Lab and field estimates of active time of chemical alarm cues of a cyprinid fish and an amphipod crustacean

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Summary

Predation risk is detected by the presence of cues released passively during a predation event. Refinement of risk assessment could occur if prey have the ability to assess cue age. Here, we test for antipredator behavioural responses to chemical alarm cues of varying ages. Fathead minnows, *Pimephales promelas*, give an antipredator response to alarm cues derived from conspecifc skin extract that has been aged at 18°C for 0 (fresh) and 3 h, but not after 6 h. Alarm cues from crushed conspecific *Gammarus lacustris* (Crustacea: Amphipoda), showed a similar chemical longevity. A field test of minnow alarm cues produced broadly similar results for conspecific fathead minnows and heterospecific responses by northern redbelly dace, *Phoxinus eos*. Close agreement among all three data sets suggests either a common duration of predation risk or a common molecular basis of chemical cues across aquatic taxa. When we heated skin extract to denature and remove half of the constituent proteins, we inactivated biological activity of alarm cue suggesting that protein is required for skin extract to function as an alarm cue in minnows. Protein degradation may be a means of assessing age and, therefore, ecological relevance of chemical information in behavioural decision-making.

Keywords: active time, chemical alarm cues, predator–prey, fathead minnow, amphipod.

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Introduction

Predation is a pervasive agent of selection that shapes prey behaviour, morphology and life history. Consequently, prey have evolved sensitive mechanisms to detect predation risk including the ability to discern gradations in the nature and relative degree of risk (Lima & Dill, 1990). Public information about the presence of predation risk can be detected through vocalizations (e.g., Sherman, 1977; Shelley & Blumstein, 2005; Templeton & Greene, 2005), visual warning displays and subtle postural changes (Sheenan et al., 1994; Leal et al., 1997; Brown et al., 1999; Stankowich, 2008), seismic (Gregory et al., 1986) and vibratory mechanostimuli (Kirchner et al., 1994; Cocroft, 1996; Randall et al., 2000) and a variety of chemical stimuli (Smith, 1992; Kats & Dill, 1998; Dicke & Grostal, 2001). Relative to other sensory modalities, chemical information is relatively slow to spread through the environment and persists for greater periods of time. This raises interesting questions about longevity of chemical information in terms of cue detectability, and behavioural responses to these cues, relative to the presence/absence of risk, and behavioural trade-offs with predation risk.

Damaged prey tissue releases a suite of chemical alarm cues that are released only in the context of a predatory attack. The ecological importance of chemical alarm cues in mediating predator–prey interactions in aquatic communities is well established (Smith, 1992; Chivers & Smith, 1998). Ecological function of these cues is determined by parameters affecting cue detection: (1) active space — the volume of water perceived as dangerous by prey, and (2) active time — the duration for which chemical information elicits this perception. In the fathead minnow, *Pimephales promelas*, it has been estimated from a dilution series conducted in lab aquaria that 1 cm^2 skin activates 58 000 l, or a sphere with a radius of 2.3 m (Lawrence & Smith, 1989). Subsequent field studies corroborated this estimate. Natural populations of fathead minnows and northern redbelly dace (*Phoxinus eos*) avoid an area with a radius of 2 to 8 m in response to the amount of cue contained in 2 cm^2 of minnow skin (Wisenden, 2008).

Active time of aquatic chemical alarm cues has thus far received scant attention. Ferrari et al. (2008) estimated active time for overt responses by larval woodfrogs (*Rana sylvatica*) to injury-released chemical alarm cues of conspecifics to be between 5 min and 4 h. Cue aged 2 h elicited responses intermediate between fresh cue and cue aged 4 h. In another study, alarm cues

of tadpoles of green frogs (*Rana clamitans*) (Fraker, 2009) or bullfrogs (*Rana catesbeiana*) (Peacor, 2006) released when tadpoles are eaten by odonate predators persisted between 48 and 72 h. *Physa* snails respond to cues released by sunfish (*Lepomis gibbosus*) fed a diet of snails with a half-life of 41 h (Turner & Montgomery, 2003). Hazlett (1999) showed that crayfish *Orconectes virilis* respond to predator odour of snapping turtle (*Chelydra serpentina*) after the odour aged for 1 h at room temperature but not after 2 h. Here, we present lab and field data investigating active time of chemical alarm cues in two species, the fathead minnow (*Pimephales promelas*) and the amphipod crustacean (*Gammarus lacustris*), which are both under strong selection from multiple predators to detect and respond to chemical information about predation risk. Both species are abundant in the vicinity of Moorhead, MN, USA and both have been used in past studies of chemical alarm cues, both in the lab and in the field (e.g., Chivers & Smith, 1998; Wisenden et al., 1999, 2001).

We follow-up on these behavioural assays of the effect of cue age with an experiment on the chemical nature of minnow alarm cue, specifically, the hypothesis that the active ingredient(s) of minnow alarm cue is protein-dependent. This information may provide insight into mechanisms of biochemical decay of alarm cues once released into the environment. Purine-N-oxides, such as hypoxanthine 3-N oxide and pyridine-N-oxide, are known to induce alarm behaviour in a range of fish species in the superorder Ostariophysi (Pfeiffer et al., 1985; Brown et al., 2000, 2001a,b, 2003). However, intensity of cross-species reactions among ostariophysans (Schütz, 1956) and other fish groups (Mirza & Chivers, 2001; Mirza et al., 2001) decline with increasing phylogenetic distance suggesting a gradation of species-specific alarm cues rather than one molecule conserved across all species. Kasumyan & Ponomarev (1987) hypothesized that alarm molecules form a labile complex with protein, and that minor modifications of the protein component result in species-specific alarm cues. Moreover, polypeptides commonly form signalling molecules in marine invertebrates (Rittschoff, 1990; Decho et al., 1998).

Materials and methods

Lab-based estimate of active time of minnow and amphipod alarm cues

Lab-reared fathead minnows were acquired from the US Environmental Protection Agency (Duluth, MN, USA). Skin extract was collected by killing

22 fathead minnows (mean total length ± SE = 41.8 ± 1.2 mm) by cervical dislocation with a razor blade. Skin fillets were removed from each side of each fish, measured to the nearest mm and placed in 100 ml dechlorinated tap water on a bed of crushed ice. A total area of 47.4 cm^2 of skin was collected. The solution was homogenized with a hand blender to simulate predator attack and release chemical alarm cues from damaged epithelial tissue. The homogenate was filtered through a loose wad of polyester fibre to remove large pieces of connective tissue, diluted to a final volume of 480 ml for a final concentration of approximately 1 cm^2 skin per 10 ml dose. The stock solution was aliquoted into 45 doses of 10 ml each. Fifteen doses were placed immediately into a freezer (−20°C). These doses formed the test cue for the fresh frozen treatment. Fifteen aliquots of blank dechlorinated water (control) were also placed in the freezer at this time. The remaining doses of alarm cue were left on the lab bench at room temperature (18°C). Fifteen doses were placed into the freezer after 3 h at room temperature, and the last 15 doses were placed in the freezer after 6 h at room temperature.

A single EPA fathead minnow was placed in each of a battery of 37-l test aquaria and maintained on a diet of flake food. There was no difference in fish size among treatment groups (TL = 48.3 ± 1.19 mm, $N = 60$, $F_{3,28} = 0.289$, $p = 0.833$). Water temperature was 18°C and photoperiod was 12L : 12D. Test aquaria contained a thin layer of naturally-coloured gravel and a sponge filter. A 5 × 5 cm grid was drawn on the front pane for determining fish position and activity. One end of a 2-m-long stimulus injection tube was wedged into the lift tube of an air-powered sponge filter. The other end of the injection tube extended about 1 m out from the test aquaria where the experimenter could attach a 60-ml syringe to surreptitiously inject test stimuli. Turbulence created by airflow in the lift tube of the sponge filter masked hydrostatic pressure changes associated with stimulus injection. The injection tube was rinsed by withdrawing and discarding 60 ml tank water, twice. A third 60 ml of tank water was withdrawn and retained. This water was used to flush test stimuli completely into the test aquarium. Injection tubes were replaced with fresh tubing for each trial. We recorded fish activity as the number of grid lines crossed in 8 min before and after the introduction of 10 ml of test stimuli. Stimulus injection required about a minute to complete during which time no data were recorded. We recorded vertical distribution as the sum score of the horizontal row occupied by the test fish at 15-s intervals during the pre- and post-injection observation periods. We

conducted 15 trials per treatment. Antipredator responses in general, and of minnows specifically, involve a reduction in activity and movement to the bottom (Lawrence & Smith, 1989; Lima & Dill, 1990). These behaviours reduce the probability of predation (Mathis & Smith, 1993; Wisenden et al., 1999).

Adult *Gammarus lacustris* (Crustacea: Amphipoda) were collected in January 2008 from Lake Chautauqua, located approximately 1 km southeast of Fergus Falls, MN, USA. Holes were drilled through the ice using a gas-powered auger, and *G. lacustris* were collected using small dip nets. *Gammarus lacustris* were transferred to the MSUM aquatic research facility and kept in a 19-l aquarium with a gravel substrate, aeration and sponge filter. Oak (*Quercus* sp.) and maple (*Acer* sp.) leaves served as shelter and food.

A stock solution of *G. lacustris* alarm cue was made by reducing 77.0 g of adult *G. lacustris* (about 100 individuals) to a fine pulp using a hand blender and diluting the pulp with 616 ml dechlorinated tap water. The resulting concentration of *G. lacustris* alarm cue was 0.125 g/ml. Stock solution was aliquotted in 2 ml doses. Some aliquots (fresh treatment) were transferred were frozen immediately (−20°C). Other aliquots were held at room temperature (22°C) for 3 h or 6 h before being transferred to the freezer for storage. A control treatment was made from 2-ml aliquots of dechlorinated tap water, which was frozen at −20°C until needed.

Trials were conducted in plastic containers (16 × 25 × 18 cm high) with a transparent front panel and translucent side panels. A fine gravel substratum was added to the bottom of the aquaria, and one plastic leaf (resembling a maple leaf) was placed in the centre of the bottom of the aquarium as a shelter object. A 2 × 2 cm grid comprising four rows and eight columns was drawn on the front panel to quantify activity and vertical distribution. A single *G. lacustris* was placed into each test container and allowed at least 3 h to acclimate prior to being tested. *G. lacustris* were observed for a 5-min pre-stimulus period during which activity (number of grid lines crossed) and vertical distribution (vertical position sampled every 15 s) were recorded. One of the four treatment cues was then dribbled into the aquarium via a syringe held 1–2 cm above the water surface and moved in an oval-shaped pattern starting in the front left corner and moving clockwise until back to the starting position. This ensured that the test cues were distributed evenly throughout the test container. Activity and vertical distribution were measured for a 5-min post-stimulus measurement. We conducted 15 trials

for each of the four cue treatments using a new individual *G. lacustris* for each of the 60 trials.

Field-based estimates of active time of fathead minnow alarm cue

The site for the field test of active time was Budd Lake, MN, USA, within the park boundaries of Itasca State Park at the headwaters of the Mississippi River (Itasca is derived from *veritas caput*, true head). Budd Lake is a small lake (ca. 5 ha) that contains only two species of fish: northern redbelly dace (*Phoxinus eos*) and fathead minnows. Adult fathead minnows were captured from Budd Lake and transferred to the Itasca Biological Field Station (University of Minnesota). Chemical alarm cues were prepared from skin extract prepared as described above for the laboratory experiment. A total of 28 fish (TL = 53.0 ± 0.99 mm) were killed by cervical dislocation. Skin fillets (104.62 cm^2) were collected and transferred to a beaker of well water on a bed of crushed ice, then blended with a hand blender, filtered through a loose wad of polyester wool, and diluted to a final volume of 530 ml. Aliquots of 10 ml were transferred from the stock solution to individual blocks of cellulose sponge (ca. 2.5 × 2.5 × 2.5 cm) each with a length of stainless steel wire running through it. Each sponge block contained the approximate equivalent of 2.05 cm^2 of minnow skin. Fifteen sponge blocks were frozen (−20°C) immediately, 15 blocks were placed in the freezer after 3 h at room temperature (ca. 22°C), and 15 blocks were placed in the freezer after 6 h at room temperature. A fourth treatment, a blank well-water control, was prepared by infusing 15 sponge blocks with well water and freezing at −20°C until needed.

On the day of testing (11 June 2008), individual sponge blocks were transported to the field site inside plastic bags buried in crushed ice to ensure that test blocks remained frozen until placed in the lake. A field team of eight people (two per sponge treatment) was required to manage the logistics of the field test. Standard minnow traps (6 × 6 mm metal mesh shaped into a cylinder, 50 cm long, 22 cm in diameter, with one entrance at each end) were individually fitted with one of the sponges by attaching the piece of wire in the sponge block to the inside centre of the trap equidistant from the trap entrances. Traps were placed around the perimeter of the lake in depths of 0.5–1.0 m spaced 8–10 m apart. Traps were set in groups of four representing one of each of the sponge treatments. We waited 5 min before setting the next

group of four traps, and so on, so that fifteen replicate groups were set over a span of one hour and fifteen minutes. When 2 h had elapsed from the time of setting the first group of traps, we retrieved that set of four traps, and spent 5 min recording the number of dace and minnows captured in each trap. This method ensured that set duration for each trap was standardized among treatments. If, for example, more than 5 min was required to process the catch of one set of traps then the fishing time for all four sponge groups was delayed equally. Because of large numbers of fish captured, duration of traps gradually increased from 120 min for the first group of traps, to 193 min for the final (15th) group of traps (mean set time $= 159 \pm 5$ min, $N = 15$).

Biochemical stability of minnow alarm cue

Wild-caught fathead minnows were purchased from a commercial outlet based in Moorhead, MN, USA (TL $= 53.3 \pm 1.3$ mm). Minnows were held in 190-l aquaria at 18°C on a 12L : 12D cycle and fed commercial flake food. We prepared skin extract as described above. A total of 83 cm^2 of skin was homogenized, filtered, diluted to a final volume of 830 ml, and frozen in 10-ml aliquots at −20°C until needed. The final concentration of skin extract was approximately 1 cm^2 per 10-ml dose. To prepare unheated extract, 10-ml aliquots of skin extract were thawed, chilled on ice for 5 min and centrifuged at 30 000× *g* for 10 min at 4°C. The supernatant was used as the centrifuged, unheated stimulus. To prepare heat-treated extract, 10-ml aliquots of skin extract were thawed, placed in a 90°C water bath for 7 min and then placed on ice for 5 min. The samples were then centrifuged at 30 000× *g* for 10 min at 4°C, resulting in a small pellet of precipitated denatured protein. The supernatant was used as the test stimulus for the centrifuged, heated stimulus. Skin extract stimuli (heated and unheated) were prepared immediately before testing. Control stimulus was dechlorinated tap water frozen in 10-ml aliquots at −20°C.

Behavioural assay of cue efficacy to evoke antipredator response generally followed the protocol described for the first experiment. Individual minnows from the stock population were placed in 37-l aquaria filled with dechlorinated tap water and a thin layer of naturally coloured gravel. A grid (5 × 5 cm) was drawn on the front panel to quantify behaviour. Cue introduction was by means of an airline tube that released cue beside an airstone affixed to the back centre of the test aquarium. Water currents generated by the airstone distributed cue throughout the test aquarium. Test fish were given at

least 48 h to acclimate to test aquaria before testing. Before each trial, skin extract was thawed and prepared as described above. The test protocol was 5 min of pre-stimulus observation, followed immediately by 1 min of stimulus injection, followed immediately by 5 min of post-stimulus observation ($N = 15$ per treatment). We scored activity by summing the number of grid lines crossed by minnows during each observation period. Time near the bottom was scored by summing the number of point samples at 10-s intervals for which the test fish was located in the bottom row of the grid (within 5 cm of the bottom).

Protein concentration of skin extract was determined by dye-binding protein assay (Bradford, 1976) using bovine serum albumin as a standard. We compared protein concentration for thawed skin extracts that were (1) not heated, not centrifuged, (2) not heated, centrifuged or (3) heated, centrifuged.

Results

Lab-based estimates of active time of minnow and amphipod alarm cues

All behavioural data from fathead minnows and *Gammarus* were normally distributed (Kolmogorov–Smirnov test, $p > 0.10$ for all). Change in behaviour (post stimulus minus prestimulus) showed a significant effect of cue treatment on minnow activity ($F_{3,56} = 28.92$, $p < 0.001$, Figure 1A) and time spent near the bottom ($F_{3,56} = 11.04$, $p < 0.001$, Figure 1B). Pair-wise post-hoc comparisons showed that change in activity and vertical distribution were significantly greater for fresh extract than extract aged 3 h and that extract aged 3 h induced a significantly more intense response than extract aged 6 h (Duncan's test, $p < 0.05$). However, extract aged 6 h did not differ significantly from water control trials ($p > 0.05$).

The behavioural response of *Gammarus* to chemical alarm cues was remarkably similar to those shown by fathead minnows (change in activity: $F_{3,56} = 9.65$, $p < 0.001$, Figure 2A; change in vertical distribution: $F_{3,56} = 7.19$, $p < 0.001$, Figure 2B). Duncan's post-hoc pair-wise comparisons of change in activity showed: Fresh $>$ (3 h, 6 h) $>$ (6 h, Water). Duncan's post-hoc pair-wise comparisons for change in vertical distribution showed: Fresh $<$ (3 h, Water) $<$ 6 h.

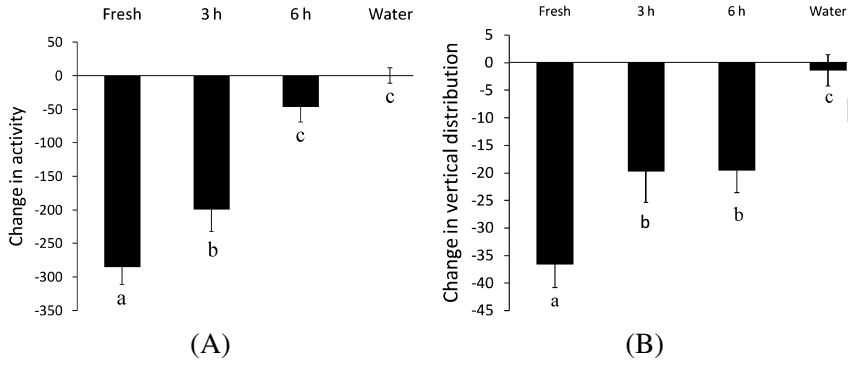

Figure 1. Mean (± 1SE) change in (A) activity (number of lines crossed in 5 min) and (B) vertical distribution by individual fathead minnows exposed to one of four treatment cues: minnow skin extract that was frozen freshly, frozen after aging at room temperature for 3 h, frozen after aging for 6 h, or dechlorinated water control. Letters below bars indicate the outcome of post-hoc pair-wise comparisons. Shared letters do not differ ($p > 0.05$).

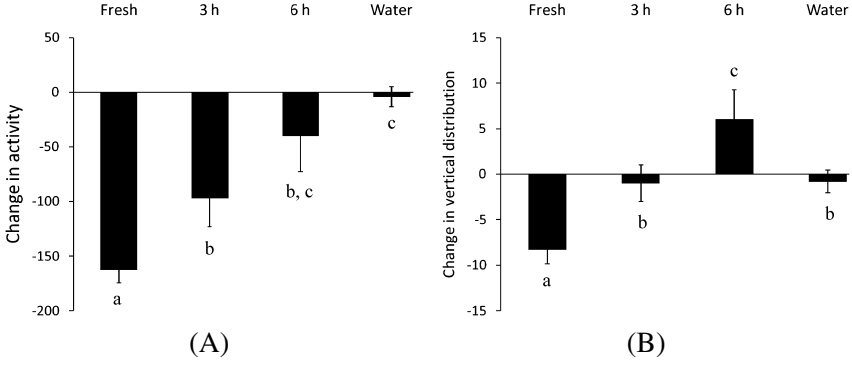

Figure 2. Mean (± 1SE) change in (A) activity (number of lines crossed in 5 min) and (B) vertical distribution by individual amphipod crustaceans (*Gammarus lacustris*) exposed to one of four treatment cues: fresh-frozen dilute *Gammarus* alarm cue, cues aged 3 h, cue aged 6 h, and dechlorinated water control. Letters below bars indicate the outcome of post-hoc pair-wise comparisons. Shared letters do not differ ($p > 0.05$).

Field-based estimates of active time of fathead minnow alarm cue

Trends in the field data broadly mirrored trends in the lab data, but large variation undermined statistical power. In addition, high fish density resulted in a synergism between chemical alarm cues and the visual presence of fish inside traps. Fish density in Budd Lake is very high. In two to three hours, 60 minnow traps caught 3390 northern redbelly dace and 4639 fathead minnows for

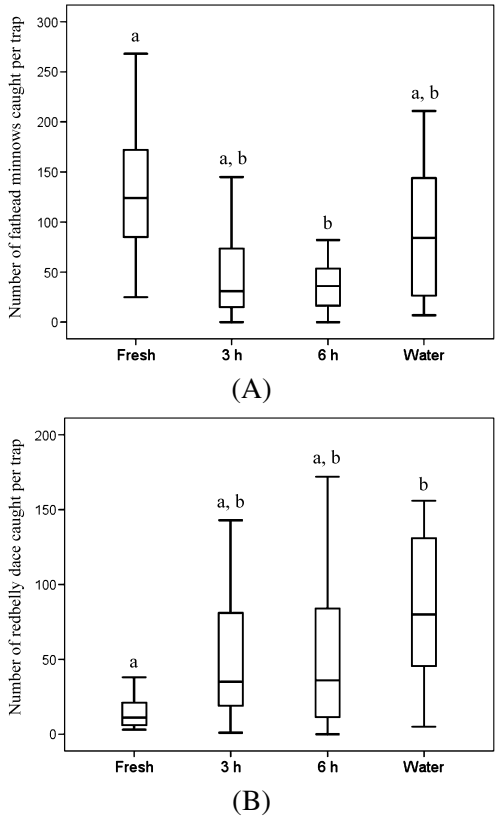

Figure 3. Median (± quartiles, range) of the number of (A) fathead minnows and (B) northern redbelly dace captured per trap in Budd Lake in traps chemically labeled with fresh skin extract, skin extract aged 3 h and 6 h, or water control.

a total of 8029 fish (134 ± 13 fish per trap). Field data met the requirements of normality form for fathead minnows (K–S $Z = 1.09$, $p = 0.182$) but not for northern redbelly dace (K–S $Z = 1.60$, $p = 0.012$). Therefore, all catch data were analyzed using non-parametric Kruskal–Wallis ANOVA.

The number of fathead minnows captured per trap differed significantly among treatment groups ($KW_3 = 14.6$, $p = 0.002$; Figure 3A). Post-hoc pair-wise comparisons ($p < 0.05$, Siegel & Castellan, 1988) between treatments revealed that the treatments ranked as follows: (Fresh, Water, 3 h) > (Water, 3 h, 6 h). The number of redbelly dace captured per trap also differed significantly among treatment groups ($KW_3 = 16.8$, $p = 0.001$; Figure 3B). Post-hoc pairwise comparisons ($p < 0.05$) between treatments

revealed that the treatments ranked as follows: (Fresh, 3 h, 6 h) < (3 h, 6 h, Water).

Biochemical stability of minnow alarm cue

Change in activity data (K–S $Z = 1.06$, $p = 0.214$) and change in vertical distribution data (K–S $Z = 1.35$, $p = 0.052$) were normally distributed. Change in the activity levels of fathead minnows depended on cue treatment ($F_{2,44} = 4.84$, $p = 0.013$; Figure 4A) with Water = Heated < Non-heated (Duncan's test, $p < 0.05$). Change in vertical distribution showed similar trends ($F_{2,44} = 17.69$, $p < 0.001$, Water = Heated < Non-heated, Figure 4B).

Data for measures of protein concentration were normally distributed (K–S $Z = 0.94$, $p = 0.337$) and differed significantly among treatments ($F_{2,27} = 19.19$, $p < 0.001$; Figure 5) with Centrifuged, Heated < Not centrifuged, Not heated = Centrifuged, Not heated (Duncan's test, $p < 0.05$). Protein concentration in heated and centrifuged extract was reduced by 47.9% compared to unheated, uncentrifuged extract, and by 48.9% compared to unheated, centrifuged extract. Heat treatment increased the pH of the supernatant from 8.231 ± 0.0046 ($N = 10$) for unheated extract to 8.552 ± 0.0056 ($N = 10$) for heated extract ($t_{18} = 5.69$, $p < 0.001$). This minor change likely resulted from loss of CO_2 from solution but the pH shift

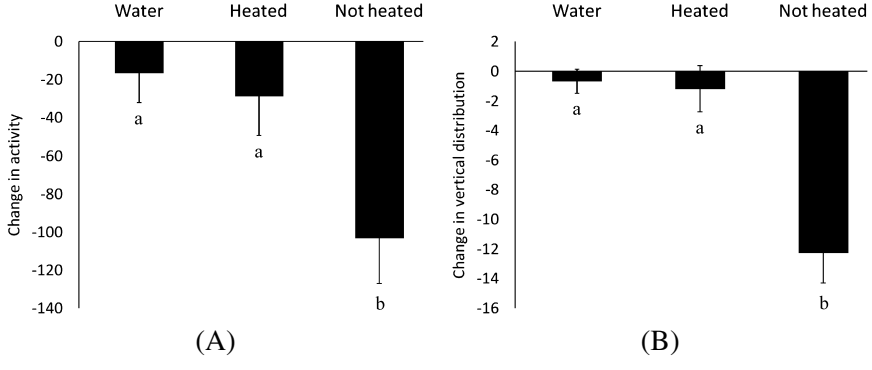

Figure 4. Mean (± 1SE) change in (A) activity (number of lines crossed in 5 min) and (B) vertical distribution by individual fathead minnows exposed to one of three treatment cues: dechlorinated water control (negative control), minnow skin extract that was heated to 90°C for 5 min, or minnow skin extract that was not heated (positive control). Letters below bars indicate the outcome of post-hoc pair-wise comparisons. Shared letters do not differ ($p > 0.05$).

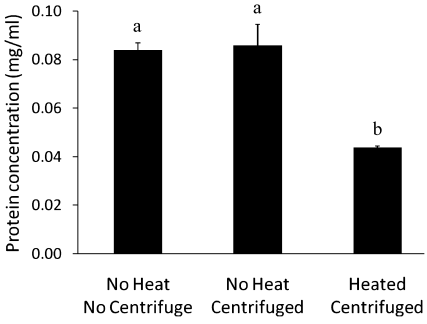

Figure 5. Mean (± 1SE) concentration of protein in aliquots of minnow skin extract that were (1) not heated and not centrifuged, (2) not heated and centrifuged or (3) heated and then centrifuged to denature and precipitate out protein. Letters above bars indicate the outcome of post-hoc pair-wise comparisons. Shared letters do not differ ($p > 0.05$).

was not sufficiently large or in the range of values that would affect structure of hypoxanthine 3-N oxide (Scheinfeld, 1969; Brown et al., 2000).

Discussion

Lab-based estimates of active time of minnow and amphipod alarm cues

Lab data estimated that the duration of biological activity of injury-released chemical alarm cues of minnows and amphipods is between 3 and 6 h at 18°C. These estimates agree generally with the estimate of 2–4 h of active time of injury-released chemical alarm cue of frog tadpoles determined in the field (Ferrari et al., 2008). Broadly similar estimates of active time for minnows, amphipods and tadpoles may reflect convergent selection on chemical cues for properties of chemical stability, the ability to be transported by water, olfactory receptors available to detect them, or ecological constancy in aquatic habitats in duration of predation risk. One may predict that aquatic predators may generally employ predictable temporal patterns. There could be selection on prey to use biochemical breakdown products of chemical alarm cues as a biochemical clock to assess time since cue release and discount predation risk accordingly. Natural populations of fathead minnows and brook stickleback (*Culaea inconstans*) avoid areas where alarm cues have been released for more than 2 h but less than 4 h (Wisenden et al., 1995). These new data differ from those of Wisenden et al. (1995) in that

cues were pre-aged before behavioural assays were conducted. In both instances, duration of response was a few hours, suggesting that both cognitive (learning) and biochemical (cue decay) processes may be involved in assessment of predation risk.

Alarm responses to chemical cues have been studied in *Gammarus* in the lab (Wudkevich et al., 1997; Wisenden et al., 1999) and in the field (Dahl et al., 1998; Wisenden et al., 2001). *Gammarus* responded to fresh alarm cues with an antipredator response but switched to foraging behaviour in response to cue aged 6 h. The apparent feeding response of *Gammarus* to cue aged 6 h is similar to those seen in a previous study (Wisenden et al., 1999). This suggests that food cues (i.e., amino acids, etc.) may persist or remain relevant longer than alarm cues, and that *Gammarus* opportunistically scavenge on dead conspecifics when the risk of predation has passed. If alarm cues of *Gammarus* are protein-based, biochemical degradation of alarm cues into constituent amino acids would soon render an alarm cue into a food cue. More experimentation is needed in this area.

Field-based estimates of active time of fathead minnow alarm cue

Recognition and response to heterospecific alarm cues occur commonly among sympatric cyprinids (Chivers & Smith, 1998) and specifically between the two species at this study site (Wisenden & Barbour, 2005; Wisenden, 2008). Field data for northern redbelly dace conform generally to lab estimates of active time in fathead minnows. In the field, redbelly dace showed partial avoidance of traps with cue pre-aged for 3 and 6 h, in that only the water traps were different from traps with fresh alarm cue. This suggests that field responses may be more sensitive to risk than lab-reared fish from the Environmental Protection Agency.

Field data for fathead minnows underscore the perils of attempting to replicate lab results in the field. Variation in catch data was very large making interpretation of the results speculative. Moreover, fathead minnow catch data contain a counterintuitive result; the catch rate of fish was highest in traps that contained fresh alarm cue. This result likely occurred because the unusually high catches experienced on our test day elicited a synergism between the effect of alarm cues and the effect of social cohesion (Wisenden et al., 2003). A brief explanation of this phenomenon is as follows. Two forces affect trap recruitment: (1) the presence of chemical alarm cues inhibits trap

entry (Mathis & Smith, 1992), while (2) the presence of conspecifics inside a trap induces trap entry (Wisenden et al., 2003). When chemical alarm cues are presented simultaneously with a fish shoal in a trap, the two opposing tendencies do not offset each other. One of the suite of antipredator behaviours induced by the presence of alarm cues is to increase shoal cohesion — with fish inside the trap (a shoal) — with the net result of causing greater numbers of fish to enter shoal-baited traps than would be the case if alarm cues are absent (Wisenden et al., 2003). We do not know enough about the conditions that invoke a synergistic reaction to explain why minnows gave this response but dace did not. In the previous study (Wisenden et al., 2003) both minnows and dace responded synergistically. Setting aside the catch rates of fathead minnows in traps labelled with fresh alarm cue, the remaining field data (for fathead minnows and redbelly dace) are broadly consistent with lab estimates of active time and provide further support for an estimate of active time between 3 and 6 h.

Advection, or mass movement of water (current), exerts an overriding influence on both active space and active time. Even in wind-sheltered lakes such as Budd Lake, advection dilutes cues below the threshold of sensitivity long before 2 h (Wisenden, 2008) or 3 h (current study). In the experiments reported here, we endeavoured to manipulate only one variable — cue age — and left all others constant to study the effect of cue age. We controlled for advection in lab experiments by standardizing turbulence used to mix and disperse in test aquaria. Advection was controlled in the field experiment also. In Budd Lake, capricious currents occur that vary moment by moment, and with basin and shoreline morphology (Wisenden, 2008). However, in the field experiment treatment cues were distributed randomly with respect to these factors and, therefore, did not contribute to treatment differences in catch rates. The role of sensory adaptation in determining active time was not considered in this study design. In laboratory experiments, all cues were pre-aged, and all behavioural assays were of short duration and of similar length for all treatments. Therefore, sensory adaptation cannot explain differences among treatments.

Biochemical stability of minnow alarm cue

Pfeiffer and colleagues proposed that hypoxanthine 3-N oxide was the active compound in the skin of Ostariophysan fishes (minnows, characins, catfish, suckers) that elicited alarm reactions from conspecifics (Pfeiffer et al.,

1985). Brown and colleagues (Brown et al., 2000, 2001a,b, 2003; Kelly et al., 2006) demonstrated biological activity of hypoxanthine 3-N oxide for eliciting alarm reactions from Ostariophysan fish, and showed that the NO side group on the hypoxanthine molecule was responsible for this activity (Brown et al., 2000). Concentrations of hypoxanthine 3-N oxide as low as 0.4 nM are sufficient to evoke an alarm reaction in fathead minnows (Brown et al., 2001a). However, these data alone do not explain heterospecific response intensities that vary with phylogenetic distance (Schütz, 1956). Our data provide a mechanism to explain heterospecific responses, and a mechanism for biochemical degradation over time. The heat treatment we employed was not sufficient to affect hypoxanthine 3-N oxide, nor did the pH shift enough to alter its structure (Brown et al., 2000). Our findings are consistent with Lebedeva et al. (1975) and Kasumyan & Ponomarev (1987) in that alarm function is associated with molecules in skin extract of large molecular weight. Fractions of skin extract that produce alarm reactions in minnows have molecular masses of about 1100 Da and greater than 1500 Da (Lebedeva et al., 1975; Kasumyan & Ponomarev, 1987). In contrast, the molecular mass of hypoxanthine 3-N oxide is 132 Da. It may be the case that proteins or polypeptides function for intracellular storage (stabilisation), or as a carrier (activation, solubility, transport) of a small signalling molecule, such as a pyridine-N-oxide. If hypoxanthine 3-N oxide, or similar, is bound to polypeptides then reduction of protein by the heat treatment may have precipitated hypoxanthine 3-N oxide out of solution. Alternatively, hypoxanthine 3-N oxide might not be the signalling molecule but is simply a molecular mimic that binds to the same olfactory receptors as polypeptide alarm cues. Further discussion of physiological mechanisms awaits research on specific olfactory receptors involved in mediating alarm reactions, and the chemical nature of the cues themselves,

The potential role of protein in minnow alarm cue concurs generally with reports in the literature of semiochemicals relating to predation risk (Rittschof, 1990; Decho et al., 1990, Fraker et al., 2009). Polypeptides serve as a source of environmental information for kairomones of amoebas (Kusch, 1999), ciliates (Wicklow, 1997), crustaceans (Rittschof, 1990), molluscans (Rittschof, 1990) and amphibians (Lutterschmidt et al., 1994). Fraker et al. (2009) recently concluded that two separate polypeptides from skin extract of tadpoles are required to elicit alarm behaviour. Additional evidence in support of the protein hypothesis is that protein content of European minnows

Phoxinus phoxinus skin is low in winter, high in summer, and proportional to the efficacy of the alarm function of skin extract (Lebedeva et al., 1975).

Biochemical breakdown of chemical cues is often aided by microbial action (Decho et al., 1990; Peacor, 2006). These processes may explain the disparity between estimates of active time in alarm cues of tadpoles to 5 min to 2 h when conducted under field conditions (Ferrari et al., 2008) but more than 36 h when conducted in the lab (Peacor, 2006; Fraker, 2009). Peacor (2006) found that cue degradation in pond water (containing microbial fauna) was 2 days, half that of cue held in well water (3–4 days). In contrast, our data generated similar results for lab and field estimates of active time of minnow alarm cue. Perhaps proteases that act on minnow alarm cue are present in other components of minnow skin extract, whereas tadpole cues may breakdown only upon contact with extrinsic proteases. More likely, the explanation for the disparity between short active times (Ferrari et al., 2008; this study) and long active times (Turner & Montgomery, 2003; Peacor, 2006; Fraker, 2009) is that short activity time occur when alarm cues (from injured prey tissue) are presented alone while long activity times occur when alarm cues are presented simultaneously with predator odour (kairomones).

Synthesis

Antipredator response to injury-released chemical alarm cues is a basic and ubiquitous phenomenon among aquatic taxa from protozoa to amphibia (Chivers & Smith, 1998; Wisenden, 2003). It is reasonable to postulate that alarm cues arose from passively released by-products of damaged tissue (Wisenden & Stacey, 2005; Wisenden & Chivers, 2006). However, olfactory receptors for amino acids selected for feeding behaviour (e.g., Nikonov & Caprio, 2001) were not likely to have been co-opted to detect olfactorally-conspicuous components of injured skin because the medial olfactory tract is responsible for detecting alarm cues, whereas food cues are typically mediated by olfactory receptors that reside in the lateral olfactory bundles (Hamdani et al., 2000).

Taken together, the data presented here make a case for a general ecological constancy of the duration of predation risk or activity of chemical alarm cues, across disparate taxa. The estimate of several hours seems to be evident in lab and field contexts, and the cue appears dependent on protein, either as the active ingredient, or as a carrier or activator of some type. Estimates of

active time (current study) and active space (Wisenden, 2008) extend beyond a simple stimulus-receptor physiological response, because active time and space of alarm cues incorporate learning (Chivers & Smith, 1995) and social interactions among individuals in a shoal (Mathis et al., 1996). Further research is needed to resolve these questions.

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