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Predator detection in *Lymnaea stagnalis*

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Summary

Laboratory-reared *Lymnaea* are capable of detecting and responding to the scent of a crayfish predator. The present investigation is a first attempt to characterize multiple stress-related behavioural responses resulting from predator detection and to depict the neurophysiological correlates of one of these illustrated behaviours. Snails respond to crayfish effluent (CE) by increasing the following behaviours: aerial respiration, exploratory/searching phase and sensitivity to the shadow-elicited full-body withdrawal response. In contrast, when snails detect CE they decrease both their righting response time when dislodged from the substratum and their basal cutaneous oxygen consumption. Interestingly, basal heart rate does not change in response to CE exposure. Finally, we directly measured the activity of the neuron that initiates aerial respiratory behaviour, RPeD1, in semi-intact preparations. Naïve snails exposed to CE prior to recording demonstrated both a significantly reduced spontaneous firing rate and fewer bouts of bursting activity compared with non-exposed snails. These data show that laboratory-reared *Lymnaea* that have never experienced a natural predator are still capable of detecting and responding to the presence of a historically sympatric predator. These data open a new avenue of research, which may allow a direct investigation from the behavioural to the neuronal level as to how an ecologically relevant stressful stimulus alters behaviour.

Key words: *Lymnaea*, crayfish, sympatric predator, defensive behaviours, predator–prey interactions.

Introduction

The most effective way to avoid becoming prey is to avoid the predator. Prey animals have developed morphological and behavioural traits to defend themselves against predation at each stage of predator–prey interaction and utilize the most appropriate sensory modalities to detect, localize and identify predators. Many examples of species-typical behaviours labelled ‘vigilance’ or ‘risk assessment’ have been demonstrated (Apfelbach et al., 2005). In aquatic environments, chemical cues are important signals between and among species (Alexander and Covich, 1991; Burks and Lodge, 2002; Chivers and Smith, 1998; Dodson, 1988) and provide a means by which organisms can gather information about their spatio-temporal environment. Prey species utilize olfactory detection of chemical cues to assess the risk of predation and thus adjust their phenotype appropriately (Hoverman and Relyea, 2007; Kats and Dill, 1998; Turner et al., 2000).

Many organisms, including gastropods, have been shown to use kairomones [predator-derived chemical cues (Covich et al., 1994; Jacobsen and Stabell, 2004; Turner et al., 2000)] for risk assessment, and as a consequence of this detection increase the use of spatial or temporal refugia when predators are recognized (Rigby and Jokela, 2000; Vermeij and Covich, 1978). *Lymnaea stagnalis* responds to both fish and crayfish predator-released kairomones and, depending on the specific predator detected, utilizes a functionally appropriate response by either sheltering under crevices or crawling above the waterline (Dalesman et al., 2006; Rundle and Bronmark, 2001).

Learning about predators through pairing predation directly with the predator or via damaged conspecific alarm cues or both has been noted in several invertebrates including gastropods (Chivers et al., 1996; Hazlett et al., 2002; Langerhans and Dewitt, 2002; Rochette et al., 1998). However, the majority of these studies utilized wild-caught animals, which makes it impossible to determine whether predator-induced defence responses were innate or the result of prior experience (Dalesman et al., 2006). To surmount this predicament, we set up an experimental protocol where only snails from a population that has been maintained in the laboratory since the early 1950s (i.e. over 250 generations) would be used. These snails would therefore have never experienced a natural predator such as crayfish.

We have extensively utilized the *Lymnaea* model system to elucidate the underlying neuronal mechanisms of: (1) the central pattern generator (CPG) underlying aerial respiration; (2) how activation of the whole-body withdrawal behaviour inhibits respiratory behaviour; (3) how learning and memory are encoded within the nervous system; (4) how juvenile behaviours differ from adult behaviour; and (5) how an environmentally relevant toxin (H_2S) alters adaptive behaviours (Lukowiak et al., 1996; McComb et al., 2002; Parvez et al., 2006; Rosenegger et al., 2004; Syed et al., 1992b; Syed and Winlow, 1991). We now

set out to determine, using this model system, how the detection of a predator alters behaviour and the activity of neurons that control different aspects of the behaviours.

Despite much investigation into predator–prey interactions in gastropods and specifically predator detection in *Lymnaea stagnalis*, few studies have examined empirically the behavioural and physiological manifestations of predator detection in this model organism. Our present study addressed the following questions. (1) Do laboratory-reared *Lymnaea* respond with predictable behavioural changes when exposed to predator effluent? (2) Does exposure to predator effluent induce metabolic changes in *Lymnaea*? (3) Does predator detection alter neuronal activity in key neurons involved in predator-induced defensive behaviours? Here our data unequivocally demonstrate that lab-bred *Lymnaea* are capable of detecting and responding in a functionally appropriate manner to the scent of a natural predator, the crayfish.

Materials and methods

Snails

Lymnaea stagnalis (L.) were bred and raised in the snail facility at the University of Calgary from a strain of *Lymnaea* originally obtained from *Vrije Universeit* in Amsterdam. The ancestors of these snails were obtained from canals in a polder located near Utrecht in the early 1950s and since then they have not been exposed to naturally occurring predators (except neurobiologists!). Snails were maintained in home aquaria at room temperature (20–22°C) and fed lettuce *ad libitum* until experimental treatment.

Righting response, exploratory behaviour and shadow withdrawal

We first sought to determine whether exposure to crayfish effluent (CE) affected non-withdrawal defensive responses; therefore, we investigated two measures of prey vigilance. When dislodged from the substrate and inverted onto the dorsal surface of the shell, snails perform a righting behaviour to regain contact with the substrate. We measured the time to right of individual snails, first in pond water (PW) then, after 24 h, in CE water and boiled CE (BC) following another 24 h rest interval. Individual snails were placed in a 9 cm Petri dish filled with 1 cm of PW, CE or BC water for a 10 min acclimation period. A wooden stick was used to dislodge each snail and manoeuvre it onto its dorsal surface. Each snail was dislodged 5 times and the average righting time was taken in each treatment. To ensure that direction of treatment was not a factor, treatments were run both in the order outlined and in reverse.

As another measure of vigilance to predator presence, we determined the time to explore after full-body withdrawal in each treatment. When disturbed, snails withdraw into their shell and will re-emerge after a period and begin to explore when placed in PW. Snails were placed in a 1 l beaker filled with 500 ml of PW, CE or BC for 2 h, then withdrawn from the water and immediately placed into a 9 cm Petri dish filled with PW. The time to explore, as determined when the snail's tentacles and head were extended and the snail began to actively crawl along the substrate, was measured.

A third measure of predator detection that we analysed was to measure the shadow-induced withdrawal response in *Lymnaea*. When performing aerial respiration at the air–water interface, *Lymnaea* will close their pneumostome (the respiratory orifice) and partially withdraw into their shell, and thus inhibit aerial respiration, when a shadow passes over the pneumostome opening (Stoll, 1973). We measured the shadow response of snails in PW, CE and BC by passing a shadow over each snail during a bout of aerial respiration. A 35 W halogen light was placed 50 cm above 500 ml of PW, CE or BC in a 1 l beaker. To increase the rate of aerial respiration, the water was made hypoxic by bubbling N_2 through it to displace the oxygen for 20 min prior to placing the snails in the beaker. This is a standard procedure often utilized during operant conditioning of the aerial respiratory behaviour (for details see Lukowiak et al., 1996; Lukowiak et al., 2003). Snails were placed in the beaker and allowed to acclimate for 2 h. A 2 cm diameter piece of cardboard was attached to the end of a stick and placed approximately 2–3 cm over an aerial respiring snail for 2–5 s. Withdrawal was scored as successful if the snail completely closed the pneumostome under the shadow within the 5 s interval. Each snail was tested 3 times and the mean response was taken.

Brief description of aerial respiratory behaviour

Lymnaea are bimodal breathers obtaining oxygen either through cutaneous respiration (i.e. directly through the skin) or through aerial respiration via a rudimentary lung (i.e. gas exchange with the atmosphere). To perform aerial respiration, the snail must surface and open its pneumostome while contracting and relaxing the appropriate respiratory muscles. A more detailed description has been given previously (Lukowiak et al., 2003).

Breathing observations

To determine whether exposure to a crayfish predator affected the respiratory behaviour, snails were first placed in 500 ml of room temperature eumoxic pond water (PW) and then, after a 24 h rest interval, placed in 500 ml of either CE or BC for 0.5 h. The time and duration of the pneumostome openings were noted during each of the 0.5 h periods. From these measurements, the number of openings, total breathing time and average breathing time were calculated.

All experiments were done 'blind' such that the examiner was unaware of the treatment being given.

Heart rate and O_2 consumption

Heart rate was measured visually by observing the heart beating directly through the shell. Snails were placed in a clear glass beaker containing 500 ml of PW or CE. The heartbeat was counted for 3× 1 min intervals and the mean was taken. Two separate experimental procedures were utilized. (1) Snails were allowed to acclimate in PW or CE for 10 min and then the heart rate was counted. (2) Snails were placed in PW overnight and the heart rate was measured in the morning, then the PW was siphoned out of the beaker and CE was siphoned back in, such that the flow did not physically disturb the snails; the heart rate was then measured in the same snails immediately after CE was added. These two protocols were used to ensure that physically

disturbing the snails was not the cause of heart rate alterations and therefore were analysed separately. Since the two protocols gave consistent results only data from the latter are reported here.

Oxygen consumption was measured for snails in either PW or CE in a closed system. Individual snails were placed in a 50 ml Erlenmeyer flask filled with either PW or CE and all the air was flushed out when the rubber stopper was placed over the opening of the flask. An oxygen electrode was placed through the stopper and recordings were taken every 10 s for 20 min. Snails were rested for 24 h and then retested in the opposite treatment. Snails in both CE and PW were compared with 'blank' controls where no snail was placed in the chamber, to account for electrode O_2 consumption. Trials were stopped after 18–20 min to ensure that the level of oxygen in the chamber did not decrease beyond 90% saturation. O_2 measurements were analysed using OxyView (PST3-V5.32 02/2004 by PreSens). Experiments were done at room temperature (22°C).

Semi-intact preparations

The preparations were dissected similar to methods previously described (Inoue et al., 2001; McComb et al., 2003; Spencer et al., 1999) except that only the penis was removed, the head/foot complex and buccal mass were left fully intact. Preparations were pinned down in individual recording dishes with their dorsal sides uppermost. The central ring ganglia (CNS) were pinned to the dish directly through the foot musculature, dorsal-side up. The outer sheath surrounding the CNS was removed using fine forceps. Standard electrophysiological techniques were used as previously described in *Lymnaea* semi-intact preparations. Intracellular recordings were obtained using sharp glass microelectrodes filled with saturated K_2SO_4 solution. Tip resistances of the microelectrodes used for recordings ranged from 20 to 50 MΩ. Intracellular signals were amplified via a NeuroData amplifier and displayed simultaneously on a Macintosh PowerLab/4SP (ADInstruments) and a Hitachi oscilloscope. Recordings were analysed and stored using the PowerLab software. See McComb et al. (McComb et al., 2003; McComb et al., 2005) for fuller details.

Statistics

We analysed water treatment effects on snail behaviour data with repeated measures analysis of variance. All repeated measures data were tested for equal variance using Mauchly's test for sphericity. In cases where sphericity could not be assumed, we used the conservative adjusted Greenhouse-Geisser *P* values. For cases in which we identified a significant interaction between the repeated factor and water treatment, we used repeated contrasts to identify which water treatment pairs differed significantly. For oxygen consumption experiments, we paired averaged time-matched O_2 saturation values for each snail in both PW and CE treatments. Electrophysiological data were analysed using ANOVA with Tukey's *post hoc* test to detect cases in which we identified a significant interaction. Non-homogenous data (number of spikes per 10 min interval) were square-root transformed to homogenize data prior to ANOVA. All statistics were performed on SPSS version 11.0.4 for Macintosh.

Results

Righting response, exploratory behaviour and shadow withdrawal

It must be emphasized that in all experiments reported here, snails did not come into direct contact with the predator but only came into contact with water taken from the aquarium where the crayfish were maintained (i.e. the crayfish effluent, CE). It seemed logical to hypothesize that if *Lymnaea* could sense the presence of a predator (i.e. CE), defensive behaviours should be enhanced. Therefore, we asked the question: when placed in a vulnerable position (i.e. with the ventral part of the foot exposed and away from the substrate), do snails alter their righting behaviour in CE? We found that snails exposed to CE significantly decreased their righting time compared with snails in PW or BC control groups. That is, when snails were placed inverted upon their shells, they took a significantly shorter time to flip over and regain their foothold on the substrate when in CE compared with PW or BC (Fig. 1).

We next sought to determine whether snails significantly altered their exploratory behaviour in CE, by measuring the time it took for snails to re-emerge from their shells after a small perturbation. We exposed snails to one of the three treatments (PW, CE, BC; see Materials and methods) for 2 h then removed them from the water and placed them into a Petri dish filled with PW. The time from when the snail was first placed in the Petri dish until the snail began to crawl on the substrate was recorded. We found that snails previously exposed to CE significantly increased their overall time to begin exploring (Fig. 2). That is, the CE-treated snails took significantly longer to emerge from their shells following a minor disturbance compared with the time following PW or BC exposure.

Our next measure of predator detection was to determine whether exposure to CE would affect the sensitivity of *Lymnaea* to the shadow-induced pneumostome withdrawal response. Snails were placed in 500 ml of hypoxic CE or PW and allowed to acclimate, after which the shadow treatment began. We found that snails in CE elicited a full pneumostome withdrawal more often when presented with a passing shadow than did snails in PW. Together, our data demonstrate that snails significantly

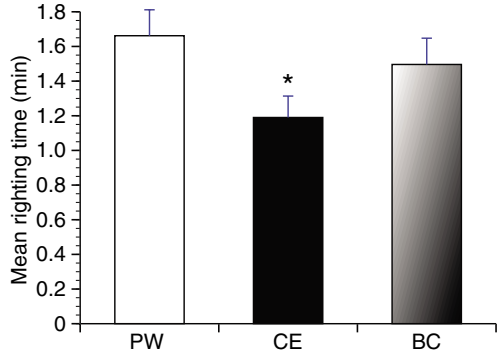

Fig. 1. The change in mean (±s.e.m.) righting response time after exposure to pond (PW, 1.66±0.15 min), crayfish (CE, 1.19±0.12 min) or boiled crayfish (BC, 1.5±0.15 min) water (N=36). PW and BE means are not significantly different from each other (P>0.05) but are significantly different (*P<0.05) from CE treatment (repeated measures ANOVA).

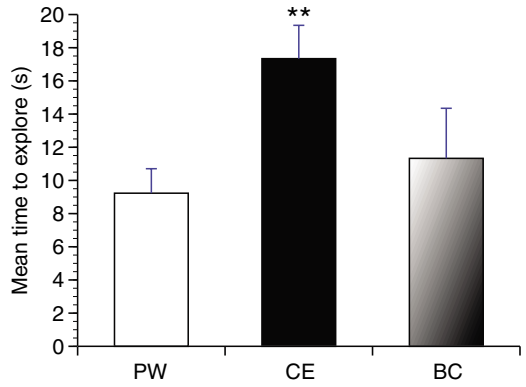

Fig. 2. The mean (±s.e.m.) time to explore for snails placed in PW after a 2 h exposure to PW, CE or BC. Time to explore in the CE treatment was 17.34±2.01 s compared with 9.23±1.476 s in PW and 11.33±3.018 s in BC treatment (N=54, **P<0.01, one-way ANOVA).

decrease their righting response and significantly increase their exploratory and shadow withdrawal responses following exposure to CE (Fig. 3).

Breathing observations

We next determined whether aerial respiratory behaviour was altered in *Lymnaea* exposed to CE. Previous reports indicate that when pulmonate snails are in the presence of a crayfish predator they tend to spend more time near the surface of the water (Dalesman et al., 2006; Turner et al., 2000; Turner and Montgomery, 2003). We hypothesized that if our laboratory-reared *Lymnaea* were capable of detecting crayfish kairomone, and as a consequence spent more time at the air–water interface, they may show a significant alteration in aerial respiratory behaviour. We therefore measured the number of pneumostome openings and the total breathing time in PW, CE and BC. These data are plotted in Fig. 4. As can be seen, the number of pneumostome openings (Fig. 4A) and the total breathing time (Fig. 4B) were significantly increased in CE (P<0.01, N=65) compared with controls. Interestingly, the mean breathing time for each pneumostome opening was not significantly different as a result of CE exposure (Fig. 4C, P=0.144, N=65). We conclude that snails can detect the presence of a crayfish

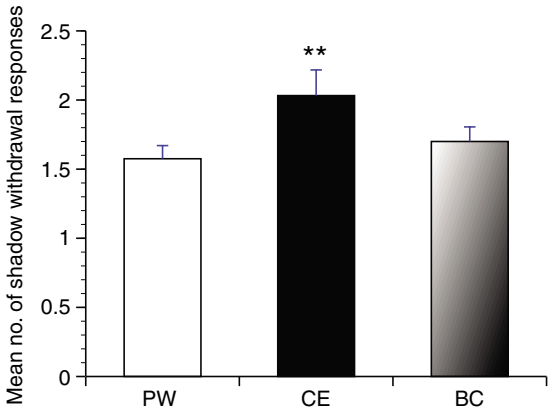

Fig. 3. The mean (±s.e.m.) number of elicited shadow responses of snails in the PW and CE conditions. Snails in CE elicited full pneumostome withdrawal more often when presented with a passing shadow than did snails in PW. Specifically, the mean (±s.e.m.) number of shadow withdrawal responses in PW was 1.00±0.09, whereas snails in CE closed their pneumostomes on average 1.60±0.07 times (N=22, **P<0.01, repeated measures analysis).

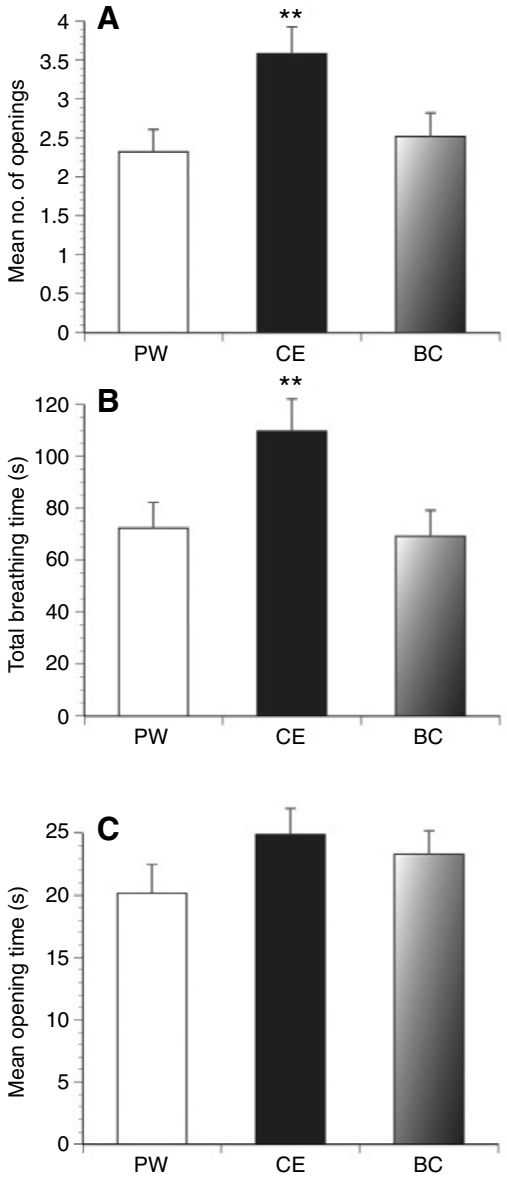

Fig. 4. The mean (±s.e.m.) number of pneumostome openings (A), total breathing time (B) and mean breathing time (C) of snails in each of the three water treatments. (A) Number of pneumostome openings in PW (2.32±0.29) compared with that in CE (3.58±0.35) and BC (2.52±0.30). The number for CE is significantly higher (**P<0.01, N=65) than that for either PW or BC, which were not significantly different from each other (one-way ANOVA). (B) The total breathing time in PW (72.5±9.84 s), CE (109.8±12.29 s) and BC (69.20±10.01 s; N=65). Again, CE results were significantly higher (**P<0.01, N=65) than those for either PW or BC, which were not significantly different from each other (one-way ANOVA). (C) The mean breathing time was not significantly different in any of the groups.

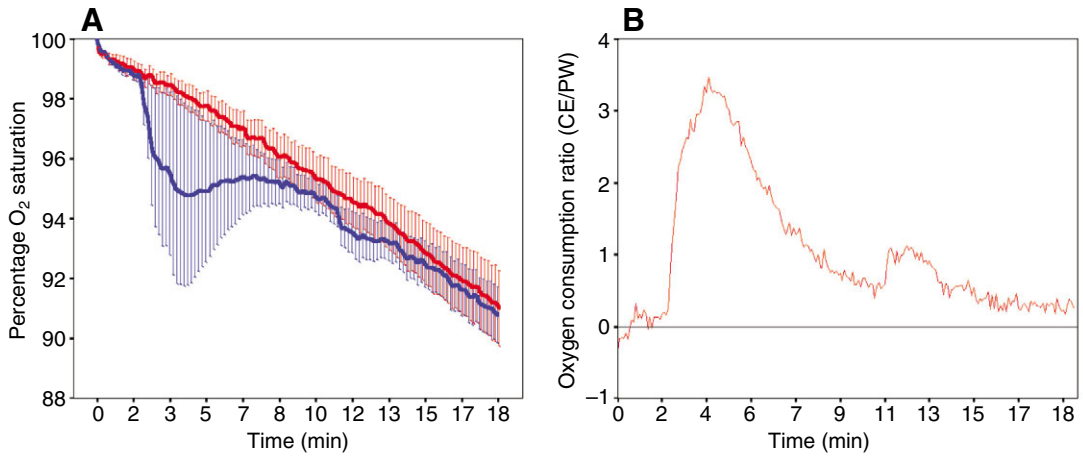

Fig. 5. Oxygen consumption of snails cutaneously respiring in either PW or CE. (A) Total averaged data (means ± 1 s.e.m.) demonstrating oxygen depletion in a closed chamber for snails in either CE (top red trace) or PW (bottom blue trace) measured as percentage O_2 saturation. The two traces are significantly different (repeated measures analysis for time-linked measurements on each snail, N=9, P<0.001). (B) The ratio of CE to PW O_2 consumption for each snail at each time interval, reference line at 0.

predator and increase the number of times they open their pneumostome to breathe, and thereby increase the total breathing time when exposed to CE.

Heart rate and O_2 consumption

Considering the respiratory behavioural data (see above), we next sought to determine whether predator detection was causing a change in either cardiac or respiratory physiology of the snails. We found that heart rate was not significantly different in CE from that in PW. That is, there was no significant change in heart rate (mean ± s.e.m., 29.01±1.93 in CE and 29.57±2.13 in PW, N=28, P=0.34, data not shown).

Non-aerial oxygen consumption (i.e. cutaneous respiration) was measured in both PW and CE by analysing the rate of oxygen decrease over time in a closed system (see Materials and methods). We found that the mean O_2 consumption was not significantly different between the two treatments (i.e. PW *vs* CE) over the course of the experiment. However, we found a significant increase in O_2 consumption in PW-treated snails between 2 and 8 min (Fig. 5A). It is of note that between the 4 and 8 min time points the averaged data give the appearance of a small increase in percentage saturation. However, in fact the oxygen level in the chamber does not increase for any one animal and this apparent increase in percentage saturation is explained by the noticeable increase in variation of animal behaviour during this time period (see Discussion). By taking the ratio of CE to PW O_2 consumption for each snail at each time interval, a striking 3-fold increase in O_2 consumption in the PW-treated snails compared with CE-treated snails was detected during this time interval (Fig. 5B). Therefore, we found that there was a time-dependent increase in O_2 consumption in PW that was not apparent in CE. That is, snails in CE do not consume O_2 as rapidly as snails in PW during the initial stage of the experiment. This suggests to us that in *Lymnaea* the so-called compensatory mechanisms described by Taylor et al. (Taylor et al., 2003) during exposure to a hypoxic challenge are suppressed by the detection of the predator.

Electrophysiological activity of RPeD1

Finally, we examined the activity of RPeD1 in semi-intact preparations that had previously been treated with PW, CE or BC. We chose RPeD1 because this is the neuron that (1) initiates the rhythmic activity that drives aerial respiratory behaviour and (2) is inhibited during the defensive full-body withdrawal response. We found significant reductions in three measures of

Table 1. *Intrinsic membrane properties of RPeD1 neurons in semi-intact brain preparations*

Intrinsic membrane properties	PW	CE	BC	P
Activity (spikes per 10 min)	94.46±15.96	23.06±5.70	83.20±6.94	<0.01
Total sample frequency (Hz)	0.16±0.02	0.058±0.012	0.12±0.08	<0.01
Resting membrane potential (mV)	–63.36±1.24	–61.27±1.16	–62.57±1.55	NSD
Mean spikes per burst	8.91±1.76	2.02±0.38	8.57±1.87	<0.01
Burst frequency (Hz)	1.55±0.267	1.11±0.14	1.91±0.18	NSD
After-hyperpolarization (mV)	–5.81±1.52	–5.78±0.11	–5.44±1.67	NSD
Spike amplitude (mV)	83.82±1.18	85.43±1.61	83.88±1.46	NSD
Burst duration (s)	12.64±2.86	4.52±0.77	9.78±1.22	<0.01

Values for pond water (PW), crayfish effluent (CE) and boiled CE (BC) are means ± s.e.m. P<0.01 indicates that CE treatment is significantly different from both PW and BC control groups, and PW and BC are not significantly different from each other. NSD, no significant difference. (All data represent raw non-transformed data.)

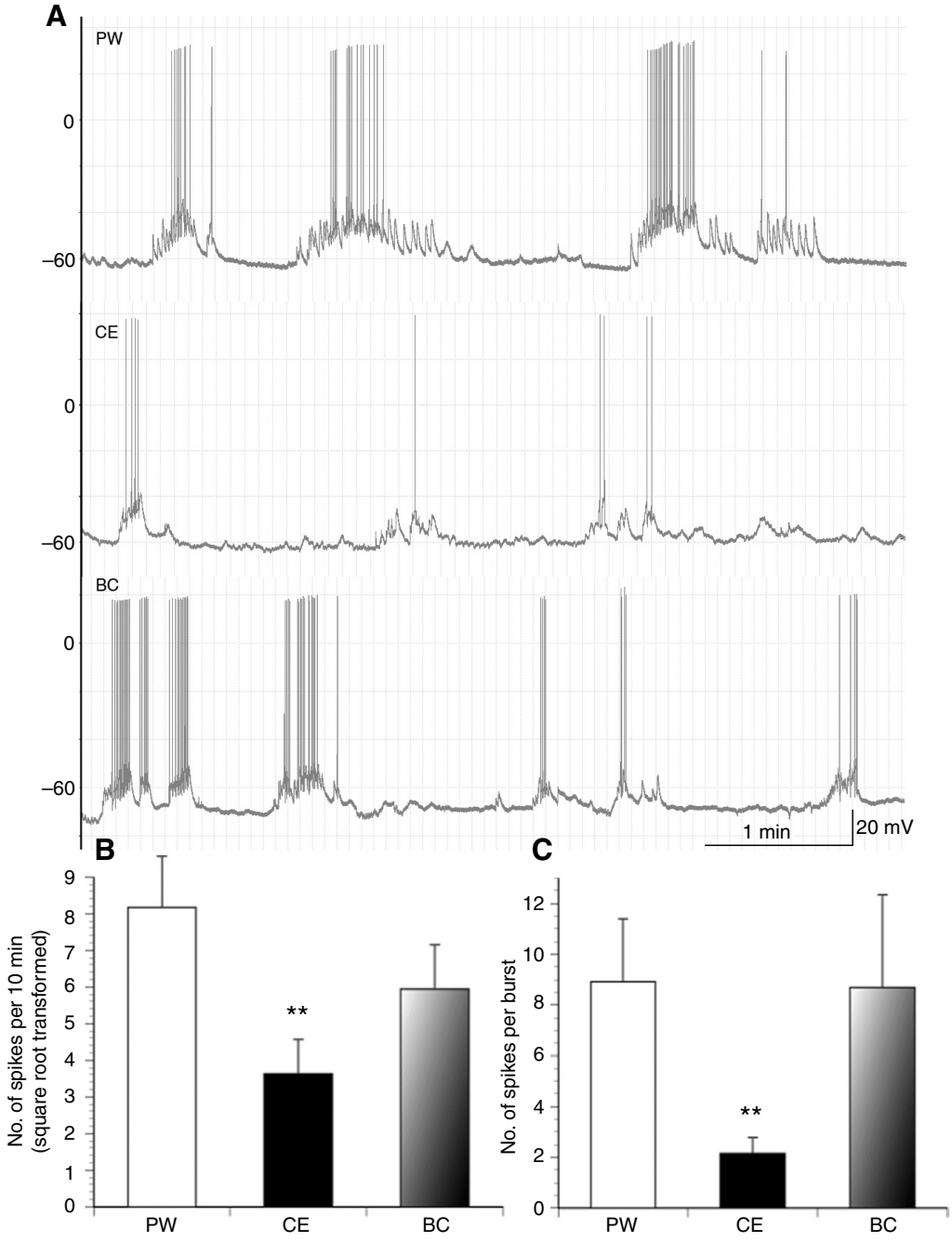

Fig. 6. (A) Representative electrophysiological recordings from RPeD1 in semi-intact preparations taken after intact snails were exposed to PW (top), CE (middle) or BC (bottom) treatments. All traces demonstrate spontaneous firing activity, and bursting activity. (B) Summary data for mean (±s.e.m.) spiking activity per 10 min (square-root transformed; N=14). Results for CE (3.63±0.93) are significantly lower (**P<0.01, N=14) than those for either PW (8.17±1.40) or BC (5.94±1.20), which were not significantly different from each other (one-way ANOVA). (C) Mean (±s.e.m.) number of spikes per burst. Again, results for CE (2.14±0.62) were significantly lower (**P<0.01, N=14) than those for either PW (8.91±2.49) or BC (8.69±3.65), which were not significantly different from each other (one-way ANOVA).

activity in the CE-treated animals compared with either the PW- or BC-treated snails (Fig. 6). The spontaneous firing activity, bursting activity and number of spikes per burst in CE-treated snails were significantly reduced compared with those in the PW- and BC-treated snails. Representative samples of recordings taken from RPeD1 in PW, CE and BC are presented in Fig. 6A, whilst summary data are presented in Fig. 6B,C. As is readily apparent, exposure of the intact snail to CE for 2 h before dissection was sufficient to significantly alter on-going electrical activity in RPeD1. We found no significant differences in other electrophysiological parameters (see Table 1) such as resting membrane potential, action potential amplitude or after-hyperpolarization.

Discussion

We have demonstrated that laboratory-reared *Lymnaea*, over 250 generations since the early 1950s, have maintained their capacity to detect crayfish predators via a kairomone, as evidenced by significant changes in certain defensive behaviours, physiological changes and changes in electrophysiological parameters in a key neuron, RPeD1. We showed that *Lymnaea* significantly increase aerial respiratory activity (which occurs when snails are at the air–water interface) when exposed to the effluent of *Procambarus clarkii* scent (i.e. CE; Fig. 4). This finding is consistent with the observation that snails often crawl out of the water when presented with shell-crushing predators such as crayfish (Alexander and Covich, 1991; Levri, 1998;

McCarthy and Fisher, 2000; Turner, 1997). However, our inability to detect changes in heart rate for snails in the presence of CE suggests that the increase in aerial respiratory behaviour may simply be a result of spending more time near the air–water interface and not due to increased respiratory demand. In support of this suggestion are our data showing that snails in PW initially consume more O_2 via cutaneous respiration than they do in CE (Fig. 5); although at the end of the experiment the two groups had consumed the same amount of O_2. Therefore, it appears that detection of the predator alters the so-called 'compensatory' response of an organism to hypoxia. As has been pointed out in other publications (Taylor et al., 2003; Taylor et al., 2001), organisms when sensing a decrease in P_{O_2} initially increase behaviours (the 'compensatory response') such as heart rate and respiratory rate to counteract the fall in P_{O_2}. Snails that sensed the predator did not do this and therefore we expect that these adaptive responses may represent an attempt to minimize O_2 requirements, and thereby lessen or avoid hypoxic impairment or damage (Hochachka et al., 1996). How this suppression of the compensatory response is mediated in the snail is not clear but will be the subject of further investigation. The above results combined with our findings of significant suppression of exploratory behaviour and a significant increase in the righting response following CE exposure (Figs 1 and 2) suggests that snails detecting the predator (i.e. increased risk assessment) alter their activities in a manner consistent with behavioural decisions that would prove beneficial to survivorship (i.e. 'keeping a low profile' or getting out of harm's way quicker). This is not surprising given that predator detection via kairomones not only gives information regarding predator presence but also potentially gives information regarding the proximity, physiological state and even diet of potential predators (Dalesman et al., 2006; Kats and Dill, 1998; Wisenden, 2000).

Exposure to predator odour from crayfish (i.e. CE) also caused a significant increase in the shadow response compared with controls (Fig. 3). Further, the righting response time of snails exposed to CE was decreased compared with either pond or boiled crayfish water (Fig. 1). Collectively these data sets indicate that snails actively reduced the duration of vulnerability on perceiving the presence of a predator. Increases in anti-predator responses such as vigilance during times of predator presence have been demonstrated in many aquatic organisms in which prey respond appropriately to factors such as predator density (Wiackowski and Staronska, 1999), distance (Turner and Montgomery, 2003), size of predator and prey vulnerability (Alexander and Covich, 1991; Cotton et al., 2004; Dewitt et al., 1999). Multiple prey traits allowing for functional responses to predator threat are not uncommon in aquatic systems as multiple traits provide a battery of possible defences against predation. *Lymnaea* and other pulmonates are capable of altering their defensive responses appropriately according the perceived predator threat. That is, depending on 'who' the predator is (e.g. fish *vs* crayfish), a different defensive strategy will be taken. Differential habitat use under multiple predator systems demonstrates both increased vigilance and the differentiation between predator threats, allowing for functional tradeoffs in predator defence (Dalesman et al., 2006; Dewitt and Langerhans, 2003).

When naïve snails were exposed to CE, the spontaneous firing activity and bursting activity of RPeD1 decreased in the semi-intact preparations compared with control snails (Fig. 6). To our knowledge this investigation provides the first evidence of neurobiological changes associated with predator detection in pulmonates. RPeD1 has been shown to be both necessary and sufficient to drive the aerial respiratory behaviour of *Lymnaea* (Syed et al., 1992a; Syed and Winlow, 1991) and is subordinate to the defensive full-body withdrawal behaviour (Inoue et al., 1996; Syed and Winlow, 1991). It is therefore not surprising that the activity pattern of this neuron is altered in the manner described when the crayfish predator is detected. Neurobiological investigation of the shadow response in *Lymnaea* has demonstrated that this process is mediated by peripherally located dermal photoreceptors and is susceptible to the habituation phenomenon (Stoll, 1973). It is therefore feasible that detection of a predator alters both central (RPeD1) and peripherally located components of the aerial respiratory network. The apparent conflict in our data showing an increase in respiratory behaviour and a decrease in the activity of RPeD1 may be explained by an up-regulation in the efficacy of peripheral inputs onto downstream components of the respiratory network, which would therefore require less input from RPeD1 to initiate the respiratory rhythm. Further investigation into both the location and activity of these chemosensory receptors is ongoing in our laboratory. Previously it has been demonstrated that there is an age-dependent change in suppressive input from the pneumostome area to CNS neurons, such as RPeD1 (McComb et al., 2005). Thus, we do not find it surprising that alterations in the peripheral nervous system function as a result of predator detection, and may play an important role in the mediation of aerial respiratory behaviours. The interaction between the central and peripheral nervous systems of molluscs, especially as regards mediation of adaptive behaviours, is complicated, interesting and controversial (Lukowiak and Colebrook, 1988; Lukowiak and Jacklet, 1972).

Lymnaea stagnalis has been extensively investigated as a model demonstrating the neuronal basis of cue-associated learning (Benjamin et al., 2000; Haney and Lukowiak, 2001; Sugai et al., 2007; Sugai et al., 2006); however, ours is the first study to investigate how a naturally occurring and ecologically relevant chemical cue alters neuronal function in *Lymnaea*. Further, the neuronal and molecular changes associated with operant conditioning of the respiratory behaviour in *Lymnaea* have been demonstrated in RPeD1 and therefore make this neuron the prime candidate for investigation of the neuronal mechanisms of 'fear conditioning' in an identified neuron.

Together, our data demonstrate numerous defensive behavioural and physiological responses that *Lymnaea* utilize following detection of a predator scent. The data also unequivocally show that this instinct has been maintained in the lab-reared snails over many generations and may allow investigations at the neuronal level into how such instinct is mediated. These behaviours are robust and repeatable in the laboratory setting and support both laboratory and field investigations demonstrating that *Lymnaea stagnalis* not only detect predator kairomones but also respond in the appropriate manner to decrease the probability of predation. The identity of

these kairomones has yet to be elucidated; however, chemical communication within decopod crustaceans is well described (Ameyawakumfi and Hazlett, 1975; Atema, 1986; Blake and Hart, 1993), and a metabolic component within the urine has been shown to play a role in dominance hierarchies (Breithaupt and Eger, 2002; Schneider et al., 1999). It is therefore likely that a component within the urine is a candidate for the kairomone involved in these snail–crayfish interactions.

The mechanisms of predator detection, which presumably are crucial to survival, remain hard to elucidate because of the difficulty in demonstrating causal links (Bolhuis and Macphail, 2001) between ecologically relevant behaviours (Kavaliers and Choleris, 2001) and neural substrates driving these behaviours. We have begun to form these links by investigating ecologically relevant chemical cues and the associated neuronal networks that are affected by predator detection. Since the molecular events in a single neuron (RPeD1) in *Lymnaea* have been shown to be necessary for long-tem memory formation, reconsolidation, extinction and forgetting (for reviews, see Lattal et al., 2006; Parvez et al., 2006), it is now possible to directly investigate how an ecologically relevant stress stimulus that has been maintained in laboratory-rearing conditions affects learning and memory at the level of a single neuron.

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