responses of *Baetis* to potential kairomones before and after addition of the cues (Bioassays 2, 3 and 4) by using the rate of change in the number of *Baetis* observed on exposed surfaces of substrates over the course of the bioassay before (as a baseline) versus after adding the cue as the response variable (increased number exposed = high risk; decreased number exposed = low risk). This variable is a proxy for effect size and was calculated as follows: [(ln numbers exposed at the end of the experiment − ln numbers exposed before cues were added)/days of the bioassay]. Then, we used ANOVA to compare rates of change of *Baetis* observed on exposed surfaces between treatments, and post hoc tests using Bonferroni corrections to correct for experiment-wise error caused by multiple pairwise comparisons, thereby testing for the effects of treatments on differences in refuge use of *Baetis* before and after adding the cue.

In all cases, the data obtained to quantify prey responses to predator cues (changes in foraging behaviour and refuge use) met normality assumptions (Kolmogorov–Smirnov test ($Z = 0.129$, $P = 0.200$; $Z = 0.847$, $P = 0.384$; $Z = 1.645$, $P = 0.096$; $Z = 0.901$, $P = 0.391$; $Z = 0.867$, $P = 0.440$, respectively for Bioassays 1–5) and homogeneity of variance (Levene's test = 1.220, $P = 0.335$; Levene's test = 1.372, $P = 0.255$; Levene's test = 0.720, $P = 0.705$; Levene's test = 0.616, $P = 0.653$; Levene's test = 1.408, $P = 0.257$, respectively for Bioassays 1–5)). All tests were conducted with SPSS STATISTICS 25.0 (IBM Corp, 2017).

Ethical Note

The care and protocols for using fish and invertebrates for experiments conducted at RMBL were approved by the RMBL Animal Welfare Committee. Protocols adhered to guidelines proposed by RMBL professional societies and complied with federal, state and local laws regarding the use of invertebrate and vertebrate animals in research.

No anaesthesia was used during collection of fish mucus to avoid negative effects on viability of skin mucus microorganisms (A. Landeira-Dabarca, M. Álvarez, & B. Peckarsky, personal observation). However, stress was minimized by quick handling and allowing the fish to recover completely in an aerated bucket with fresh stream water (generally 5–10 min) before releasing them to their original locations at Carballedo Hatchery (Galicia, NW Spain) and the Pitkin Fish Hatchery (Gunnison County, CO, U.S.A.). No mortality occurred in the sampled animals.

The mayflies used in this study were collected with a D-net and kept cold during transport and later under experimental conditions (see details above). After the experiments, individuals were euthanized in a freezer at −20 °C.

RESULTS

Chemical Characterization of Salmonid Skin Mucus

We collected 1.48 ± 0.08 g (mean ± SE) of fresh skin mucus for each Atlantic salmon, which is equivalent to 6 ± 0.26 mg (mean ± SE) of dry mucus. Therefore, water content made up to 99.2% of the fresh weight of mucus. This amount of mucus contained 0.21 ± 0.04 mg (mean ± 1SE) of proteins and 5.05 ± 0.95 mg of sulphated sugars, which provides a good estimate of the amount of GAGs (e.g. CS). Moreover, quantification of the two subunits (monosaccharides) of the GAGs revealed a hexosamine concentration of 0.39 ± 0.07 mg/ml and a concentration of uronic acid of 0.42 ± 0.08 mg/ml (mean ± 1SE).

Bioassay 1: Fish Mucus Dose

There were significant differences in the refuge use of *B. rhodani* between treatments ($F_{3,20} = 6.055$, $P = 0.006$). Post hoc comparisons showed that, compared to controls, the number of *B. rhodani* observed on exposed substrate surfaces decreased significantly when the larvae were subjected to at least 0.3 g/ml stock solution of fresh mucus ($P = 0.018$; Fig. 2). This stock concentration of fresh mucus corresponds to an estimated concentration of 0.33 mg/ml of fresh mucus in the microcosms.

Bioassay 2: Effects of Fish Mucus Bacteria

Exposure of mucus to the UV treatment significantly and indiscriminately reduced (94–100%) the cultivable bacterial community of the skin mucus of Atlantic salmon ($F_{1,6} = 11\ 022.34$, $P < 0.001$). There were no differences between treatments in the number of *B. rhodani* foraging on exposed substrate surfaces before adding the cues ($F_{3,20} = 3.333$, $P = 0.950$). Therefore, any differences in the rates of change in the abundance of *B. rhodani* larvae foraging on exposed substrate surfaces between treatments could be attributed to the effect of the addition of cues. There were significant differences between treatments in the rates of change in the abundance of *B. rhodani* foraging on exposed surfaces of tiles before versus after cues were added ($F_{3,20} = 3.867$, $P = 0.030$). Post hoc comparisons showed that refuge use of *B. rhodani* increased significantly compared to controls when they were exposed to either (1) fresh salmon mucus ($P = 0.008$) or (2) salmon mucus treated with UV ($P = 0.032$; Fig. 3). Therefore, *B. rhodani* responded to fish mucus cues even if mucus-dwelling bacteria were not present.

Bioassay 3: Role of Fish Mucus Components

As in Bioassay 2, there were no differences between treatments in the number of *B. rhodani* foraging on exposed substrate surfaces before adding the cues ($F_{6,35} = 12.733$, $P = 0.239$). However, there were significant differences between treatments in the rates of change of the number of *B. rhodani* foraging on exposed substrate surfaces before and after adding the cue ($F_{6,35} = 3.820$, $P = 0.001$). Post hoc comparisons revealed that, compared to controls, the number of *B. rhodani* foraging on exposed substrate surfaces decreased significantly when they were treated with (1) fresh salmon mucus ($P = 0.008$), (2) lyophilized mucus ($P = 0.008$) and (3) lyophilized mucus deprived of the protein fraction (i.e. treated with PK; $P = 0.002$; Fig. 4). Therefore, *B. rhodani* only responded to cues that included the saccharide fraction of fish mucus, but not to the protein fraction or the full structure of mucus glycosaminoglycan (CS-GAG; Fig. 4).

Bioassay 4: Role of Fish Mucopolysaccharides

As in Bioassays 2 and 3, there were no significant differences between treatments in the number of *B. bicaudatus* foraging on exposed substrate surfaces at the start ($F_{4,60} = 3.383$, $P = 0.496$). However, there were significant differences between treatments in the rates of change in the number of *B. bicaudatus* foraging on exposed substrate surfaces before versus after adding the cues ($F_{4,60} = 5.371$, $P = 0.001$). Specifically, post hoc comparisons showed that risky behaviour of *Baetis* was reduced significantly (declined over the bioassay) compared to controls when *Baetis* were treated with (1) fresh mucus from brook trout ($P = 0.001$) and (2) hexosamine ($P = 0.003$), but not when subjected to the full structure of mucus glycosaminoglycan (CS-GAG) or uronic acid (Fig. 5). These outcomes demonstrate that, in addition to responding to a natural kairomone in the form of fresh mucus from brook trout, *B. bicaudatus* also responded to one of the two

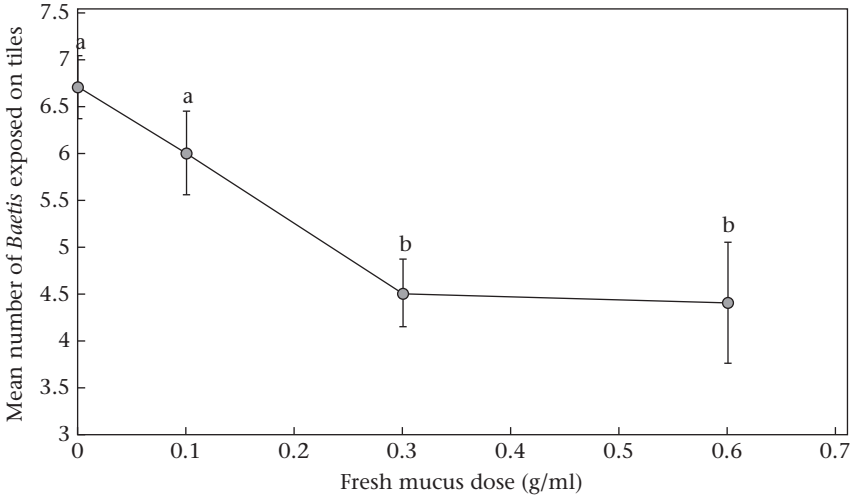

Figure 2. Dose–response curve describing the magnitude of the predator avoidance response of *B. bicaudatus* (estimated as number of mayfly larvae foraging on exposed tile surfaces) as a function of exposure to fresh mucus of Atlantic salmon. Concentrations of stock solutions of mucus ranged from 0 to 0.6 g/ml of cue corresponding to an estimated kairomone concentration in the volume of microcosms from 0 to 0.66 mg/ml.

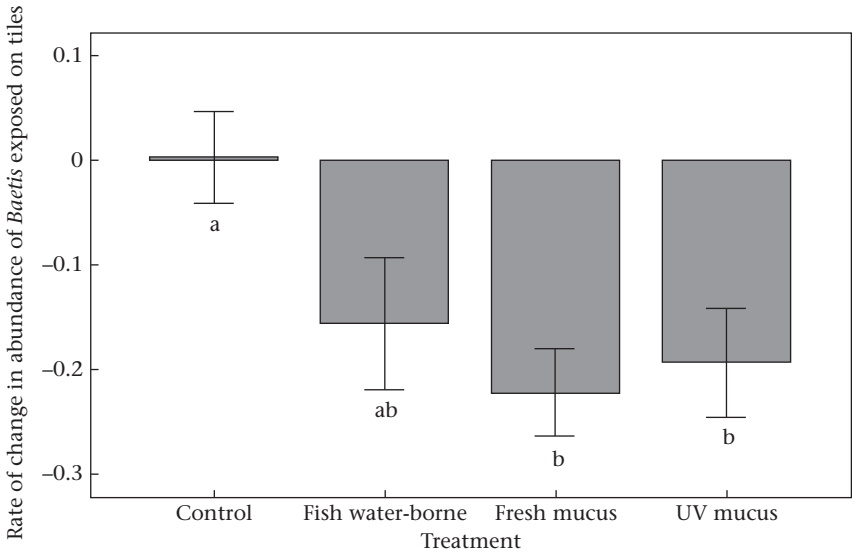

Figure 3. Rate of change (mean ± 1SE) in the number of *B. rhodani* foraging on exposed substrate surfaces over the course of the bioassay before versus after adding the following cues as treatments: fishless water (control); water-borne cue from Atlantic salmon; fresh Atlantic salmon mucus; UV-sterilized mucus. More negative rates of change indicate a reduction in risky responses to predator cues, thereby corresponding to stronger predator avoidance behaviour. Different lowercase letters (a, b) denote significant differences between treatments ($P < 0.05$).

commercial monosaccharides (hexosamine), which is a microbially mediated breakdown product of fish mucus glycosaminoglycans (Fig. 1).

Bioassay 5: Hexosamine Dose

There were significant differences between dose treatments in the mean number of *B. bicaudatus* foraging on exposed tile surfaces ($F_{5,60} = 3.290$, $P = 0.021$). Post hoc comparisons showed that, compared to controls (0 mg/ml), the number of *B. bicaudatus* observed on exposed substrate surfaces decreased significantly (i.e. mayflies reduced risky foraging behaviour) when treated with stock solutions of at least 0.225 mg/ml of the hexosamine (N) acetyl-glucosamine ($P = 0.035$), with no greater response to higher doses (Fig. 6). This outcome demonstrates the existence of a threshold of hexosamine needed to stimulate predator avoidance by *B. bicaudatus*.

DISCUSSION

As in previous studies (e.g. Brönmark & Hansson, 2000; Peckarsky et al., 2008; Winkelmann et al., 2008; Álvarez et al.,

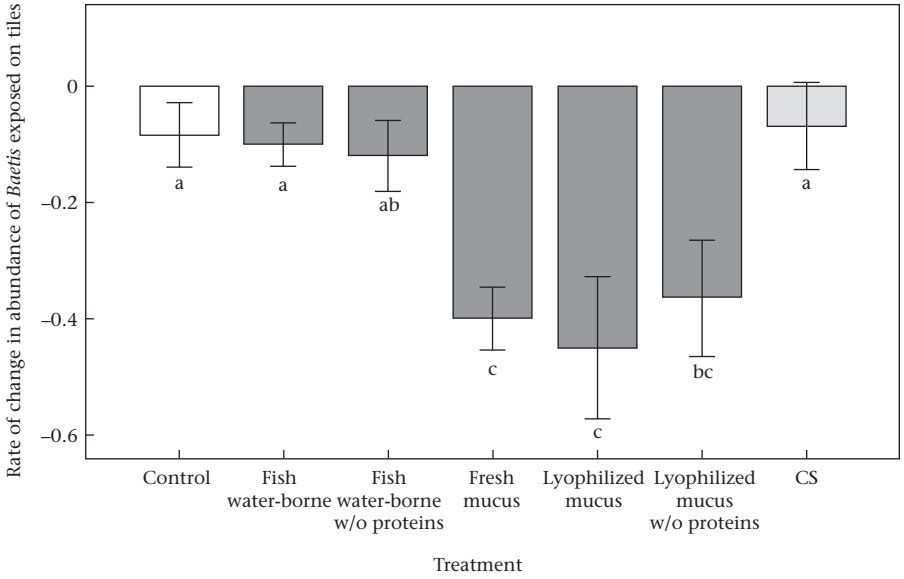

Figure 4. Rate of change (mean ± 1SE) in the number of *B. rhodani* foraging on exposed substrate surfaces over the course of the bioassay before versus after adding the following cues as treatments: fishless water (control); water-borne cue from Atlantic salmon; water-borne cue from Atlantic salmon without the protein fraction; fresh Atlantic salmon mucus; lyophilized Atlantic salmon mucus; lyophilized Atlantic salmon mucus without the protein fraction; chondroitin sulphate (CS). More negative rates of change indicate a reduction in risky responses to predator cues, thereby corresponding to stronger predator avoidance behaviour. Different lowercase letters (a, b, c) denote significant differences between treatments ($P < 0.05$).

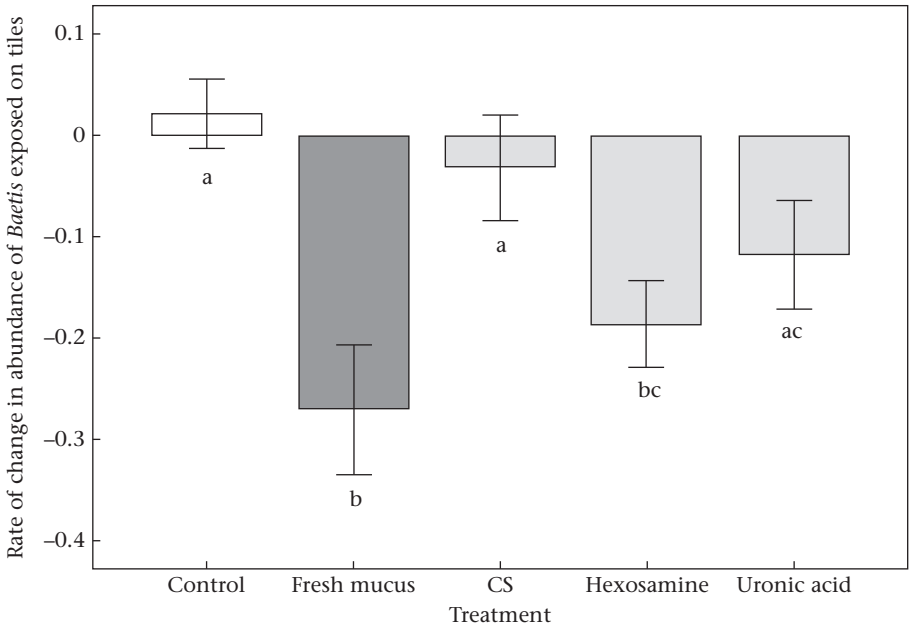

Figure 5. Rate of change (±1SE) in the number of *B. bicaudatus* foraging on exposed substrate surfaces over the course of the bioassay before versus after adding the following cues: fishless water (control); fresh brook trout mucus: chondroitin sulphate (CS); and the two main breakdown products of glycosaminoglycan, hexosamine and uronic acid. More negative rates of change indicate a reduction in risky responses to predator cues, thereby corresponding to stronger predator avoidance behaviour. Different lowercase letters (a, b, c) denote significant differences between treatments ($P < 0.05$).

2014), we observed that fish water-borne chemical cues and fish mucus (Álvarez et al., 2014) stimulated predator avoidance responses of two species of *Baetis* larvae to natural salmonid predators, confirming that fish skin mucus constitutes the origin of the water-borne predator cue. Furthermore, the threshold dose of Atlantic salmon skin mucus at which we observed a measurable predator avoidance response was 0.33 mg/ml (estimated kairomone concentration per volume of microcosms), which corresponds to 1.3 μg/ml glycosaminoglycans (GAGs). A threshold dose of predator cue has also been reported for other predator–prey interactions that involve fish as a predator (for example Pohnert and Von Elert (2000) involving *Daphnia*, Rittschof and Cohen (2004) and Forward and Rittschof (1999) with shrimp larvae, Cohen and Forward (2003) with crab larvae and Mirza and Chivers (2003) using fish as prey).

The unexpected lack of a behavioural response of *B. rhodani* to treatments using water-borne cues originating from live Atlantic salmon in Bioassays 2 and 3 could potentially be attributed to dilution of the experimental cue below the threshold needed to stimulate a response (only 1 ml of cue was added once and not continuously). Similarly, subthreshold cues have been demonstrated to fail to affect prey traits in other studies (Ferrari, Trowell, Brown, & Chivers, 2005; Siepielski, Fallon, & Boersma, 2016), such as habitat selection by aquatic beetles (Trekels & Vanschoenwinkel,

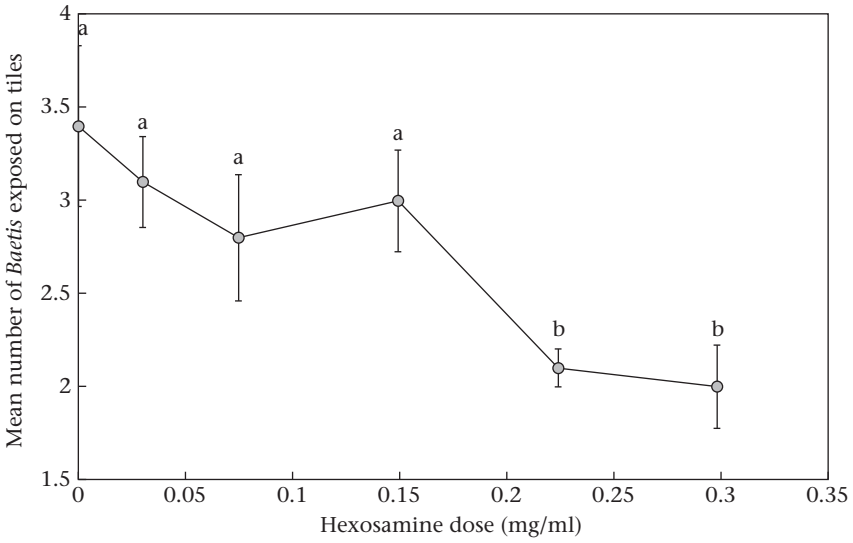

Figure 6. Dose–response curve describing the magnitude of the predator avoidance response of *B. bicaudatus* (estimated as number of mayfly larvae foraging on exposed tile surfaces) as a function of treatment with the hexosamine (N) acetyl-glucosamine at different concentrations (from 0 to 0.3 mg/ml stock cue solution, which corresponds to 0 to 3.25 ng/ml estimated kairomone concentration calibrated by microcosm flow). Different lowercase letters (a, b) denote significant differences between treatments ($P < 0.05$).

2017), size of *Culex* larvae (Jourdan et al., 2016), and morphology (Tollrian, 1995) or life history of *Daphnia* (Pestana, Baird, & Soares, 2013).

The hypothesis that microorganisms, such as mucus-dwelling bacteria closely associated with the fish predator, are involved in chemical cue production has been gaining traction in studies of predator–prey interactions in freshwater systems (Akkas et al., 2010; Beklioglu et al., 2006; Boriss, Boersma, & Wiltshire, 1999, Forward & Rittschof, 1999, ; Rahman et al., 2000; Ringelberg & Van Gool, 1998). However, our results testing the effect of salmon mucus without bacteria (UV treatment in Bioassay 2) indicate that the bacteria themselves are not the kairomone stimulating predator avoidance responses. Nevertheless, our bioassays are consistent with the hypothesis that chemicals released by well-documented breakdown activity of bacteria as commensals in fish skin mucus act as kairomones, if compounds previously produced by bacterial activity persist in the mucus. However, some studies have shown that kairomones associated with bacterial activity can completely degrade in 24 h unless more substrate is added (Boriss et al., 1999; Loose et al., 1993). Therefore, not only the dose of the predator cue but also the timing of the availability of the cue determines whether prey can detect the presence of the predator (Van Buskirk, Krügel, Kunz, Miss, & Stamm, 2014).

As in other studies (Von Elert & Pohnert, 2000), we observed a predator avoidance response of *Baetis* to lyophilized and fresh mucus, which is consistent with the hypothesis that the kairomone involved in the fish predator–mayfly prey interaction is a water-soluble molecule and nonvolatile. This characterization of the properties of the kairomone is an important distinction from other systems, because recent studies on predator-released kairomones that deter insect oviposition described a volatile substance that is possible to detect from above the water surface (Hurst, Kay, Brown, & Ryan, 2010; Silberbush et al., 2010). However, as further evidence that kairomones recognized by mayflies are not volatile substances, ovipositing females of *Baetis bicaudatus* (Encalada & Peckarsky, 2011) and *Callibaetis ferrugineus hageni* (Caudill, 2003) do not discriminate between fish and fishless water, even though both species suffer high mortality in aquatic habitats containing brook trout.

In contrast to other studies, results from Bioassay 3 enabled us to rule out the protein fraction of the mucus as a critical component of the kairomone used by *Baetis* to detect salmonid predators. Many infochemicals in freshwater ecosystems that have been identified as protein in nature mediate the communication between individuals of the same species (e.g. pheromones: Brönmark & Hansson, 2000; Burks & Lodge, 2002; Ferrari, Wisenden, & Chivers, 2010).

Also contrary to the results of other studies that used fish as both predators and as prey (e.g. Brown, Paige, & Godin, 2000; Faulkner et al., 2017; Mathuru et al., 2012), our results showed that the complete molecule of chondroitin sulphate-GAG (a long hydrocarbon structure) was not the kairomone causing *Baetis* to respond to predatory salmonids (Bioassays 3 and 4). Instead, *B. bicaudatus* significantly reduced risky foraging activity when exposed to one of the monosaccharide units that comprise the CS molecule, i. e hexosamine, and showed only a weak (nonsignificant) response to the other, uronic acid. These two GAG subunits together (i.e. disaccharides) have been shown to contribute to the kairomone in previous studies testing responses of other invertebrate prey species to predators (e.g. Forward & Rittschof, 1999; Rahman et al., 2000; Rittschof & Cohen, 2004); but to our knowledge, this is the first time these subunits have been tested separately.

Nevertheless, the active component of predator fish kairomone molecules (whether it is a charged sulphated, aminated, acetylated and/or carboxylic groups) is still under debate (Akkas et al., 2010; Cohen & Forward, 2003; Forward & Rittschof, 1999, 2000; Rahman et al., 2000). Many of these studies reported that kairomone molecules without the acetyl-amine functional groups did not trigger prey responses (e.g. Cohen & Forward, 2003), which may explain the stronger predator avoidance response of *B. bicaudatus* to hexosamine (with amino and acetyl groups) compared to their weaker response to uronic acid (with carbonyl and carboxylic acid functional groups). Interestingly, we observed that the predator avoidance response of *Baetis* was stronger when exposed to natural fish mucus than to single commercial monosaccharides, which agrees with other studies investigating the nature of the active components of infochemicals (Mathuru et al., 2012; Rahman et al., 2000). This observation suggests that although monosaccharides may be an active component of the kairomone, the predator avoidance response of *Baetis* was stronger when combined with other components of natural mucus.

In addition, the significant prey responses to a subunit rather than to the complete GAG structure provides further support to the hypothesis that bacterial activity breaking up these long hydrocarbon structures is necessary for kairomone release (Beklioglu et al., 2006; Forward & Rittschof, 1999; Ringelberg & Van Gool, 1998; Rittschof & Cohen, 2004). However, more studies are needed to explore this possibility.

Few studies on chemical communication between aquatic organisms have calculated the dose needed to stimulate a predator avoidance response by prey. We estimated 0.225 mg/ml (stock solution) of the monosaccharide hexosamine as the threshold of the kairomone emanating from fish needed to reduce risky foraging behaviour in *Baetis*. Cohen and Forward (2003) reported a similar concentration of CS and/or HA disaccharides (0.22 mg/ml) needed to stimulate diel vertical migration in crab larvae and Rahman et al. (2000) observed that 0.26 mg/ml of HA commercial disaccharide caused predator avoidance responses in snails. This hexosamine concentration corresponds to a kairomone concentration of 2.43 ng/ml calibrated by the flow through the microcosms, which is in the range of other mucus components released into water, such as cortisol ($1.39 \pm 0.98 - 5.28 \pm 1.96$ ng/ml, Guardiola et al., 2016), which is similar in size and composition to hexosamine. Moreover, exposing *Baetis* to kairomone concentrations above the observed threshold did not change their behavioural response to fish, as previously demonstrated in other prey–predator interactions (Cohen & Forward, 2003; Forward & Rittschof, 1999; Mirza & Chivers, 2003; Pohnert & Von Elert, 2000; Rittschof & Cohen, 2004).

In summary, this study reports new evidence regarding the mechanism, origin, nature and identity of the chemical compounds utilized in interspecific communication between fish and aquatic insects. Our bioassays enabled us to conclude and contribute to the increasing evidence that the amino sugars that are bacterially mediated breakdown products of the long chains of skin mucus glycosaminoglycans, primarily hexosamine (Fig. 1) are critical components of the fish kairomones that elicit a predator avoidance response in *Baetis* larvae.

Our research reinforces the potential for independent evolution of generalized predator avoidance responses to compounds that are strong infochemicals in the aquatic environment by many species of aquatic prey that recognize similar kairomones and at similar threshold doses (Rahman et al., 2000). In addition, the observation of similar predator avoidance responses by North American *B. bicaudatus* and European *B. rhodani* to a wide variety of fish species (Álvarez et al., 2014) suggests that kairomones originating from skin mucus of different species of fish may be common and similar (McKelvey & Forward, 1995; Ringelberg & Van Gool, 1998; Álvarez et al., 2014). We speculate that this generalized response by prey could be an advantage in a changing world with

introduction of invasive and potentially novel predators of *Baetis*. In contrast, general predator avoidance responses may result in loss of fitness of prey species that are responding to nonpredatory fish species, thereby allocating unnecessary time to predator avoidance (Pestana et al., 2013; Polačik & Janáč, 2017; Šmejkal et al., 2018).

Acknowledgments

We thank the 2013 Benthettes (Marge Penton, Steve Horn, Kara Cromwell, Jasmine Hamilton and Mencía Moreno) for their help implementing the bioassays at the Rocky Mountain Biological Laboratory, fish mucus sampling and for contributing ideas to the manuscript. Additionally, we thank the staff of the Pitkin's Hatchery (Colorado, U.S.A.) and to the staff of the Carballedo's Hatchery (Pontevedra, Spain) for providing fish for this study. We are also very grateful to A. Puime for his support and help during the bioassays at UVigo and to P. Rodriguez-Lozano for his input to the manuscript. We also thank Microbial Biotechnology group from UVigo, especially C. Sieiro, B. García-Fraga and A. Fernández-Da Silva, and Genetic- XB2 group from UVigo, especially P. Morán and L. Covelo-Soto for help with this study. This paper was improved by input from numerous anonymous referees. This study was supported by a project funded by the Spanish Ministry of Science and Innovation (National Program for Fundamental Research, CGL 2009-07904) to MA and a National Science Foundation grant (DEB 0516035) and Kingsdale fellowship and fellowships from the Rocky Mountain Biological Laboratory to B.L.P. The work was finalized with support from University of South Bohemia, Faculty of Science & SoWa (LM2015075, EF16_0130001782-SoWa Ecosystems Research) to A.L.D.

References

Akkas, S. B., Kepenek, A. O., Beklioglu, M., & Severcan, F. (2010). Molecular approach to the chemical characterization of fish-exuded kairomone: A fourier transform infrared spectroscopic study. *Aquatic Sciences, 72*(1), 71–83. https://doi.org/10.1007/s00027-009-0114-2.

Álvarez, M., Landeira-Dabarca, A., & Peckarsky, B. L. (2014). Origin and specificity of predatory fish cues detected by *Baetis* larvae (Ephemeroptera; Insecta). *Animal Behaviour, 96*, 141–149. https://doi.org/10.1016/j.anbehav.2014.07.017.

Bansil, R., & Turner, B. S. (2018). The biology of mucus: Composition, synthesis and organization. *Advanced Drug Delivery Reviews, 124*, 3–15. https://doi.org/10.1016/j.addr.2017.09.023.

Beklioglu, M., Telli, M., & Gozen, A. G. (2006). Fish and mucus-dwelling bacteria interact to produce a kairomone that induces diel vertical migration in *Daphnia*. *Freshwater Biology, 51*(12), 2200–2206. https://doi.org/10.1111/j.1365-2427.2006.01642.x.

Blumenkrantz, N., & Asboe-Hansen, G. (1973). New method for quantitative determination of uronic acids. *Analytical Biochemistry, 54*, 484–489. https://doi.org/10.1016/0003-2697(73)90377-1.

Boriss, H., Boersma, M., & Wiltshire, K. H. (1999). Trimethylamine induces migration of waterfleas. *Nature, 398*(6726), 382.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*(1–2), 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Brönmark, C., & Hansson, L. A. (2000). Chemical communication in aquatic systems: An introduction. *Oikos, 88*(1), 103–109. https://doi.org/10.1034/j.1600-0706.2000.880112.x.

Brown, G. E., Paige, J. A., & Godin, J. G. J. (2000). Chemically mediated predator inspection behaviour in the absence of predator visual cues by a characin fish. *Animal Behaviour, 60*, 315–321. https://doi.org/10.1006/anbe.2000.1496.

Bucciarelli, G. M., & Kats, L. B. (2015). Effects of newt chemical cues on the distribution and foraging behavior of stream macroinvertebrates. *Hydrobiologia, 749*(1), 69–81. https://doi.org/10.1007/s10750-014-2146-4.

Burks, R. L., & Lodge, D. M. (2002). Cued in: Advances and opportunities in freshwater chemical ecology. *Journal of Chemical Ecology, 28*(10), 1901–1917. https://doi.org/10.1023/A:1020785525081.

Caudill, C. C. (2003). Empirical evidence for nonselective recruitment and a source-sink dynamic in a mayfly metapopulation. *Ecology, 84*(8), 2119–2132. https://doi.org/10.1890/0012-9658(2003)084[2119:EEFNRA]2.0.CO, 2.

Chapman, D. W. (1966). Food and space as regulators of salmonid populations in streams. *American Naturalist, 100*(913), 345–357. https://doi.org/10.1086/282427.

Cohen, J. H., & Forward, R. B., Jr. (2003). Ctenophore kairomones and modified aminosugar disaccharides alter the shadow response in a larval crab. *Journal of Plankton Research, 25*(2), 203–213. https://doi.org/10.1093/plankt/25.2.203.

Encalada, A. C., & Peckarsky, B. L. (2011). The influence of recruitment on within-generation population dynamics of a mayfly. *Ecosphere, 2*(10), 107. https://doi.org/10.1890/ES11-00103.1.

Farndale, R. W., Buttle, D. J., & Barrett, A. J. (1986). Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochemical and Biophysics Acta, 883*, 173–177. https://doi.org/10.1016/0304-4165(86)90306-5.

Faulkner, A. E., Holstrom, I. E., Molitor, S. A., Hanson, M. E., Shegrud, W. R., Gillen, J. C., et al. (2017). Field verification of chondroitin sulfate as a putative component of chemical alarm cue in wild populations of fathead minnows (*Pimephales promelas*). *Chemoecology, 27*, 233–238. https://doi.org/10.1007/s00049-017-0247-z.

Ferrari, M. C., Trowell, J. J., Brown, G. E., & Chivers, D. P. (2005). The role of learning in the development of threat-sensitive predator avoidance by fathead minnows. *Animal Behaviour, 70*(4), 777–784. https://doi.org/10.1016/j.anbehav.2005.01.009.

Ferrari, M. C., Wisenden, B. D., & Chivers, D. P. (2010). Chemical ecology of predator–prey interactions in aquatic ecosystems: A review and prospectus. *Canadian Journal of Zoology, 88*, 698–724. https://doi.org/10.1139/Z10-029.

Filisetti-Cozzi, T. M., & Carpita, N. C. (1991). Measurement of uronic acids without interference from neutral sugars. *Analytical Biochemistry, 197*, 157–162. https://doi.org/10.1016/0003-2697(91)90372-Z.

Flecker, A. S. (1992). Fish predation and the evolution of invertebrate drift periodicity: Evidence from neotropical streams. *Ecology, 73*(2), 438–448. https://doi.org/10.2307/1940751.

Forward, R. B., Jr., & Rittschof, D. (1999). Brine shrimp larval photo-responses involved in diel vertical migration: Activation by fish mucus and modified amino sugars. *Limnology & Oceanography, 44*(8), 1904–1916. https://doi.org/10.4319/lo.1999.44.8.1904.

Forward, R. B., Jr., & Rittschof, D. (2000). Alteration of photoresponses involved in diel vertical migration of a crab larva by fish mucus and degradation products of mucopolysaccharides. *Journal of Experimental Marine Biology and Ecology, 245*, 277–292. https://doi.org/10.1016/S0022-0981(99)00169-0.

Guardiola, F. A., Cuesta, A., & Esteban, M.Á. (2016). Using skin mucus to evaluate stress in gilthead seabream (*Sparus aurata* L.). *Fish & Shellfish Immunology, 59*, 323–330. https://doi.org/10.1016/j.fsi.2016.11.005.

Hawkers, J. W. (1974). The structure of fish skin. I. General organization. *Cell and Tissue Research, 149*(2), 147–158. https://doi.org/10.1007/BF00222270.

Hurst, T. P., Kay, B. H., Brown, M. D., & Ryan, P. A. (2010). *Melanotaenia duboulayi* influence oviposition site selection by *Culex annulirostris* (Diptera: Culicidae) and *Aedes notoscriptus* (Diptera: Culicidae) but not *Culex quinquefasciatus* (Diptera: Culicidae). *Environmental Entomology, 39*(2), 545–551. https://doi.org/10.1603/EN09138.

IBM Corp. (2017). *IBM SPSS Statistics for Windows*. Armonk, NY: IBM Corp Version 25.0. .

Ichikawa, T., & Ishihara, K. (2011). Protective effects of gastric mucus. In P. Tonino (Ed.), *Gastritis and gastric cancer – New insights in gastroprotection, diagnosis and treatments* (pp. 3–24). Amsterdam, The Netherlands: InTech.

Jourdan, J., Baier, J., Riesch, R., Klimpel, S., Streit, B., Müller, R., et al. (2016). Adaptive growth reduction in response to fish kairomones allows mosquito larvae (*Culex pipiens*) to reduce predation risk. *Aquatic Sciences, 78*(2), 303–314. https://doi.org/10.1007/s00027-015-0432-5.

Kats, L. B., & Dill, L. M. (1998). The scent of death: Chemosensory assessment of predation risk by prey animals. *Écoscience, 5*, 361–394. https://doi.org/10.1080/11956860.1998.11682468.

Landeira-Dabarca, A., Álvarez, M., & Molist, P. (2014). Food deprivation causes rapid changes in the abundance and glucidic composition of the cutaneous mucous cells of Atlantic salmon *Salmo salar* L. *Journal of Fish Diseases, 37*, 899–909. https://doi.org/10.1111/jfd.12184.

Landeira-Dabarca, A., Sieiro, C., & Álvarez, M. (2013). Change in food ingestion induces rapid shifts in the diversity of microbiota associated with cutaneous mucus of Atlantic salmon *Salmo salar*. *Journal of Fish Biology, 82*(3), 893–906. https://doi.org/10.1111/jfb.12025.

Loose, C. J., Von Elert, E., & Dawidowicz, P. (1993). Chemically-induced diel vertical migration in *Daphnia*: A new bioassay for kairomones exuded by fish. *Archiv für Hydrobiologie, 126*(3), 329–337.

Malmqvist, B. (1988). Downstream drift in madeiran levadas: Tests of hypotheses relating to the influence of predators on the drift of insects. *Aquatic Insects, 10*(3), 141–152. https://doi.org/10.1080/01650428809361323.

Mathuru, A. S., Kibat, C., Cheong, W. F., Shui, G., Wenk, M. R., Friedrich, R. W., et al. (2012). Chondroitin fragments are odorants that trigger fear behavior in fish. *Current Biology, 22*, 538–544. https://doi.org/10.1016/j.cub.2012.01.061.

McIntosh, A. R., & Peckarsky, B. L. (1999). Criteria determining behavioural responses to multiple predators by a stream mayfly. *Oikos, 85*, 554–564. https://doi.org/10.2307/3546705.

McIntosh, A. R., & Peckarsky, B. L. (2004). Are mayfly anti-predator responses to fish odour proportional to risk? *Archiv für Hydrobiologie, 160*, 145–151. https://doi.org/10.1127/0003-9136/2004/0160-0145.

McIntosh, A. R., Peckarsky, B. L., & Taylor, B. W. (1999). Rapid size-specific changes in the drift of *Baetis bicaudatus* (Ephemeroptera) caused by alterations in fish odour concentration. *Oecologia, 118*, 256–264. https://doi.org/10.1007/s004420050726.

McIntosh, A. R., Peckarsky, B. L., & Taylor, B. W. (2002). The influence of predatory fish on mayfly drift: Extrapolating from experiments to nature. *Freshwater Biology, 47*, 1497–1513. https://doi.org/10.1046/j.1365-2427.2002.00889.x.

McKelvey, L. M., & Forward, R. B., Jr. (1995). Activation of brine shrimp nauplii photoresponses involved in diel vertical migration by chemical cues from visual and non-visual predators. *Journal of Plankton Research, 17*(12), 2191–2206. https://doi.org/10.1093/plankt/17.12.2191.

Mirza, R., & Chivers, D. P. (2003). Predator diet cues and the assessment of predation risk by juvenile brook charr: Do diet cues enhance survival? *Canadian Journal of Zoology, 81*(1), 126–132. https://doi.org/10.1139/z02-225.

Mitchell, M. D., Bairos-Novak, K. R., & Ferrari, M. C. (2017). Mechanisms underlying the control of responses to predator odours in aquatic prey. *Journal of Experimental Biology, 220*, 1937–1946. https://doi.org/10.1242/jeb.135137.

Paterson, R. A., Pritchard, D. W., Dick, J. T., Alexander, M. E., Hatcher, M. J., & Dunn, A. M. (2013). Predator cue studies reveal strong trait-mediated effects in communities despite variation in experimental designs. *Animal Behaviour, 86*, 1301–1313. https://doi.org/10.1016/j.anbehav.2013.09.036.

Peckarsky, B. L., & Cowan, C. A. (1995). Microhabitat and activity periodicity of predatory stoneflies and their mayfly prey in a western Colorado stream. *Oikos, 74*, 513–521. https://doi.org/10.2307/3545997.

Peckarsky, B. L., Kerans, B. L., Taylor, B. W., & McIntosh, A. R. (2008). Predator effects on prey population dynamics in open systems. *Oecologia, 156*(2), 431–440. https://doi.org/10.1007/s00442-008-1004-3.

Pestana, J. L. T., Baird, D. J., & Soares, A. M. V. M. (2013). Predator threat assessment in *Daphnia magna*: The role of kairomones versus conspecific alarm cues. *Marine and Freshwater Research, 64*(8), 679–686. https://doi.org/10.1071/MF13043.

Pohnert, G., & Von Elert, E. (2000). No ecological relevance of trimethylamine in fish-*Daphnia* interactions. *Limnology & Oceanography, 45*(5), 1153–1156. https://doi.org/10.4319/lo.2000.45.5.1153.

Polačik, M., & Janáč, M. (2017). Costly defense in a fluctuating environment—sensitivity of annual *Nothobranchius* fishes to predator kairomones. *Ecology and Evolution, 7*, 4289–4298. https://doi.org/10.1002/ece3.3019.

Rahman, Y. J., Forward, R. B., Jr., & Rittschof, D. (2000). Responses of mud snails and periwinkles to environmental odors and disaccharide mimics of fish odor. *Journal of Chemical Ecology, 26*(3), 679–696. https://doi.org/10.1023/A:1005428221952.

Reissig, J. L., Strominger, J. L., & Leloir, L. F. (1955). A modified colorimetric method for the estimation of N-acetylamino sugars. *Journal of Biological Chemistry, 217*, 959–966.

Reverter, M., Tapissier-Bontemps, N., Lecchini, D., Banaigs, B., & Sasal, P. (2018). Biological and ecological roles of external fish mucus: A review. *Fishes, 3*(4), 41. https://doi.org/10.3390/fishes3040041.

Ringelberg, J., & Van Gool, E. (1998). Do bacteria, not fish, produce 'fish kairomones'? *Journal of Plankton Research, 20*(9), 1847–1852. https://doi.org/10.1093/plankt/20.9.1847.

Rittschof, D., & Cohen, J. H. (2004). Crustacean peptide and peptide-like pheromones and kairomones. *Peptides, 25*(9), 1503–1516. https://doi.org/10.1016/j.peptides.2003.10.024.

Rosier, R. L., & Langkilde, T. (2011). Behavior under risk: How animals avoid becoming dinner. *Nature Education Knowledge, 2*(11), 8.

Sar, N., & Rosenberg, E. (1989). Fish skin bacteria: Production of friction-reducing polymers. *Microbial Ecology, 17*(1), 27–38. https://doi.org/10.1007/BF02025591.

Scrimgeour, G. J., Culp, J. M., & Cash, K. J. (1994). Anti-predator responses of mayfly larvae to conspecific and predator stimuli. *Journal of the North American Benthological Society, 13*(2), 299–309. https://doi.org/10.2307/1467248.

Shephard, K. L. (1994). Functions for fish mucus. *Reviews in Fish Biology and Fisheries, 4*(4), 401–429. https://doi.org/10.1007/BF00042888.

Siepielski, A. M., Fallon, E., & Boersma, K. (2016). Predator olfactory cues generate a foraging–predation trade-off through prey apprehension. *Royal Society Open Science, 3*(2). https://doi.org/10.1098/rsos.150537, 150537.

Silberbush, A., Markman, S., Lewinsohn, E., Bar, E., Cohen, J. E., & Blaustein, L. (2010). Predator-released hydrocarbons repel oviposition by a mosquito. *Ecology Letters, 13*(9), 1129–1138. https://doi.org/10.1111/j.1461-0248.2010.01501.x.

Silbert, J. E., Bernfield, M., & Kokenyesi, R. (1997). A special class of glycoproteins. In J. Montreuil, J. F. G. Vliegenthart, & H. Schachte (Eds.), *Glycoproteins II* (pp. 1–31). London, U.K.: Elsevier.

Šmejkal, M., Ricard, D., Sajdlová, Z., Čech, M., Vejřík, L., Blabolil, P., et al. (2018). Can species-specific prey responses to chemical cues explain prey susceptibility to predation? *Ecology and Evolution, 8*(9), 4544–4551. https://doi.org/10.1002/ece3.4000.

Tikkanen, P., Muotka, T., & Huhta, A. (1996). Fishless-stream mayflies express behavioural flexibility in response to predatory fish. *Animal Behaviour, 51*(6), 1391–1399. https://doi.org/10.1006/anbe.1996.0142.

Tollrian, R. (1995). Predator-induced morphological defenses: Costs, life history shifts, and maternal effects in *Daphnia pulex*. *Ecology, 76*(6), 1691–1705. https://doi.org/10.2307/1940703.

Trekels, H., & Vanschoenwinkel, B. (2017). When fear kicks in: Predator cues initially do not but eventually do affect insect distribution patterns in a new artificial pond cluster. *Hydrobiologia, 790*(1), 157–166. https://doi.org/10.1007/s10750-016-3027-9.

Van Buskirk, J., Krügel, A., Kunz, J., Miss, F., & Stamm, A. (2014). The rate of degradation of chemical cues indicating predation risk: An experiment and review. *Ethology, 120*(9), 942–949. https://doi.org/10.1111/eth.12266.

Van de Winkel, J. G. J., Toin, H. M. S., van Kupevelt, M., Janssen, H. M. J., & Lock, R. A. (1986). Glycosaminoglycans in the skin of rainbow trout (*Salmo gairdneri*). *Comparative Biochemistry & Physiology, 85*, 473–475. https://doi.org/10.1016/0305-0491(86)90030-1.

Von Elert, E., & Loose, C. J. (1996). Predator-induced diel vertical migration in *Daphnia*: Enrichment and preliminary chemical characterization of a kairomone exuded by fish. *Journal of Chemical Ecology, 22*(5), 885–895. https://doi.org/10.1007/BF02029942.

Von Elert, E., & Pohnert, G. (2000). Predator specificity of kairomones in diel vertical migration of *Daphnia*: A chemical approach. *Oikos, 88*(1), 119–128. https://doi.org/10.1034/j.1600-0706.2000.880114.x.

Weissburg, M., Smee, D. L., & Ferner, M. C. (2014). The sensory ecology of nonconsumptive predator effects. *American Naturalist, 184*(2), 141–157. https://doi.org/10.1086/676644.

Winkelmann, C., Petzoldt, T., Koop, J. H., Matthaei, C. D., & Benndorf, J. (2008). Benthivorous fish reduce stream invertebrate drift in a large-scale field experiment. *Aquatic Ecology, 42*(3), 483–493. https://doi.org/10.1007/s10452-007-9101-7.