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The behavioural response of adult *Petromyzon marinus* to damage-released alarm and predator cues

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Using semi-natural enclosures, this study investigated (1) whether adult sea lamprey *Petromyzon marinus* show avoidance of damage-released conspecific cues, damage-released heterospecific cues and predator cues and (2) whether this is a general response to injured heterospecific fishes or a specific response to injured *P. marinus*. Ten replicate groups of 10 adult *P. marinus*, separated by sex, were exposed to one of the following nine stimuli: deionized water (control), extracts prepared from adult *P. marinus*, decayed adult *P. marinus* (conspecific stimuli), sympatric white sucker *Catostomus commersonii*, Amazon sailfin catfish *Pterygoplichthys pardalis* (heterospecific stimuli), 2-phenylethylamine (PEA HCl) solution, northern water snake *Nerodia sipedon* washing, human saliva (predator cues) and an adult *P. marinus* extract and human saliva combination (a damage-released conspecific cue and a predator cue). Adult *P. marinus* showed a significant avoidance response to the adult *P. marinus* extract as well as to *C. commersonii*, human saliva, PEA and the adult *P. marinus* extract and human saliva combination. For mobile *P. marinus*, the *N. sipedon* washing induced behaviour consistent with predator inspection. Exposure to the *P. pardalis* extract did not induce a significant avoidance response during the stimulus release period. Mobile adult female *P. marinus* showed a stronger avoidance behaviour than mobile adult male *P. marinus* in response to the adult *P. marinus* extract and the adult *P. marinus* extract and human saliva combination. The findings support the continued investigation of natural damage-released alarm cue and predator-based repellents for the behavioural manipulation of *P. marinus* populations in the Laurentian Great Lakes.

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Key words: 2-phenylethylamine; alarm chemicals; antipredator behaviour; chemical ecology; predator–prey interactions; sea lamprey.

INTRODUCTION

Sea lamprey *Petromyzon marinus* L. 1758 are ectoparasitic primitive fish that invaded most of Laurentian Great Lakes in the early 20th century (Smith & Tibbles, 1980) and precipitated large declines in a variety of native fish populations, especially lake trout *Salvelinus namaycush* (Walbaum 1792) (Applegate, 1951; Smith & Tibbles, 1980; Eshenroder *et al.*, 1992). Control of this invasive pest presently relies on lampricides

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used in tributary streams where *P. marinus* spawn (Brege *et al.*, 2003), low-head barrier dams to prevent access to spawning grounds (Lavis *et al.*, 2003) and trapping of adult *P. marinus* during their upstream migration (Twohey *et al.*, 2003). Due to rising lampricide costs and an effort to diversify the control programme, the Great Lakes Fishery Commission has encouraged the development and use of alternative control methods that are effective and environmentally benign (Great Lakes Fishery Commission, 2011). One of the recently suggested approaches involve using chemosensory alarm cues as natural repellents that could be used for the behavioural manipulation of migratory phase adult *P. marinus* populations for control purposes (Imre *et al.*, 2010).

Indeed, recent evidence suggests that *P. marinus* recognize and respond to chemosensory alarm cues. Wagner *et al.* (2011) demonstrated in a semi-natural experimental study that the proportion of adult *P. marinus* on the side of a laboratory stream channel scented with decaying *P. marinus* extract was significantly lower compared to the control treatment. Whether adult *P. marinus* responded to damage-released alarm cues or substances released by tissue decay was unclear, because the putrefaction extract used by Wagner *et al.* (2011) was a mixture of extracts from freshly killed animals as well as animals left to decay for 1, 2 and 5 days. In a follow-up, larger study, Bals & Wagner (2012) addressed the latter uncertainty and demonstrated that adult *P. marinus* avoided the odour of freshly killed and decayed conspecifics, as well as the odour of a decayed phylogenetically close species, silver lamprey *Ichthyomyzon unicuspis* Hubbs & Trautman 1937, but not the odour of freshly killed or decayed bluegill *Lepomis macrochirus* Rafinesque 1819, a more distantly related species. Whether the avoidance response to the odour of decayed *I. unicuspis* is due to similarity of alarm chemicals specific to this lineage or whether *P. marinus* had the opportunity to learn to associate these alarm chemicals with danger due to previous exposure is not clear (Pollock *et al.* 2003; Pollock & Chivers 2004).

In spite of this evidence indicating that *P. marinus* responds to chemosensory alarm cues, it is unknown whether *P. marinus* shows any avoidance response to predator odours. *Petromyzon marinus* in its native habitat (Atlantic Ocean and tributary rivers used for spawning) falls prey to a large variety of predators, including several fish-eating bird species, northern water snake *N. sipedon*, fox *Vulpes vulpes*, mink *Neovison vison*, muskrat *Ondatra zibethicus*, raccoon *Procyon lotor* and several fish species (Scott & Crossman, 1998; Kircheis, 2004). Even though *P. marinus* in the Great Lakes is not native to these ecosystems, it is likely that it retained an innate ability to respond to chemosensory cues released by potential predators (Blumstein, 2006). In addition, several of the predators (both terrestrial and aquatic) known to prey on anadromous *P. marinus* are also present in and around the Great Lakes. Curiously, only a single unpublished study has investigated the behavioural response of *P. marinus* to predator odours and suggested that *P. marinus* show an avoidance response to human saliva (R. Namespetra, unpubl. data).

The goal of this study was to investigate the behavioural response of adult *P. marinus* to damage-released alarm cues prepared from conspecific *P. marinus* (adult *P. marinus* and decayed adult *P. marinus*), heterospecific fishes [sympatric white sucker *Catostomus commersonii* (Lacépède 1803) and Amazon sailfin catfish *Pterygoplichthys pardalis* (Castelnau 1855)], three different predator cues [*N. sipedon* washing, human saliva and 2-phenylethylamine (PEA HCl)] and a conspecific damage-released alarm cue and predator cue combination (adult *P. marinus* extract and human saliva mixture). Human saliva has common components with the saliva of other mammals and it is

likely to be perceived by *P. marinus* as a chemical cue released by mammalian predators during prey handling. The PEA HCl is a trace amine present in the urine of various mammalian predator species and has been recently shown to induce a strong avoidance response in rodents and herbivore species (Ferrero *et al.*, 2011). The combination of conspecific damage-released alarm cue and predator cue was included because the combination carries more information and it could be perceived as a stronger threat. It was hypothesized that adult *P. marinus* are highly responsive to predatory threats using chemosensory alarm cues in their environment. It was predicted that (1) adult *P. marinus* will exhibit an avoidance response to the conspecific damage-released alarm cues and predator odours, as well as to their combination, and (2) *P. marinus* will avoid the sympatric *C. commersonii* extract (members of the same prey guild), but not the allopatric *P. pardalis* extract. As a member of the superorder Ostariophysi, *C. commersonii* are known to have a specialized alarm pheromone produced in the skin cells. On the other hand, the *P. pardalis* is an armoured ostariophysan that has lost the ability to produce specialized alarm substances in their skin (Helfman *et al.*, 2009) and *P. marinus* did not have previous exposure to them. These predictions were tested in an experimental study conducted in in-ground semi-natural laboratory stream channels.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

The US Fish and Wildlife Service provided c. 4500 upstream migrating adult *P. marinus* collected by trapping from the Cheboygan, Ocqueoc, Manistique and St Mary's Rivers between May and July 2012. The experimental animals were held in 10001 flow-through tanks receiving chilled Lake Huron water (mean \pm s.d. temperature = $11.0 \pm 2.6^\circ$ C and pH = 7.9 ± 0.1) at the US Geological Survey Hammond Bay Biological Station before and during the experiment. All experimental subjects were photoreversed. The holding tanks were fitted with light-emitting diode (LED) lights on timers to manage the photoperiod of the experimental subjects. Both during photoreversal and during the actual experiment, animals were kept at a 16L:8D photoperiod with lights being turned off at 0600 hours. Experimental subjects were given a minimum of 3 days to acclimate to the change in their photoperiod. The 3 day photoreversal period was considered adequate based on a laboratory study by Kleerekoper *et al.* (1961) who found that photoperiod can be re-established in *P. marinus* after exposing the experimental animals to one to two artificial diurnal light cycles.

STIMULUS PREPARATION

The stimulus solutions used in the experiment were tissue extracts from *C. commersonii* (female $n = 1$; total length (L_T) = 420 mm; wet mass (M_W) = 1161 g; collected from Ocqueoc River), *P. pardalis* (female $n = 1$; $L_T = 350$ mm; $M_W = 332$ g; obtained from a local aquarium fish retailer), adult *P. marinus* (male $n = 1$; $L_T = 480$ mm; $M_W = 194$ g; female $n = 1$; $L_T = 513$ mm; $M_W = 235$ g; obtained from Manistique River), decayed adult *P. marinus* (male $n = 1$; $L_T = 505$ mm; $M_W = 269$ g; female $n = 1$; $L_T = 494$ mm; $M_W = 246$ g; collected from Manistique River), washing from *N. sipedon* (males $n = 2$; L_T , mean \pm s.d. = 770 ± 14 mm; female $n = 1$; $L_T = 1180$ mm; collected around the Hammond Bay Biological Station), human saliva (collected on three occasions from several adult human males and females at the Hammond Bay Biological Station), 99% pure PEA HCl (www.PureBulk.com), deionized water and a combination of human saliva and adult *P. marinus* extract.

Tissue donor subjects were killed with a quick blow to the head (use of experimental and tissue donor subjects was approved by the Algoma University Animal Care Committee; AUP

#: 2012-II-01). Tissue extracts were prepared by homogenizing the respective tissues (where applicable) in deionized water and using an aqueous solution of the filtrate as a stimulus to mimic the cue released as a result of a natural predation event. For each tissue extract solution, 160 g of all tissue types was collected from the anterior end of the decapitated bodies of the animals and homogenized with 800 ml of deionized water. The homogenate was then filtered, diluted to 2 l total volume with deionized water, placed in 50 ml aliquots and stored frozen at -20°C . The decayed adult *P. marinus* were prepared by placing euthanized animals in 1 l high-density polyethylene bottles and left to decay aerobically at ambient temperature for 48 h. Both the fresh and the decayed adult *P. marinus* extract were prepared using equal amounts of male and female tissue.

Nerodia sipedon washing was prepared by placing all three snakes in a shallow, high-density polyethylene enclosure containing 1 l of deionized water. They were kept in the enclosure for 24 h at room temperature. The water from the enclosure was placed in 50 ml aliquots and stored at -20°C . All *N. sipedon* were released unharmed back to the habitat where they were collected.

Human saliva was collected from several males and females (employees and researchers at the Hammond Bay Biological Station). Approximately 5–15 ml of saliva was collected from each donor. The collected saliva was pooled and then stored at 4°C until needed. Saliva was kept for a maximum of 18 h.

Water collected from the stream channels was used to dilute odorant solutions for dispensing. Five hundred millilitres of diluted solution were prepared for each replicate/treatment. The tissue extracts (*C. commersonii*, *P. pardalis*, adult *P. marinus* and decayed adult *P. marinus*), *N. sipedon* washing and deionized water were diluted to 5% by volume. The PEA HCl solution added to the stream channels was 0.32 M. Human saliva was diluted to 1% by volume. The saliva and adult *P. marinus* extract combination odorant was prepared at 1 and 5%. All stimulus solutions to be dispensed into the laboratory stream channels were prepared immediately prior to use to minimize degradation.

The concentration of the tissue extract stimuli was substantially smaller than the tissue extract preparations used in a recent study (Bals & Wagner, 2012) that examined the behavioural response of adult *P. marinus* using the same experimental system (experimental stream channels located at the Hammond Bay Biological Station, in Millersburg, Michigan, U.S.A.); less tissue was used (160 g v. 220–310 g) in at least double the rinsate:extract volume (2 l v. 1 l or less) that was filtered and added to the stream channels at half the concentration (diluted to 5 v. 10% in stream water before being delivered to the stream channels for any given replicate) than in Bals & Wagner's (2012) study.

EXPERIMENTAL PROTOCOL

To investigate the behavioural response of adult *P. marinus* to the suite of damage-released heterospecific and conspecific cues as well as predator cues, a semi-natural experiment was performed in two parallel, in-ground laboratory stream channels located at Hammond Bay Biological Station over 11 experimental days, between 26 May and 14 July 2012. The experimental room had no windows, hence received no outside natural light. The stream channels received $0.01\text{ m}^3\text{ s}^{-1}$ of Lake Huron water. Ten replicates of 10 adult *P. marinus* (five groups of 10 males and five groups of 19 females) were observed for each of the nine stimuli. Male and female replicates were acclimated and tested in separate stream channels, to avoid any potential chemosensory and behavioural interactions between the sexes.

Petromyzon marinus were placed in cages (overturned baskets) separated by sex, in the two parallel laboratory stream channels, a minimum of 3 h before being released and observed. This allowed the future experimental subjects to acclimate to the environmental conditions characteristic to the laboratory stream channels. All replicate groups of animals to be tested in a given day of trials were deposited in the acclimation area at the same time, to avoid further disturbance. The acclimation area was several metres upstream of the experimental arena section of the laboratory stream channels. Before the start of two simultaneous replicates of a given treatment, two groups of 10 *P. marinus* (of opposite sex) were moved downstream and released into experimental arena section of the two stream channels (length \times width \times depth = 450 cm \times 185 cm \times 20 cm) by tipping the basket slowly over. Animals were given further 20 min to acclimate to the disturbance of being moved slowly downstream and released. After the 20 min acclimation period,

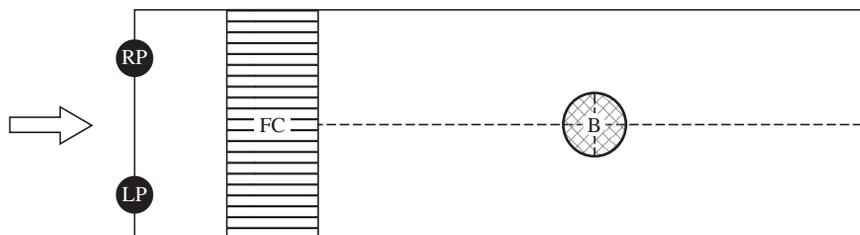


FIG. 1. View from above a laboratory stream channel (\Rightarrow , the direction of flow). The water passed through a flow conditioner (FC) made of PVC pipes to make the water flow linear and eliminate eddies. Experimental subjects were released from a basket in the centre of the stream channel (B) at the beginning of the 20 min acclimation period. Stimuli were dispensed from pumps on the right (RP) or left (LP) (---, the middle of the laboratory stream channel).

animals were observed and their distribution was noted for 20 min before the release of the stimuli (pre-stimulus observation period), as well as for 20 min during the release of the stimuli (stimulus observation period). During the stimulus observation period, peristaltic pumps (model: Masterflex L/S 7533–20; www.masterflex.com/) located 150 cm upstream of the observation area pumped 20 ml min^{-1} of stimulus downstream (Fig. 1). The release side of the stimulus (left or right) in a given stream channel was regularly alternated between subsequent pairs of replicates. A recent study using the same experimental system found that the release side of the stimulus did not influence the response of the animals to stimuli (Wagner *et al.*, 2011). After the end of a given pair of simultaneous replicates, experimental subjects were removed from the experimental arena, checked for sexual maturity and their M_W (to the nearest g; mean \pm s.d. = 229.9 ± 49.8 g) and L_T (to the nearest mm; mean \pm s.d. = 48.3 ± 3.7 cm) were recorded. In addition, experimental water temperature (mean \pm s.d. = $11.3 \pm 2.4^\circ \text{C}$) was measured for every replicate trial, while pH (mean \pm s.d. = 7.9 ± 0.1) was measured once a day.

DATA COLLECTION

All replicates were filmed with a night-vision capable Sony video camera (model: HDR-HC9; www.sony.com/) and the footage was simultaneously recorded on DVDs with Sony video recorders (model: VRD-MC6). After the experiment ended, behavioural data were collected from DVDs. The stream channel was separated into two halves (scented and non-scented half channel, Fig. 1) and the number of moving and resting animals in each half channel was recorded every 30 s based on the location of the animal's head. A moving *P. marinus* was defined as an actively swimming individual within the experimental arena. A resting *P. marinus* was defined as a motionless individual latched onto a solid surface, on the bottom or on the concrete sidewall of the experimental arena.

STATISTICAL ANALYSES

Not all animals were moving in any given replicate; hence, the proportion of all *P. marinus* and moving *P. marinus* on the scented side was calculated for all replicates. Accordingly, the experiment generated two data sets (all *P. marinus* and moving *P. marinus*). All proportion data were normally distributed (Shapiro–Wilk test, all $W \geq 0.977$, all $P \geq 0.115$) and were transformed with an arcsine transformation.

Data were analysed with two-way ANOVA. The dependent variable was the proportion of (all or moving) *P. marinus* on the scented side of the laboratory stream channel, while stimulus type and sex were treated as fixed categorical factors in the model; the interaction between the stimulus type and sex was also included.

If stimulus type, sex or their interaction was significant, two further sets of *post hoc* analyses (one for each data set, *i.e.* all *P. marinus* and moving *P. marinus*) were performed to identify

significant differences and to decompose the interaction term. Stimuli were compared to the deionized water control with one-way Dunnett's tests. If the sex or the stimulus type \times sex interaction was significant, planned comparisons were performed to identify the stimuli to which adult male and female *P. marinus* showed a significantly different avoidance response. The pre-stimulus proportion data were also analysed for both datasets as above to test the assumption that the distribution of the animals did not differ before the application of the different stimuli.

All statistical tests were performed with Statistica 10 (Statsoft Inc.; www.statsoft.com/). The significance level was set at $\alpha = 0.05$.

RESULTS

The distribution of experimental subjects did not differ between treatments before the application of the respective stimuli for either all *P. marinus* (two-way ANOVA, pre-stimulus observation period, stimulus type, $F_{8,72} = 0.870$, $P > 0.05$; sex, $F_{1,72} = 0.161$, $P > 0.05$; stimulus type \times sex, $F_{8,72} = 1.584$, $P > 0.05$) or moving *P. marinus* (two-way ANOVA, pre-stimulus observation period, stimulus type, $F_{8,72} = 1.095$, $P > 0.05$; sex, $F_{1,72} = 0.246$, $P > 0.05$; stimulus type \times sex, $F_{8,72} = 1.963$, $P > 0.05$).

The proportion of all *P. marinus* on the scented side differed across treatments during the stimulus observation period (two-way ANOVA, stimulus type, $F_{8,72} = 4.716$, $P < 0.001$). The proportion of all *P. marinus* on the scented side exposed to *C. commersonii* extract (Dunnett's test, $P < 0.05$), adult *P. marinus* extract (Dunnett's test, $P < 0.05$), human saliva (Dunnett's test, $P < 0.05$), PEA HCl (Dunnett's test, $P < 0.05$) and adult *P. marinus* extract and human saliva combination (Dunnett's test, $P < 0.001$) being significantly lower compared to the deionized water control. The decayed *P. marinus* extract (Dunnett's test, $P > 0.05$), the *N. sipedon* washing (Dunnett's test, $P > 0.05$) and the *P. pardalis* extract (Dunnett's test, $P > 0.05$) did not induce a significant change in the proportion of all *P. marinus* on the scented side during the application of stimuli, compared to the deionized water control.

On average, male and female adult *P. marinus* did not show a different distribution in response to various stimulus types (two-way ANOVA, sex, $F_{1,72} = 0.001$, $P > 0.05$); however, there were sex differences in avoidance response to some of the stimulus types (two-way ANOVA, stimulus type \times sex, $F_{8,72} = 2.501$, $P < 0.05$). Planned comparisons revealed that the proportion of female adult *P. marinus* was significantly higher on the scented side in response to the *N. sipedon* washing ($F_{1,72} = 7.274$, $P < 0.01$), compared to the proportion of adult male *P. marinus* on the scented side; however, it was not different from the deionized water control (Dunnett's test, $P > 0.05$).

Mean \pm s.d. proportions 0.66 ± 0.20 and 0.68 ± 0.20 of all *P. marinus* were mobile during the pre-stimulus and stimulus observation periods.

Similar to the all *P. marinus* dataset, the proportion of moving *P. marinus* on the scented side differed across treatments during the stimulus observation period (two-way ANOVA, stimulus type, $F_{8,72} = 18.088$, $P < 0.001$, Fig. 2). The proportion of moving *P. marinus* on the scented side exposed to *C. commersonii* extract (Dunnett's test, $P < 0.01$), adult *P. marinus* extract (Dunnett's test, $P < 0.05$), human saliva (Dunnett's test, $P < 0.001$), PEA HCl (Dunnett's test, $P = 0.001$) and adult *P. marinus* extract and human saliva combination (Dunnett's test, $P < 0.001$) was significantly lower, compared to the deionized water control. There was a significantly higher proportion of moving *P. marinus* on the scented side in response to the *N.*

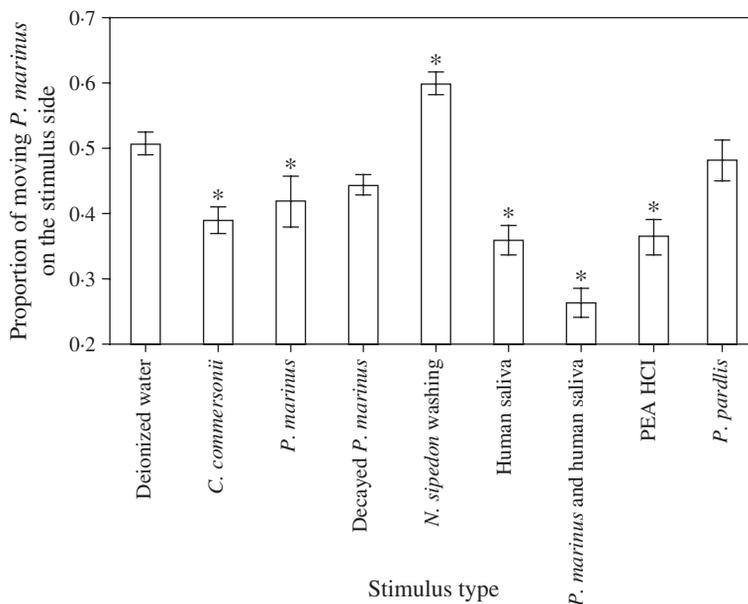


FIG. 2. Mean \pm S.E. proportion of moving *Petromyzon marinus* on the scented side during the stimulus observation period exposed to deionized water (control), *Catostomus commersonii* extract, adult *P. marinus* extract, decayed adult *P. marinus* extract, *Nerodia sipedon* washing, human saliva, adult *P. marinus* and human saliva combination, 2-phenylethylamine (PEA HCl) solution and *Pterygoplichthys pardalis* extract ($n = 10$ for each treatment). Responses to stimuli (*) are significantly different from the deionized water control (Dunnett's test). Note that non-transformed proportion data are displayed, but the analysis was performed on arcsine-transformed proportion data.

sipedon washing (Dunnett's test, $P < 0.05$) compared to the deionized water control. The decayed *P. marinus* extract (Dunnett's test, $P > 0.05$) and the *P. pardalis* extract (Dunnett's test, $P > 0.05$) did not induce a significant change in the proportion of moving *P. marinus* on the scented side during the application of stimuli, compared to the deionized water control.

Moving female adult *P. marinus*, on average, had a lower proportion on the scented side in response to various stimulus types (two-way ANOVA, sex, $F_{1,72} = 7.562$, $P < 0.01$; stimulus type \times sex, $F_{8,72} = 1.670$, $P > 0.05$) compared to moving male adult *P. marinus*. Planned comparisons showed that the proportion of moving female adult *P. marinus* was significantly lower on the scented side in response to the adult *P. marinus* extract ($F_{1,72} = 9.670$, $P < 0.01$), and the adult *P. marinus* extract and human saliva combination ($F_{1,72} = 4.269$, $P < 0.05$), compared to the proportion of moving adult male *P. marinus* on the scented side.

DISCUSSION

This is the first study to conclusively show that adult *P. marinus* strongly avoid a variety of predator odours, including human saliva and PEA HCl, and a human saliva and adult *P. marinus* extract combination. Adult *P. marinus* were also found to avoid adult

P. marinus extract. These findings strongly support the hypothesis that *P. marinus* are highly responsive to predatory threats using chemosensory alarm cues in their environment and confirm earlier suggestions by Wagner *et al.* (2011) that harm avoidance is facilitated by olfaction in *P. marinus*. Furthermore, it was demonstrated that adult *P. marinus* avoided damage-released alarm cues from a sympatric but not an allopatric species of the superorder Ostariophysi.

Damage-released chemosensory alarm cues are vital in aquatic environments in terms of relaying antipredator information to a wide variety of fish species (Brown, 2003). The presence of an avoidance response by experimental adult *P. marinus* exposed to freshly killed adult *P. marinus* whole-body extract in this study suggests that *P. marinus* respond to damage-released conspecific alarm cues. The findings corroborate the conclusions of Bals & Wagner (2012) and Pietrzakowski *et al.* (2013), who found that adult *P. marinus* avoided the odour of freshly killed adult and larval conspecifics.

A recent review by Imre *et al.* (2010) suggested that decayed adult *P. marinus* odour may function as an alarm cue for *P. marinus*. This study found that the decrease in the proportion of all adult *P. marinus* on the scented side of semi-natural stream channels exposed to decaying *P. marinus* extract was borderline significant, a behavioural response consistent with avoidance behaviour. Similarly, Wagner *et al.* (2011) and Bals & Wagner (2012) recently noted that adult *P. marinus* avoided the dilute odour of decaying conspecifics. Given that the strength of the avoidance response by *P. marinus* increases with the concentration of the stimulus (Bals & Wagner 2012), it is plausible to assume that the weaker avoidance response noted in this study may be related to the lower concentration of the stimulus in this study compared to previous studies. Overall, the results of these studies suggest that *P. marinus* respond to chemicals emitted from decayed *P. marinus* and are consistent with an alarm response to alarm cues or necromones (Wagner *et al.*, 2011).

This study also investigated the behavioural response of adult *P. marinus* to two different heterospecific stimuli, obtained from *C. commersonii*, a species sympatric with invasive *P. marinus* populations, and from *P. pardalis* that does not overlap in its distribution with *P. marinus*. Both species are members of the superorder Ostariophysi, a taxonomic group well known to harbour specialized chemicals in their skin that function as damage-released alarm cues (Pfeiffer, 1977). In contrast to *C. commersonii*, suckermouth armoured catfishes, including *P. pardalis*, are heavily armoured members of Ostariophysi that do not produce chemical alarm cues in their skin (Helfman *et al.*, 2009). *Petromyzon marinus* showed a strong avoidance response to *C. commersonii* extract. As expected, *P. marinus* did not exhibit any avoidance of the *P. pardalis* extract. The avoidance response exhibited to the *C. commersonii* extract is surprising in the light of the fact that Bals & Wagner (2012) clearly demonstrated no avoidance response by *P. marinus* to the damage-released alarm cues prepared from sympatric *L. macrochirus*. Nevertheless, the finding raises the possibility that, similar to other fish species, *P. marinus* can learn to associate alarm cues released from sympatric heterospecific individuals with the presence of predators. Heterospecific prey guild members can acquire predator recognition from being presented with a predator cue paired with alarm cues from heterospecific fishes that are a member of the same prey guild (Brown, 2003). *Catostomus commersonii* and *P. marinus* are known to co-occur (and overlap in their spawning season also) in Great Lakes tributaries (Scott & Crossman, 1998) and can fall prey to predators inhabiting riverine environments. It is plausible to assume that *P. marinus*, perhaps as larvae, were exposed to

damage-released alarm cues from *C. commersonii*, paired with olfactory predator cues. For example, Chivers *et al.* (1995) found that brook sticklebacks *Culaea inconstans* (Kirtland 1840) presented with a conspecific or fathead minnow *Pimephales promelas* Rafinesque 1820 extract paired with pike *Esox lucius* L. 1758 odour recognized the latter as a predator.

As a widespread antipredator adaptation, many prey species are averse to predator-derived odours, typically derived from skin, fur, urine, faeces and anal gland secretions (Apfelbach *et al.*, 2005). A recent experimental study by Ferrero *et al.* (2011) has identified a biogenic amine PEA HCl as a predator cue that induced innate avoidance responses in two prey species. The authors found this chemical to be present, with enriched production, across numerous mammalian carnivores (Ferrero *et al.*, 2011). Some of these carnivores produce over 3000-fold more of this chemical than the mammalian herbivores examined by Ferrero *et al.* (2011). When used as odours, biogenic amines can function as pheromones (Hashiguchi & Nishida, 2007). Hashiguchi & Nishida (2007) have recently identified a near-complete repertoire of trace amine-associated receptors (TAAR) genes in *P. marinus*, supporting the idea that *P. marinus* are able to recognize biogenic amines. PEA HCl is found in mammalian predator urine, and *P. lotor*, a known mammalian predator of *P. marinus* (Kircheis, 2004), have the highest concentration of it in their urine, compared to other North American carnivores (Ferrero *et al.* 2011). As expected, *P. marinus* presented with PEA HCl showed a strong avoidance response, compared to deionized water control. This finding is consistent with *P. marinus* recognizing and actively avoiding mammalian predator cues in their environment.

Fish species often approach predators, especially novel predators, to gain information about the riskiness of the predator (Brown *et al.*, 2000), known as predator inspection behaviour. Collecting visual cues from a predator is considered to be more risky and less reliable than chemical odours (Brown *et al.*, 2000). Compared to deionized water control, the proportion of mobile *P. marinus* increased on the side of the stream channel scented with *N. sipedon* washing, consistent with a chemically mediated predator inspection behaviour. Glowlight tetras *Hemigrammus erythrozonus* Durbin 1909 did inspect the odour of previously food-deprived Jack Dempsey cichlids *Rocio octofasciata* (Regan 1903), a novel predator odour (Brown *et al.*, 2000). Research from other fish species clearly indicated that acquired predator recognition is dependent on a predator cue being paired with conspecific alarm cues (Brown, 2003). It is plausible to assume that *P. marinus* might be treating the *N. sipedon* washing as a novel predator cue that is being inspected because the animals do not directly associate it with predation on conspecific *P. marinus*. This assumption is supported by a study by Brown (1958) that investigated the feeding habits of the *N. sipedon* and found that < 1% of the stomachs of 207 individual snakes from central New York and northern Michigan contained *P. marinus* remains.

Mammalian saliva can also function as a warning signal. For example, human saliva mixed into sand decreased predation rates on nest of diamond-backed terrapin *Malaclemys terrapin* by *P. lotor* (Burke *et al.*, 2005). Anecdotal observations by *P. marinus* control agents routinely indicated that the presence of human saliva in water induces strong fright responses by captive *P. marinus*. In addition, R. Namespeta (unpubl. data) found that *P. marinus* exhibited strong signs of distress and actively avoided undiluted human saliva when compared to river water control. The present experiment indicated that the proportion of *P. marinus* on the side of stream channels

scented with human saliva was significantly lower compared to a deionized water control. This finding is consistent with the hypothesis that *P. marinus* perceive human saliva as a mammalian predator cue, but further research is needed to ascertain what are the active ingredients of saliva that induce an avoidance response.

Adult *P. marinus* in this study showed the strongest apparent avoidance response to a combination of a mammalian predator cue (human saliva) and damage-released conspecific alarm cue (adult *P. marinus* extract). This result is consistent with the expectation that conspecific alarm cues coupled with predator cues should induce a stronger avoidance response than either of these alarm cues alone (*i.e.* sensory complementation; Elvidge *et al.* 2013), because the combination carries more information (identifies not only that a conspecific individual was predated upon but also reveals the identity of the predator) and as such it could be perceived as higher level of threat (Ferrari *et al.* 2008; Kim *et al.*, 2009; Leduc *et al.* 2010). Indeed, *H. erythrozonus*, for example, took a longer time to initiate inspection, inspected in smaller groups and remained a larger distance from the odour source derived from *H. erythrozonus*-fed *R. octofasciata*, compared to food deprived *R. octofasciata* or *R. octofasciata* that were fed a different species of fish (Brown *et al.*, 2000). Further research should address the distinction in avoidance response strength to combined, compared to single cues and investigate the potential interaction between increased overall alarm cue concentration (additive effect) and cue type (synergistic effect).

Mobile adult female *P. marinus* showed stronger avoidance of the adult *P. marinus* extract as well as the adult *P. marinus* extract and human saliva combination than adult male *P. marinus* in the study. This finding is in contrast with that of Bals & Wagner (2012), who did not find differences in avoidance behaviour between sexes when exposed to damage-released alarm cues. A recent study by Pietrzakowski *et al.* (2013) found that, when presented with the odour of freshly killed *P. marinus* larvae, adult female *P. marinus* swam significantly longer than adult male *P. marinus*, presumably to avoid predation. The findings of Pietrzakowski *et al.* (2013) and this study suggest that females may be less willing to take risks than males. As females have a larger reproductive investment, they may be selected to better avoid predators until they reproduce. Intersexual differences in predator avoidance behaviour are well documented in other fish species. For example, dominant male darkedged splitfin *Girardinichthys multiradiatus* (Meek 1904) approach predators more often than females (Macías García *et al.*, 1994) and are more frequently attacked and consumed by predators (Macías García *et al.*, 1998).

As a next step, further research should explore the effect of the concentration of predator cues and combination cues (conspecific damage-released alarm cues and predator cues) on the strength of avoidance behaviour, potential synergistic effect of combining different alarm cues as well as the efficiency of application of the above stimuli in the wild, where *P. marinus* of both sexes migrate in streams that are variable in size, temperature, microhabitat type and flow conditions. In addition, almost all the above cues are a cocktail of a wide array of chemicals, so identifying the active ingredients is instrumental for making the application of these repellents both more effective for behavioural manipulation as well as more appropriate for industrial scale production.

In conclusion, *P. marinus* showed an avoidance response to adult *P. marinus* extract, sympatric heterospecific alarm cues (*C. commersonii* extract) as well as predator cues, like human saliva (mammalian predator cue) and PEA HCl (a chemical present in

the urine of a large number of mammalian carnivores) as well as a combination of damage-released conspecific alarm cue and predator cue (adult *P. marinus* extract plus human saliva). *Nerodia sipedon* washing induced a behavioural response by mobile adult *P. marinus* consistent with predator inspection. *P. marinus* showed no response to tissue extract from *P. pardalis*, an exotic catfish. Mobile adult female *P. marinus* showed a stronger avoidance behaviour than mobile adult male *P. marinus* in response to the adult *P. marinus* extract and the adult *P. marinus* extract and human saliva combination. Overall, as suggested by Imre *et al.* (2010), damage-released alarm cues now present a realistic potential of being used as a tool in controlling *P. marinus* populations in the American Great Lakes.

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