Cohabitation enhances the avoidance response to heterospecific alarm cues in a freshwater snail

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In aquatic habitats prey often show maximum responsiveness to predators when they are exposed to kairomones and alarm cues in combination. As alarm cues can derive from heterospecific as well as conspecific animals, prey may need to fine tune their responses depending on the taxa with which they co-occur. We used juveniles of the freshwater gastropod, Lymnaea stagnalis L. (great pond snail) to test whether the response to heterospecific alarm cues could be enhanced by cohabitation in the absence of predators, and therefore without the potential for cue association learning. Snails were raised from oviposition alongside one of four other pulmonate species, differing in their phylogenetic relatedness to L. stagnalis, that is, from intragenus to intrasuborder. The antipredator response to heterospecific alarm cues paired with fish kairomones was then compared with the response of L. stagnalis reared in isolation. Cohabitation increased the response to heterospecific alarm cues paired with fish kairomones only when L. stagnalis had developed in the presence of closely related (Lymnaea fusca and Radix balthica) rather than distantly related heterospecifics (Physella acuta and Planorbarius corneus). Hence, in L. stagnalis, learning to recognize heterospecific alarm cues can occur without the need for cue association but the learnt response is constrained by species' relatedness. This result suggests that differential responses observed in studies based on wild-caught animals in response to heterospecific alarm cues may be explained by learning through cohabitation alone.

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Predators can impact on prey fitness, both directly through predation and indirectly where an avoidance response reduces the opportunities for prey to feed or reproduce (Lima & Dill 1990). Hence, prey species should use all available information to gauge predation risk correctly and respond appropriately. In the aquatic environment, where visual methods of predator recognition are impaired by poor visibility, prey frequently rely on chemical cues to recognize predation threat (Dodson et al. 1994). These chemicals may be kairomones derived from the predator (Kats & Dill 1998), or alternatively disturbance or alarm (damage) cues from prey (Chivers & Smith 1998), although a combination of both kairomones and alarm cues has frequently been demonstrated to elicit the strongest antipredator response (Dodson et al. 1994; Chivers & Smith 1998). Prey species may have an innate ability to detect predator kairomones (Hirvonen et al. 2000; Dalesman et al. 2006;

Turner et al. 2006; Orr et al. 2007) but may also learn to recognize predators through cue association following a predation event (Chivers & Smith 1995; Chivers et al. 1996; Rochette et al. 1998; Kelley & Magurran 2003; Dalesman et al. 2006; Turner et al. 2006).

A key aspect of how prey species respond to predators is their ability to match their responses to the predation threat in any given habitat. For alarm cues, in order for this matching to be most effective prey should recognize not just chemicals from conspecifics but also those from other cohabiting species within the same guild. This ability to respond to heterospecific cues has been demonstrated in several aquatic taxa, and there is good evidence that alarm cues may be phylogenetically conserved, allowing recognition of alarm cues from closely related species (Snyder 1967; Pfeiffer 1977; Stenzler & Atema 1977; Hirvonen et al. 2000; Brown & Magnavacca 2003; Schoeppner & Relyea 2005), with the degree of response proportional to the relatedness of the prey species (Schoeppner & Relyea 2005; Dalesman et al. 2007a). This link between responsiveness and relatedness appears to have innate elements, with juveniles showing stronger responses to more closely related heterospecifics without the need for prior experience (Schoeppner & Relyea 2005; Dalesman et al. 2007a). Local adaptation of innate avoidance response has been demonstrated in the freshwater gastropod Lymnaea stagnalis, which

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shows stronger innate responses to alarm cues from other prey species found in sympatry with the responding population than to allopatric species (Dalesman et al. 2007a).

There is also evidence that, in the short term, prey species can learn to recognize heterospecific alarm cues. Mirza & Chivers (2003), for example, showed that fathead minnows, *Pimephales promelas*, learned to respond to cues from brook sticklebacks, *Culaea inconstans*, following a period of conditioning with water from predatory yellow perch, *Perca flavescens*, fed on sticklebacks and minnows. Prey taxa may also respond to heterospecific alarm cues following a period of cohabitation in their natural environment, a process that has been suggested for larval damselflies (*Enallagma* spp.) responding to cues from fathead minnows (Chivers et al. 1996). Pollock et al. (2003) also showed that fathead minnows that had cohabited with brook sticklebacks for 5 years responded in a similar way to conspecific and heterospecific (i.e. stickleback) cues, suggesting that developmental plasticity may have led to a change in behaviour. As active predators were present in each of these cases the learnt recognition of heterospecific alarm cues appears to have occurred through cue association.

Many of the studies that have found a relationship between relatedness and recognition of heterospecific alarm cues have used wild-caught individuals (Snyder 1967; Pfeiffer 1977; Stenzler & Atema 1977; Vilhunen & Hirvonen 2003; Schoeppner & Relyea 2005); hence the mechanism allowing enhanced recognition of alarm cues from more closely related species is unknown. Clearly the evidence suggests that some species may be able to fine tune their behavioural responses to predation risk, including information sampled from their environment about cohabiting species. What is not clear is whether the only mechanisms allowing heterospecific alarm cue recognition within local populations are innate recognition and cue association learning, or whether species may also enhance responses to heterospecific alarm cues following cohabitation without the potential for cue association. We aimed to test for the latter of these mechanisms by investigating whether the behavioural response of *L. stagnalis* to heterospecific alarm cues is enhanced through developmental plasticity following cohabitation in the absence of predation. By investigating the responses to several heterospecific taxa (ranging from intragenus to intrasuborder) we also tested whether any such alteration to avoidance behaviour is linked to phylogenetic relatedness.

METHODS

Study Organisms

Adult *L. stagnalis* (responders) used to establish the snail culture for behavioural trials were collected from South Drain, a drainage canal on the Somerset Levels, U.K. (51.18°N, 2.88°W) in April 2008. In the laboratory, adults were kept in an aquarium with 10 litres of aerated artificial pond water (ASTM 1980) with 90 mg/litre [Ca2+] (Rundle et al. 2004). They were maintained at $15 \pm 1\,°C$ under a 12:12 h light:dark cycle, and fed on iceberg lettuce and spinach. The four heterospecific species used to produce alarm cues were chosen to represent a gradient of relatedness to *L. stagnalis*: *L. fusca* is in the same genus as *L. stagnalis*, *Radix balthica* is in a separate genus within the same family (Lymnaeidae), and both *Physella acuta* and *Planorbarius corneus* represent different families (Physidae and Planorbidae, respectively) within the same suborder (Basommatophora) as *L. stagnalis*. *Lymnaea stagnalis* is known to respond to alarm cues from these heterospecific species when paired with tench, *Tinca tinca*, kairomones; however, this response is significantly lower than the response to conspecific alarm cues paired with tench kairomones (Dalesman et al. 2007a). We used juvenile F1 snails for all experiments to ensure that results were not affected by previous

experience, which has a significant effect on the antipredator behaviour of *L. stagnalis* responding to predator cues (Dalesman et al. 2006, 2009), and as this experiment was designed to test for an effect of cohabitation on the response to alarm cues we wanted to ensure that no prior learning was possible.

Tench were taken from a laboratory stock originally obtained from Emperor Tropicals & Water Garden Centre, Plymouth, U.K. They were maintained in aerated and filtered water at $15 \pm 1\,°C$ under ambient light levels in 25-litre aquaria at a stocking density of 12 fish per aquarium (0.006 kg/litre). Gravel substrate and shelters positioned in the aquarium were used to mimic natural conditions. The tench were fed Salmon Diet P20 (EWOS Ltd, Bathgate, U.K.), which contains no mollusc extract, and hence avoided the potential for dietary cues to affect the trials. No experimental manipulations were carried out on these fish and the welfare of the fish was carefully considered throughout. After the trials the tench were retained as laboratory stock.

Cohabitation Set-up

An initial population of a minimum 24 adults per snail species were held in a single aquarium to allow cross-fertilization between all individuals for 2 weeks prior to egg collection. Freshly laid egg strings were then collected once per day from adult aquaria and randomly allocated to separate 4-litre aquaria, maintained under identical environmental conditions to the adults. Each juvenile aquarium was populated using a minimum of 20 egg strings. Aquaria were set up to contain either *L. stagnalis* alone (isolated aquaria) or *L. stagnalis* plus one additional species from the four heterospecific species used (cohabitation aquaria). The same *L. stagnalis* adults were used to produce egg strings to populate both isolated and cohabitation aquaria to minimize the possibility of maternal effects differing between treatment groups. Four replicate aquaria were set up for each mixed-species group, and a further four aquaria containing *L. stagnalis* in isolation, with a stocking density of about 200 individuals per 4-litre aquarium; in aquaria with mixed species, we maintained 50% (100 individuals) each of *L. stagnalis* and a single heterospecific species. This stocking density was achieved by random thinning of the juvenile population in each aquarium once all the egg strings in that aquarium had hatched. Juvenile snails were used for behavioural trials (*L. stagnalis*) or alarm cue production (all species) once they reached a standard spire height (the distance from apex of the shell to the outer margin of the aperture) of 6 ± 0.5 mm, except in the case of *P. corneus*, a ramshorn species, where maximum spiral diameter measured 6 ± 0.5 mm.

Odour Production

Previous work has shown that naïve juvenile *L. stagnalis* rarely show antipredator behaviour in response to alarm cues presented alone (Dalesman et al. 2006, 2007b), including alarm cues from heterospecific snails (Dalesman et al. 2007a). Hence, for the purposes of testing the effect of experience on the behavioural response to alarm cues, they were always presented simultaneously with tench kairomones. Predator kairomones and alarm cues were produced in the same way in all the experiments (Dalesman et al. 2006). We used tench to produce predator kairomones as they are a known natural predator of freshwater gastropods (Brönmark 1994) and are sympatric with the population of *L. stagnalis* used as responders in this study. Juvenile snails from this population also show a high level of crawl-out behaviour in response to tench kairomones paired with conspecific alarm cues (Dalesman et al. 2007b). We produced combined predator kairomone plus alarm cue water by placing three tench (length 10 ± 1 cm) into 4 litres of aerated artificial pond water for

1 h and mixing three crushed snails (6 ± 0.5 mm) into this water immediately prior to the behavioural trial. We used crushed snails as opposed to feeding snails to tench to ensure that the alarm cue concentration paired with tench cue was standardized for all behavioural trials. The control was the addition of aerated artificial pond water with no cues added.

Behavioural Assays

Behavioural trials were carried out in June 2008 in conditions analogous to those in which the snails were maintained. In all cases *L. stagnalis* was used as the responder. Behavioural chambers consisted of a white plastic dish, 165 mm in diameter and 60 mm deep (A.W.Gregory & Co. Ltd., London, U.K.), with a longitudinally sectioned white PVC pipe, 36 mm long and 30 mm in diameter, attached open side down to the centre using nontoxic sealant (Wickes Ultimate Sealant and Adhesive, Wickes Building Supplies Ltd., London, U.K.) to provide a refuge. Juvenile snails were acclimated to behavioural chambers for 24 h in 630 ml of artificial pond water prior to behavioural assays. We then added 70 ml of cue water to each chamber at the start of the behavioural assay to give a final concentration of 10% cue water. The chamber into which each snail was placed and the treatment added was fully randomized for each behavioural trial. The position of each snail within the behavioural chambers was recorded immediately following cue addition and subsequently every 5 min for 2 h. Crawl-out behaviour is the main antipredator response of *L. stagnalis* to tench kairomones paired with alarm cues (Dalesman et al. 2006, 2007a), hence only results from crawl-out behaviour are presented here. Crawl-out behaviour was analysed using the proportion of total time spent crawled out of the water.

We used the first set of behavioural trials to assess whether cohabitation had an effect on antipredator behaviour in *L. stagnalis* in response to conspecific alarm cue paired with tench kairomones (compared with control water) using individuals from each of the cohabitation aquaria, as well as from aquaria containing *L. stagnalis* alone. A second set of behavioural trials was then used to assess whether cohabitation affected the response to heterospecific alarm cues. First, we exposed *L. stagnalis* that had been reared in isolation to tench kairomones paired with alarm cues from each of the heterospecific species and conspecifics to assess the response without experience of other species. Second, we exposed *L. stagnalis* from cohabitation aquaria to heterospecific alarm cues paired with tench kairomones; here the design was such that *L. stagnalis* individuals from a cohabitation aquarium were only exposed to alarm cues from the heterospecific species with which they had been cohabiting.

Statistical Analysis

Time spent crawled out was calculated as a proportion of the total trial duration, and arcsine-square-root transformed prior to analysis as proportional data tend towards a binomial distribution (Sokal & Rohlf 1995), although data on the figures are presented as the raw proportions. Data used during the analyses did not conform to normality despite the appropriate transformation; however, ANOVA is robust to deviations from normality, particularly when, as in these analyses, the designs are balanced with a relatively large number of treatments (Underwood 1997). All analyses were carried out using GMAV5 (Institute of Marine Ecology, University of Sydney, Sydney, Australia). Post hoc pairwise tests on response means were carried out using Student–Newman–Keuls (SNK) tests.

The effect of rearing condition on the response to conspecific alarm cues was analysed using a three-way ANOVA. Factors included in the model were: the snail species the responding *L. stagnalis* were reared alongside (*L. stagnalis*, *L. fusca*, *R. balthica*, *P. acuta* or

P. corneus), aquaria nested within species (four levels) and treatment during the behavioural trial (control or alarm cue paired with tench kairomones), with $N = 5$ replicates giving a total of 200 snails.

The response of *L. stagnalis* to heterospecific cues was split into two analyses as 'aquaria' was a non-nested factor when snails were reared in isolation, but was nested within cohabiting species when snails were reared in cohabiting conditions.

The response of *L. stagnalis* reared in isolation to heterospecific alarm cues was analysed using a three-way ANOVA with rearing aquaria (four aquaria), treatment during the behavioural trial (control or alarm cue paired with tench kairomones) and species crushed to produce the alarm cue (*L. stagnalis*, *L. fusca*, *R. balthica*, *P. acuta* or *P. corneus*) used as factors, with $N = 5$ replicates giving a total of 200 snails.

The response of *L. stagnalis* to heterospecific alarm cues following cohabitation was analysed using a three-way ANOVA with cohabiting species (*L. stagnalis*, *L. fusca*, *R. balthica*, *P. acuta* or *P. corneus*), aquaria nested within cohabiting species (four aquaria per species) and treatment during the behavioural trial (control or alarm cue paired with tench kairomones) used as factors, with $N = 5$ replicates giving a total of 200 snails.

RESULTS

Overall there were no significant effects of individual rearing aquaria on snail behaviour in the response to either conspecific or heterospecific alarm cues (Tables 1, 2). Crawl-out behaviour rarely occurred in the control groups, and was at an extremely low level when it did (Figs 1–3).

Response to Conspecific Alarm Cues

Crawl-out behaviour was significantly higher when snails were exposed to conspecific alarm cues combined with tench kairomones compared with controls ($F_{1,15} = 269.70$, $P < 0.001$; Fig. 1), but there was no significant effect of the species that *L. stagnalis* had cohabited with during development (Fig. 1).

Response to Heterospecific Alarm Cues When Reared in Isolation

Crawl-out behaviour was significantly higher in *L. stagnalis* reared in isolation in response to alarm cues paired with tench kairomones compared to controls; this effect was significantly higher when conspecific alarm cues were present relative to any of the heterospecific alarm cues ($F_{4,160} = 4.25$, $P = 0.003$; SNK: $P < 0.01$ for all pairwise comparisons; Fig. 2, Table 1), but no significant difference was found between the heterospecific treatments (SNK: $P > 0.05$ for all pairwise comparisons; Fig. 2) Finally,

Table 1
Lymnaea stagnalis reared in isolation; results from three-way ANOVA

Source	df	Mean square	F	P
Aquaria (Aq)	3	0.17	1.09	0.354
Treatment during behavioural trial (Tr)	1	11.30	70.73	<0.001
Alarm cue species (Sp)	4	0.68	4.28	0.003
Aq*Tr	3	0.18	1.09	0.353
Aq*Sp	12	0.11	0.70	0.753
Tr*Sp	4	0.68	4.25	0.003
Aq*Tr*Sp	12	0.10	0.63	0.816
Total	199			

Factors used are: rearing aquaria (four aquaria), treatment during the behavioural trial (control/alarm cues plus tench kairomone) and species used to produce alarm cue (*Lymnaea stagnalis*/*Lymnaea fusca*/*Radix balthica*/*Physa acuta*/*Planorbarius corneus*). $N = 5$ for each aquaria*treatment*species combination.

Table 2
Lymnaea stagnalis reared in cohabitation; results from three-way ANOVA

Source	df	Mean square	F	P
Species cohabiting with during rearing (SpR)	4	1.50	10.22	<0.001
Aquaria nested in SpR (Aq(SpR))	15	0.15	1.13	0.331
Treatment during the behavioural trial (Tr)	1	18.09	146.13	<0.001
SpR*Tr	4	1.47	11.84	<0.001
Tr*Aq(SpR)	15	0.12	0.95	0.507
Total	199			

Factors used are: species cohabiting with and subsequently used to produce alarm cue (*Lymnaea stagnalis*/*Lymnaea fusca*/*Radix balthica*/*Physa acuta*/*Planorbarius corneus*), rearing aquaria nested within cohabiting species and treatment during the behavioural trial (control/alarm cues plus tench kairomone). $N = 5$ for each aquaria*treatment*species combination.

there was no significant difference between any of the control groups (SNK: $P > 0.05$ for all pairwise comparisons; Fig. 2).

Response to Heterospecific Alarm Cues When Reared in Cohabitation

Crawl-out behaviour was significantly higher in snails exposed to heterospecific alarm cues combined with tench kairomones than controls for all species tested (SNK: $P < 0.05$ for all pairwise comparisons; Fig. 3); however, this difference between controls and paired cues was altered by cohabitation, such that the response to alarm cues from each species paired with tench kairomones was *L. stagnalis* = *L. fusca* > *R. balthica* > *P. acuta* = *P. corneus* ($F_{4,15} = 11.84$, $P < 0.001$; SNK: $P < 0.05$ for pairwise comparisons except where indicated as equal; Fig. 3, Table 2). No significant effect of rearing environment was found in comparisons between the control groups (SNK: $P > 0.05$ for all pairwise comparisons; Fig. 3).

DISCUSSION

This is the first study to demonstrate that cohabitation, without the potential for associative learning, affects heterospecific alarm cue recognition in an invertebrate. Previous work has demonstrated that pulmonate snails can use experience to adjust their behavioural response to a potential predator if they have had exposure to cues from that predator during development (Rundle et al. 2004; Dalesman et al. 2009), and also via shorter-term cue association (Dalesman et al. 2006). At the same time, naïve
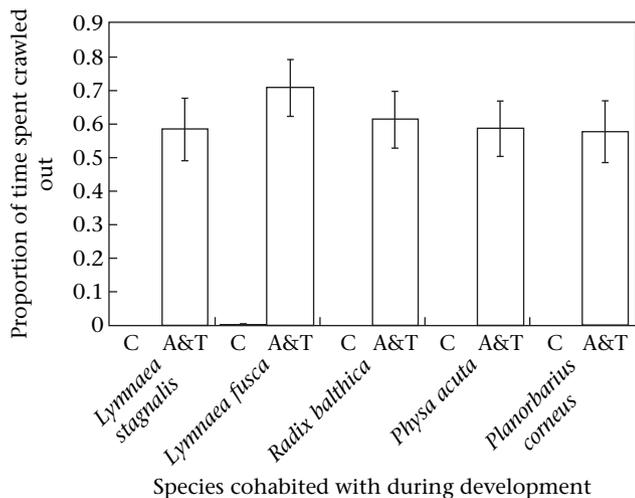
Figure 1. Mean ± SE proportion of time *L. stagnalis* spent crawled out in response to conspecific alarm cue paired with tench kairomones (A&T; □) or control (C; ■). $N = 20$ for each column.
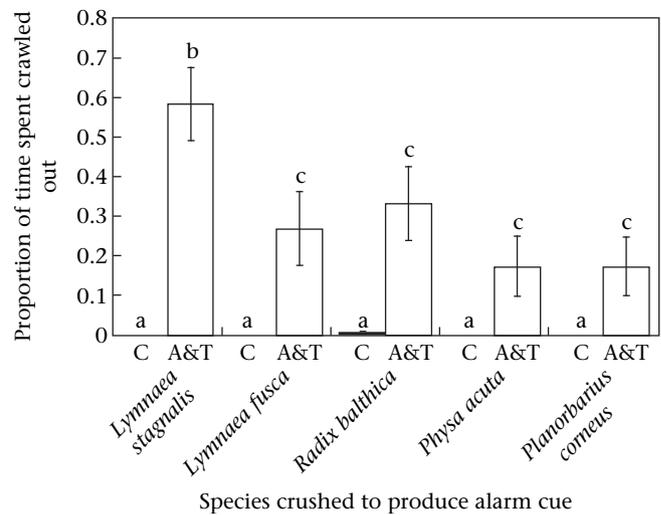
Figure 2. Mean ± SE proportion of time *L. stagnalis* reared in isolation spent crawled out in response to conspecific or heterospecific alarm cue paired with tench kairomones (A&T; □) or control (C; ■). Columns marked with different lettering (a, b, c) are significantly different from one another. $N = 20$ for each column.

L. stagnalis juveniles also respond more strongly to alarm cues from species to which they are more closely related or with which they have a sympatric distribution (Dalesman et al. 2007a). Here we have shown that an elevated behavioural response can be generated following cohabitation with another species, and that the degree of this response was linked to the relatedness of the cohabiting heterospecific. Following cohabitation, the response of *L. stagnalis* to tench kairomones paired with alarm cues from closely related species (*L. fusca* and *R. balthica*) was higher than with alarm cues from more distantly related species (*P. acuta* and *P. corneus*), such that the response to *L. fusca* alarm cue was strongest, followed by that to *R. balthica*, with the weakest responses to alarm cues from *P. acuta* and *P. corneus*.

The species chosen to provide heterospecific alarm cues in this study did not induce different responses in *L. stagnalis* without prior cohabitation, and the response to all heterospecific alarm cues was considerably lower than that in response to conspecific cues.
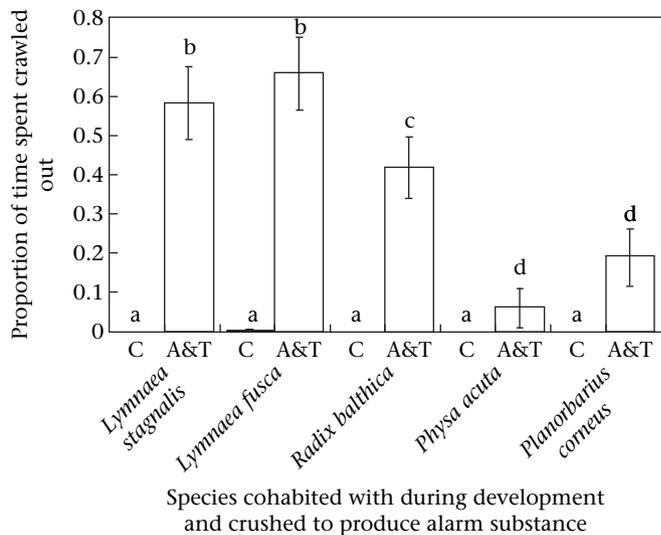
Figure 3. Mean ± SE proportion of time *L. stagnalis* reared in cohabitation spent crawled out in response to conspecific or heterospecific alarm cue paired with tench kairomones (A&T; □) or control (C; ■). Columns marked with different lettering (a, b, c, d) are significantly different from one another. $N = 20$ for each column.

This suggests that when assessing the responses to heterospecific alarm cues, environmental experience needs to be taken into account. Previous work demonstrating enhanced responses towards alarm cues from closely related compared with distantly related heterospecific species have suggested that this pattern could be caused by either adaptation (Schoeppner & Relyea 2005; Dalesman et al. 2007a) or, as demonstrated here, experience through cohabitation. The use of wild-caught individuals in a number of previous studies means that it is impossible to assess the relative contribution of innate versus learnt responses to the behaviours found (Stenzler & Atema 1977; Rochette & Dill 2000; Hazlett & McLay 2005; Turner 2008). Yet the importance of phylogenetic relatedness in aquatic invertebrates responding to heterospecific alarm cues reflects findings for vertebrates (Mirza & Chivers 2001; Brown et al. 2003; Schoeppner & Relyea 2005), although, at this stage, whether the mechanistic basis for such alarm cue recognition is the same is still unknown.

The ability to recognize alarm cues appears to be as important to gastropod prey species as predator recognition, showing both local adaptation in innate recognition and developmental plasticity. Indeed, the presence of alarm cues is required in the case of L. stagnalis to induce maximum antipredator behaviour unless they have recently experienced a predation event allowing for cue association (Dalesman et al. 2006). Closely related species are potentially more likely to share common predators than more distantly related species as their phenotypes and the environment they occupy may be more similar, and hence recognition may be conserved. Alternatively the ability to recognize alarm cues may be caused by a similarity in the chemicals released among closely related species, as is thought to be the case with alarm substance recognition in fish (Pfeiffer 1977; Hirvonen et al. 2000; Mirza & Chivers 2001; Brown et al. 2003).

The effect that cohabitation had on the recognition of heterospecific alarm cues by L. stagnalis suggests that part of the cue that is recognized and that induces the crawl-out response is released by live animals when they are undamaged. It is likely these chemicals provide an important role in species recognition and mate finding in freshwater pulmonate snails as their environment would limit the efficacy of other forms of communication (Dodson et al. 1994). Cohabitation may then increase cross-species recognition through exposure to these chemicals, but only in closely related species where chemicals are similar. Potentially, the alarm cue that L. stagnalis is responding to is a combination of species recognition chemicals and a common damaged-related chemical such as haemolymph. A similar mechanism has been proposed for the Ostariophysan alarm system, where a conserved N−O group (the release of which indicates skin damage) may be paired with species-specific compounds (Brown et al. 2003). Clearly, further work on the chemical communication systems underpinning these responses is necessary if we are to understand more fully the mechanisms resulting in such fine-tuned behavioural responses.

Acknowledgments

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