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‘Different strokes for different folks’: geographically isolated strains of *Lymnaea stagnalis* only respond to sympatric predators and have different memory forming capabilities

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SUMMARY

Gaining insight into how natural trait variation is manifest in populations shaped by differential environmental factors is crucial to understanding the evolution, ecology and sensory biology of natural populations. We have demonstrated that lab-reared *Lymnaea* detect and respond to the scent of a crayfish predator with specific, appropriate anti-predator behavioral responses, including enhanced long-term memory (LTM) formation, and that such predator detection significantly alters the electrophysiological activity of RPeD1, a neuron that is a necessary site for LTM formation. Here we ask: (1) do distinct populations of wild *Lymnaea stagnalis* respond only to sympatric predators and if so, can these traits be quantified at both the behavioral and neurophysiological levels, and (2) does the presence of a non-sympatric predator elicit anti-predator behaviors including augmentation of LTM? We tested three different populations of wild (i.e. not lab-reared) snails freshly collected from their natural habitat: (1) polders near Utrecht in The Netherlands, (2) six seasonally isolated ponds in the Belly River drainage in southern Alberta, Canada and (3) a 20-year-old human-made dugout pond in southern Alberta. We found strain-specific variations in the ability to form LTM and that only a sympatric predator evoked anti-predatory behaviors, including enhanced LTM formation and changes in RPeD1 activity.

Key words: *Lymnaea stagnalis*, long-term memory, sympatric predator, anti-predator behaviors, environmental stress.

INTRODUCTION

We previously demonstrated in laboratory-reared *Lymnaea stagnalis* that an inheritable trait, predator detection, elicits a number of anti-predator vigilance behaviors including enhanced long-term memory (LTM) formation (Orr et al., 2007; Orr and Lukowiak, 2008). We have also previously shown that there are strain-specific differences in the ability to form LTM between two populations of *Lymnaea stagnalis*, a Dutch population and an Albertan population (Orr et al., 2008). That is, we found that wild snails collected in Southern Alberta (the Belly river snails) or their laboratory reared off spring (F1 – Belly snails) possessed significantly superior LTM forming capabilities compared with either lab-reared snails (derived from snails collected in the 1950s from polders near Utrecht) or wild Dutch snails collected from the same area as those that formed the original colony. Thus, we concluded that memory-forming capabilities in *Lymnaea* were heritable. In the study described here we investigated whether there are also strain-specific differences in how *Lymnaea* respond to the detection of a predator. That is, do the different *Lymnaea* strains respond to the scent of a predator that they have never experienced? We already know that our lab-reared *Lymnaea* maintained their ability to detect a predator (Orr et al., 2007) even though they had never experienced the predator for over 250 generations. We have the opportunity to test this question in wild snails because while there are crayfish predators in The Netherlands there are no crayfish predators in Southern Alberta watersheds (Clifford, 1991). We report here that sympatric (occurring in the same geographic region) but not allopatric (i.e. non-sympatric) predators elicit anti-predator behaviors, including enhanced LTM formation. Thus, there are strain-specific differences in both behavioral and neural responses to different predator organisms.

Some understanding of population variation in cognitive traits has been gained through studies of artificial selection in rodents and insects (e.g. McGuire and Hirsch, 1977; Dukas, 2008). Yet studies that examine natural variation in behavior at the neural or genetic level and are able to associate the phenotypes with biological reasons for this variation are few and far between. This is possibly because few model organisms exist [e.g. bumble bees (Raine and Chittka, 2008)] where the opportunity to investigate cognitive trait variation in naturally occurring wild populations is possible and where the essential neural circuitry mediating learning and memory is known.

We utilize our *Lymnaea* model system to elucidate the underlying neuronal mechanisms of how associative memory formation is encoded within a three-neuron central pattern generator (CPG) circuit that drives aerial respiratory behavior following operant conditioning of this behavior (Syed et al., 1990; Syed et al., 1992b; Lukowiak et al., 1996; Lukowiak et al., 1998; Lukowiak et al., 2003; Lukowiak et al., 2008; McComb et al., 2002; McComb et al., 2005a; Parvez et al., 2006). Importantly, we have shown that one of these neurons, RPeD1 is a necessary site for LTM formation (Scheibenstock et al., 2002) as well as extinction, reconsolidation and forgetting (Sangha et al., 2003a; Sangha et al., 2003b; Sangha et al., 2005).

We show here that only a sympatric predator elicits alterations in adaptive behaviors and neurophysiological changes in RPeD1, a key neuron known to be a necessary site for LTM formation. In addition, we also found that there are strain-specific differences in memory forming abilities between different populations of Southern Alberta *Lymnaea*.

MATERIALS AND METHODS

Snails

Lymnaea stagnalis (L.) is a cosmopolitan species found worldwide in temperate regions. We used three geographically distinct populations of freshly collected snails from (1) polders near Utrecht in The Netherlands (referred to as wild Dutch; latitude, 52 deg.16′N; longitude, 5 deg.17′E and 'elevation', –1 m); (2) six seasonally isolated ponds in the Belly River drainage in Southern Alberta, Canada (referred to as Belly; latitude, 49 deg.31′N; longitude, 113 deg.16′W and elevation, 961 m); and (3) A 20-year-old human-made dugout pond (referred to as Jackson; latitude, 50 deg.44′N; longitude, 114 deg.23′W and elevation, 1254 m). The distance between the two Albertan sites is a little over 200 km. Wild *Lymnaea stagnalis* were identified using taxonomic descriptions by Clarke, and Clifford (Clarke, 1981; Clifford, 1991) as well as descriptions from other published studies in a similar localities in both The Netherlands and Alberta (Mooijvog et al., 1973; Boag and Pearlstone, 1979; Jager et al., 1979; Boag et al., 1984). In order to further ensure that both the Albertan and Dutch snails were in fact the same species, cross breeding experiments were conducted to ensure that the progeny of the initial crosses (F1s) produced viable offspring (F2s). As this was the case we concluded that these were in fact the same species. All organisms from these cross breeding experiments were destroyed and were not tested either behaviorally or electrophysiologically (dumb on our part!).

Snails were collected from ponds in Alberta and polders in The Netherlands in spring and summer of 2006, 2007 and 2008 and were then maintained in our laboratory in Calgary before use in the experiments described below.

Predators

Laboratory-reared Dutch snails detect and respond to the 'scent' of a natural sympatric crayfish predator (*Procambarus* sp.) by altering several adaptive, anti-predator behaviors (Orr et al., 2007; Orr and Lukowiak, 2008). We continued to use water containing the scent of these crayfish (crayfish effluent; CE). Crayfish are not endemic to Southern Alberta (Clifford, 1991; Proctor, 2006), that is crayfish are not a sympatric predator to Alberta *Lymnaea*. However, crayfish readily prey on Albertan *Lymnaea* in the laboratory. We therefore used an Alberta sympatric aquatic predator that is known to feed upon snails, including *Lymnaea*, the tiger salamander (*Ambystoma tigrinum*); which was obtained locally from a seasonal pond in Nose Hill Park (latitude, 51 deg.06′N; longitude, 114 deg.06′W and elevation, 1219 m) in Calgary. Water taken from the Salamander aquaria was used for the salamander effluent (SE) studies. Three tiger salamanders were collected in spring 2006 and 2007 (they are still alive in the lab) and maintained in the laboratory on a diet of live juvenile snails and worms. Tiger salamanders are not a sympatric predator of Dutch *Lymnaea*, but will prey on them in the laboratory. Thus, we use both sympatric and allopatric predators in this study.

It is important to note here that all snails involved in the experiments were never directly exposed to the predator, but were only exposed to the water from the aquarium that the predator is housed in. For discussion of direct exposure experiments involving crayfish see Orr and Lukowiak (Orr and Lukowiak, 2008). We do not know the identity of the substances in CE or SE that is sensed by *Lymnaea*; however, we do know that neither boiled CE or SE evokes the responses described in this report.

Aerial respiratory behavior

Lymnaea are bimodal breathers obtaining oxygen through either cutaneous respiration (i.e. directly through the skin) or through aerial respiration *via* a lung (i.e. gas exchange with the atmosphere). In eumoxic conditions (P_{O_2}<9975 Pa) cutaneous respiration predominates (Lukowiak et al., 1996; Taylor et al., 2001; Taylor et al., 2003). To perform aerial respiration, the snail must surface and open its pneumostome (the respiratory orifice) while contracting and relaxing the appropriate respiratory muscles. For a more detailed description see (Lukowiak et al., 2003). This behavior is driven by a three-neuron CPG that has been experimentally demonstrated to be necessary and sufficient (Syed et al., 1990; Syed et al., 1992b).

Breathing observations

To determine if exposure to a sympatric or allopatric predator altered aerial respiratory behavior, snails were placed in 500 ml of room temperature hypoxic pond water (P_{O_2}<931 Pa; PW) and then after a 24 h rest interval, either placed in 500 ml of hypoxic CE or hypoxic SE. The duration of the pneumostome openings were noted during each of the 0.5 h periods. From these measurements, the total breathing time was calculated.

Operant conditioning of aerial respiratory behavior

Snails were removed from their temporary holding aquaria and placed into a 1 l beaker containing 500 ml of hypoxic (P_{O_2}<931 Pa) water (PW, CE or SE). The water is made hypoxic by bubbling N_2 gas through the water for 20 min before introducing the snails. The animals were given a 10 min acclimatization period before the 30 min training session. Snails increase their rate of aerial respiration with a hypoxic challenge (Lukowiak et al., 1996; Lukowiak et al., 1998). Snails are operantly conditioned by applying a gentle tactile stimulus with a wooden applicator to the pneumostome (the respiratory orifice) as it begins to open. The stimulus is strong enough to cause the snails to close the pneumostome yet gentle enough that the snails do not perform the full body withdrawal response. The contingent stimulation is given during both the training session (TS) and during the test for memory (TM). This pneumostome closer response is a part of the whole-snail escape response (Inoue et al., 1996). Every time the snail opens its pneumostome and receives the stimulus during the training period, the time is recorded for future use in yoked control experiments. Yoked controls (see below) were performed for all behavioral experiments. All behavioral experiments were run concurrently and were done 'blind' where the person performing the training paradigm was unaware of the status of the cohort being tested (e.g. whether it was in PW, CE or SE).

The operant conditioning procedure we utilized consists of a single 30 min training session (TS) after which the snails are returned to their home aquaria (Sangha et al., 2003c). The snails are then tested for memory (TM; i.e. a 'savings-test') using a similar test to that of the training session except that in the case of CE- and SE-trained snails the TM was performed in PW. The time of the TM or recording is indicated as time after the TS. Each operant conditioning experiment was replicated at least twice by using two separate naïve cohorts of 10–14 snails in each trial for each experiment.

Yoked control experiments

During the training period, yoked control snails received exactly the same number and sequence of stimuli as those of the operant conditioning group; however, the stimuli were not contingent upon their pneumostome opening. However, these yoked control snails did receive a contingent stimulus to the pneumostome during the savings test session (TM). Snails that received yoked training were

treated in an identical manner to that outlined in the 'yoked operant conditioning procedure' used previously (Lukowiak et al., 1996; Lukowiak et al., 1998; Lukowiak et al., 2000; Lukowiak et al., 2003).

Semi-intact preparation

The preparations were dissected using methods similar to those previously described (McComb et al., 2005b; Orr et al., 2007; Orr and Lukowiak, 2008). The central ring ganglia (the central nervous system; 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; sheath-softening enzymes were not used as they can alter the electrophysiological properties of *Lymnaea* neurons (Hermann et al., 1997). Standard electrophysiological techniques were used as previously described in *Lymnaea* semi-intact preparations (Spencer et al., 1999; Spencer et al., 2002; McComb et al., 2003; McComb et al., 2005b). Intracellular signals were amplified using a NeuroData amplifier and displayed simultaneously on a Macintosh PowerLab/4SP (AD Instruments, Colorado Springs, CO, USA) and a Hitachi oscilloscope. Recordings were analyzed and stored using the PowerLab software (Orr and Lukowiak, 2008). Once the RPeD1 neuron was successfully impaled the cells were given a minimum 10 min stabilization period after which a 600 s trace was used for analysis. Nine electrophysiological characteristics were measured for each recording: (1) total number of action potentials (APs; spikes) per 600 s, (2) total frequency, (3) resting membrane potential, (4) number of APs per burst, (5) burst frequency, (6) after hyperpolarization of the first AP in each burst, (7) average AP peak of each burst, (8) burst duration and (9) the number of bursts per 600 s.

Statistics

We analyzed water treatment effects on snail breathing behavior data with repeated measures analysis of variance. All repeated measures data were tested for equal variance using Mauchlys test for sphericity. In cases where sphericity could not be assumed, we used the conservative adjusted Greenhouse–Geisser *P*-values. We analyzed operant conditioning effects on snail behavioral data with repeated measures analysis of variance (ANOVA) where the within-subject factors of populations were used and the between-subject factor of interval (time) were used. All repeated measures data were tested for equal variance using Mauchlys 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 the population, we used repeated contrasts to identify which treatment pairs differed significantly. Electrophysiological data were analyzed 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, etc.) were log transformed to homogenize data prior to ANOVA. All statistics were performed using SPSS, version 11.0.4 for Macintosh (SPSS Inc., Chicago, IL, USA).

RESULTS

Breathing observations

We first determined if aerial respiratory behavior was selectively altered in the three groups of collected *Lymnaea* exposed to either the crayfish (CE) or salamander (SE) predator effluent. Previous reports indicated that when pulmonate snails are in the presence of a crayfish predator they tend to spend more time near the surface of the water (Turner et al., 2000; Turner and Montgomery, 2003; Dalesman et al., 2006). Furthermore, we found that our laboratory-reared *Lymnaea* showed a significant alteration (increase) in aerial respiratory behavior with CE exposure (Orr et al., 2007). We measured the total breathing time (TBT) of the three groups of snails in a hypoxic PW, CE and SE challenge.

The TBT in hypoxic PW was not statistically different in the three geographically isolated wild snail populations (Fig. 1). However, TBT for the Dutch snails in CE was significantly increased compared with TBT in PW or SE. That is, the Dutch snails did not alter their breathing in SE. Interestingly, different results were obtained for both the Belly and Jackson snails. In these two strains CE exposure did not result in an increase in TBT compared with PW. However, when these two populations were exposed to SE, their TBT was significantly decreased compared

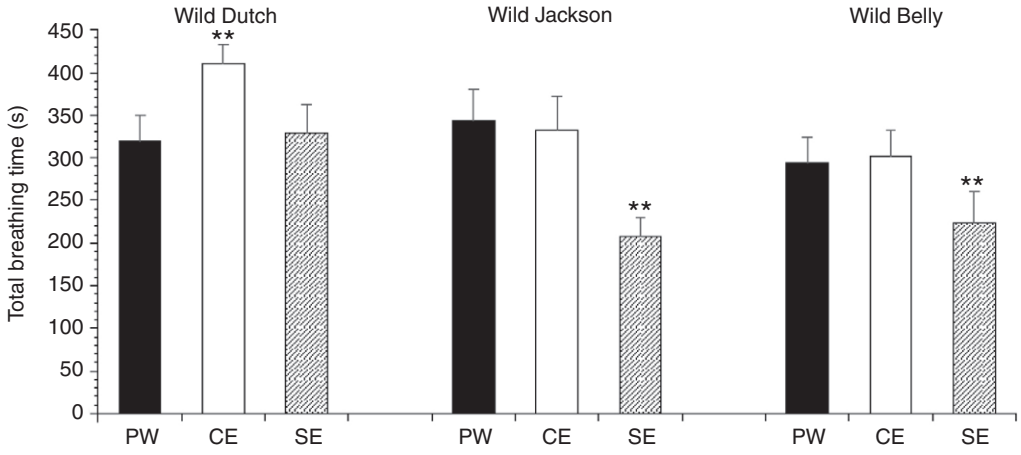

Fig. 1. Total breathing time (TBT) of three geographically separate populations of *Lymnaea stagnalis* in pond water (PW), crayfish effluent (CE) and tiger salamander effluent (SE). The TBT in PW of each population was not significantly different (N=33, P>0.05, black bars). TBT of Dutch snails (left bars) was significantly higher in the CE than in PW or SE (N=33, P<0.01). TBT in PW and SE were not significantly different (N=33, P>0.05). TBT in the Belly population (middle bars) was not significantly different in PW and CE (N=33, P>0.05) but was significantly reduced in the SE (N=33, P<0.01). TBT was similar in the Jackson and the Belly populations in that there was no significant difference between PW and CE treatments (N=33, P>0.05) but it was significantly lower in SE (N=33, P<0.01). **P<0.01.

with PW. From these behavioral data we conclude that: (1) wild Dutch snails have the capability to detect the presence of a crayfish predator and respond to its 'presence' by increasing aerial respiratory behavior; (2) wild Dutch snails do not alter their aerial respiratory behavior in response to exposure to SE; (3) both populations of Albertan snails have the capability of detecting SE and significantly decrease their aerial respiratory behavior; and (4) both populations of Albertan snails do not alter their TBT in CE and thus CE does not signal to them that there is a predator 'present'.

Operant conditioning of aerial respiration

We recently demonstrated that lab-reared *Lymnaea* (originally derived from wild Dutch snails) detect and respond to the scent of a crayfish predator (i.e. CE) with multiple predator-avoidance responses at both the behavioral and neurophysiological levels (Orr et al., 2007). We further demonstrated that predator detection enhanced long-term memory formation (LTM) at the behavioral and at the electrophysiological level in RPeD1, which is a necessary site for LTM formation (Scheibenstock et al., 2002; Orr and Lukowiak, 2008).

Here, we set out to determine if wild snails (i.e. recently collected in the three specified locations) responded in a similar manner as the lab-reared snails (Orr and Lukowiak, 2008) to detection of a predator with enhanced LTM formation. However, initially we did not believe we could adequately perform such experiments using Belly snails since their ability to form LTM is already significantly superior to Dutch snails. But, much to our surprise, we found (Fig. 2, middle panel) that the Jackson snails did not possess the superior memory forming capabilities of the Belly snails and their memory-forming capabilities were similar to the wild Dutch and the lab-reared snails (see below).

We first reconfirmed our original finding (Orr et al., 2008) that wild Dutch snails do not form LTM following a single 0.5 h operant conditioning training session in PW (Fig. 2, top row, left panel) That is, although there was memory 3 h (i.e. intermediate-term memory, ITM) after the training session there was no evidence of memory at 24 h or in the yoked control group. We next tested, for the first time, the ability of the Jackson snails to form LTM following the single 0.5 h training session (Fig. 2, middle row, left panel). As mentioned above, we were surprised that although these Albertan snails formed ITM (i.e. memory at 3 h) they did not form LTM. We then reconfirmed our original finding that the Belly snails (Fig. 2, bottom row, left panel) formed both ITM and LTM following the single 0.5 h training session. As expected they formed both ITM and LTM. We conclude that Belly snails have superior memory capabilities compared with Dutch snails, and interestingly, with Jackson snails.

We next examined what effect, if any, each predator scent (i.e. CE and SE) would have on LTM formation in the three groups following the single 0.5 h training session. We first examined the wild Dutch snails (Fig. 2, top row, middle panel). When the Dutch snails were trained in CE both ITM and LTM were formed. That is, CE enhanced the ability of the Dutch snails to form LTM following the single 0.5 h training session. Notice also that the memory at 3 h in CE was significantly better (i.e. fewer attempted openings) than in PW. However, training the Dutch snails in SE (Fig. 2, top row, right panel) did not bring about the enhancement of LTM formation, only ITM was observed. The ITM seen in SE was not statistically different from that in PW. By contrast, when we examined the response of the Jackson snails (Fig. 2 middle panel) to the predator scents we found the opposite: that CE did not enhance the ability of the Jackson snails to form LTM (middle row, middle panel). Rather when trained in SE (middle row, left panel) there was an enhancement of LTM formation. As was the case with the Dutch snails, when LTM was enhanced in the Jackson snails by SE, ITM was also statistically better than in PW. Thus, Jackson snails respond to SE and not CE whereas Dutch snails respond to CE but not to SE. Finally, we examined how the Belly snails responded to SE and CE. As can be seen (Fig. 2 lower panels), perhaps because LTM formation is already so enhanced in PW in these snails, we found that neither CE nor SE further enhanced ITM or LTM, possibly because of a 'ceiling' effect. It also has to be emphasized that in all groups under all conditions the yoked control groups (gray bars) did not exhibit LTM. We therefore concluded that detection of a sympatric predator alters memory (ITM and LTM) formation; but presenting the scent of an allopatric (i.e. non-sympatric) predator does not alter memory formation. Together the data show that memory formation, a cognitive adaptation, is only augmented when conditioning is done in the 'presence' of a sympatric predator.

Electrophysiological profile of RPeD1 from wild snails after sympatric predator exposure

We have previously demonstrated that when naïve lab-reared snails are exposed to CE, the spontaneous firing activity and bursting activity of RPeD1 decreases in the semi-intact preparations compared with control snails (Orr et al., 2007). To our knowledge this investigation was 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 behavior of *Lymnaea* (Syed et al., 1990; Syed et al., 1992a) and is subordinate to the defensive full-body withdrawal behavior (Syed and Winlow, 1991; Inoue et al., 1996). It is therefore not surprising that the activity pattern of this neuron is altered when predator scent is detected.

In the present investigation we utilized our unique ability to detect changes in the electrophysiological state of neurons hypothesized to be involved in predator defense behaviors to determine if the behavioral differences (i.e. TBT and memory forming capabilities) in the three wild populations are also manifest at the electrophysiological level in RPeD1. We recorded nine electrophysiological characteristics from this neuron in semi-intact preparations from each population immediately following a 2 h exposure to one of the three water treatments (PW, CE and SE). To ensure that the spontaneous firing properties of RPeD1 in each population of snails were directly comparable we first measured the nine listed parameters of each population after exposure to PW. We found there were no significant differences in any of the parameters between all three populations.

When measuring the intrinsic neuronal properties of RPeD1 in Dutch snails we found a significant reduction in three of the nine measured parameters when snails received CE treatment prior to recording (Fig. 3). Specifically, we found that the total number of spikes per 10 min, the number of spikes per burst and the burst duration were all significantly reduced in the CE-treated animals compared with the PW treatment (Fig. 3B–D). We were not overly surprised by these data as they recapitulate what we have previously found when investigating the Dutch-derived laboratory-reared snails. There were no significant differences in the electrophysiological characteristics of RPeD1 between the PW and SE treatments in the wild Dutch population.

In contrast to the Dutch snails, recordings from Belly snails revealed that there were no significant differences in the

physiological parameters of RPeD1 between PW- and CE-treated animals (Fig. 4). However we did find that there was a significant reduction in the spontaneous firing activity of RPeD1 when the Belly snails were exposed to SE. That is, there was a significant reduction in the total number of spikes per 10 min, the number of spikes per burst and the burst duration in SE-treated animals compared with PW- or CE-treated controls. There were no significant differences in the other six measured parameters in the SE-exposed snails.

Finally, we found that the Jackson snails demonstrated similar characteristics to the Belly snails when exposed to SE. That is, the only significant changes seen were: (1) the spontaneous electrical activity of RPeD1 showed a marked reduction in the

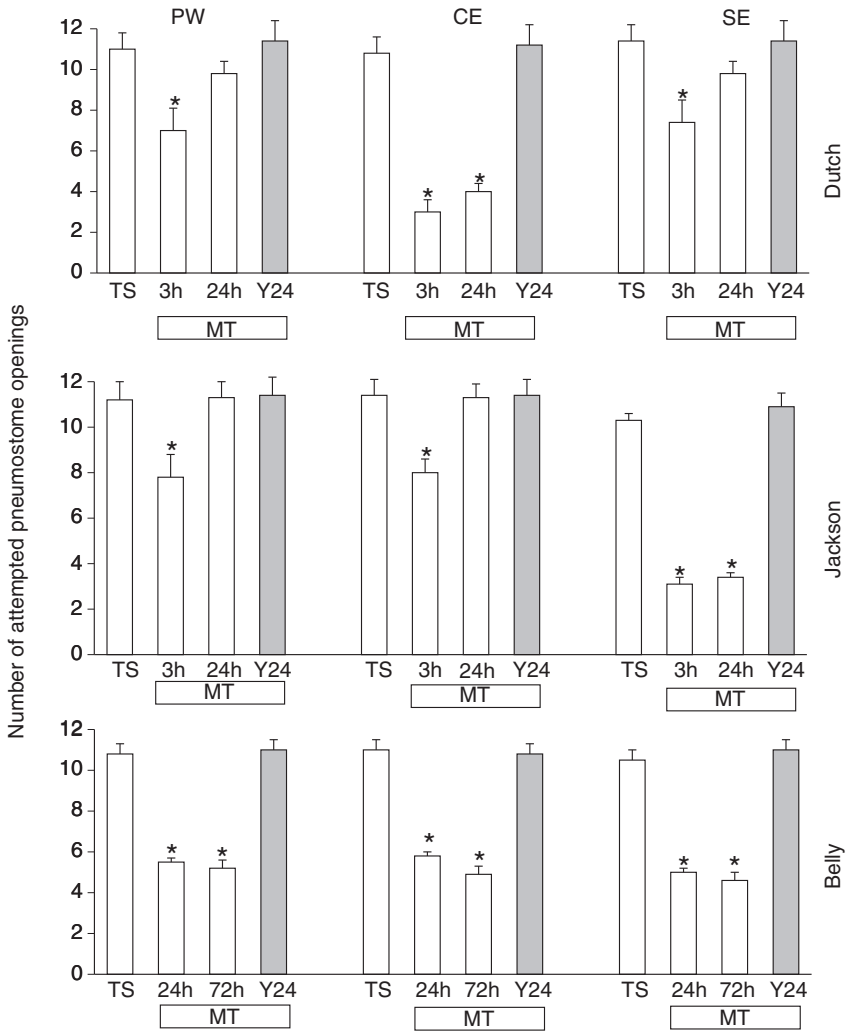

Fig. 2. Operant conditioning of the three populations of *Lymnaea stagnalis* in each of the three water treatments pond water (PW), crayfish effluent (CE) and salamander effluent (SE). Top row: Dutch snails received a single 0.5 h training session in PW (left panel) and showed intermediate-term memory (ITM; N=25, P<0.05; 24 h N=31) but not long-term memory (LTM; 24 h N=31, P>0.05, left bars). Yoked controls also did not demonstrate reduced pneumostome openings at 24 h (N=25, P>0.05, gray bar). Middle panel: Dutch snails that received a single 0.5 h training session in CE demonstrated both ITM at 3 h and LTM at 24 h (3 h N=25, P<0.01; 24 h N=31, P<0.01). In addition, the number of attempted openings (ITM) at 3 h following training is significantly lower than in PW or SE (P<0.05). Yoked controls in CE do not demonstrate LTM at 24 h (N=25, P>0.05, gray bar). Right panel: Dutch snails that received a single 0.5 h training session in SE were similar to those trained in PW, in that they demonstrate ITM at 3 h but not LTM at 24 h (3 h N=20, P<0.05; 24 h N=32, P>0.05). Yoked controls in SE also did not demonstrate LTM at 24 h (N=24, P>0.05, gray bar). Middle row: Jackson snails that received a single 0.5 h training session in PW demonstrated ITM (N=25, P<0.05) but not LTM (N=46, P>0.05). Yoked controls in PW also did not demonstrate reduced pneumostome openings at 24 h (N=35, P>0.05, gray bar). Middle panel: Jackson snails that received a single 0.5 h training session in CE demonstrated ITM at 3 h but not LTM at 24 h (3 h N=27, P<0.05; 24 h N=34, P>0.05). Yoked controls in CE also did not demonstrate LTM (N=30, P>0.05, gray bar). Right panel: Jackson snails that received a single 0.5 h training session in SE demonstrated both ITM at 3 h and LTM at 24 h (3 h N=24, P<0.01; 24 h N=42, P<0.01). In addition, the number of attempted openings at 3 h following training was significantly lower than in PW or CE (P<0.05). Yoked controls in SE did not demonstrate LTM at 24 h (N=34, P>0.05, gray bar). Bottom row: Belly snails received a single 0.5 h training session in PW and showed both ITM and LTM (3 h, N=25, P<0.05; 24 h, N=30, P>0.05). Yoked controls in PW also did not demonstrate reduced pneumostome openings at 24 h (N=30, P>0.05, gray bar). Middle panel: Belly snails that received a single 0.5 h training session in CE demonstrated both ITM at 3 h and LTM at 24 h as they did in PW (3 h N=22, P<0.05; 24 h N=32, P>0.05). Yoked controls in CE also did not demonstrate LTM (N=32, P>0.05, gray bar). Right panel: Belly snails that received a single 0.5 h training session in SE continued to demonstrate both ITM at 3 h and LTM at 24 h (3 h N=21, P<0.01, 24 h N=34, P<0.01). The number of attempted openings at both 3 h and 24 h were not significantly different in SE compared with CE and PW (P>0.05) Yoked controls in SE did not demonstrate LTM at 24 h (N=34, P>0.05, gray bar). *Significant difference from training session.

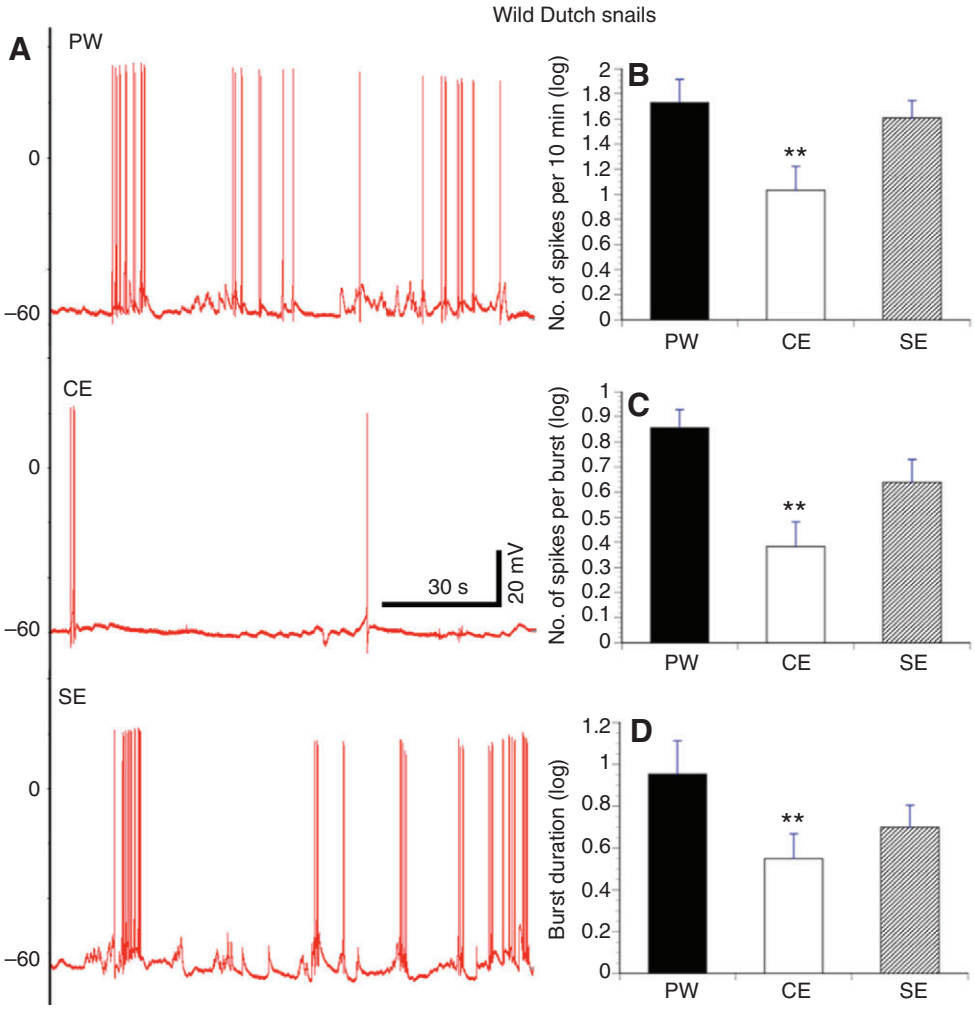

Fig. 3. (A) Representative electrophysiological recordings from RPeD1 in semi-intact preparations taken after intact Dutch snails were exposed to pond water (PW; top), crayfish effluent (CE; middle) or salamander effluent (SE; bottom) water treatments. All traces demonstrate spontaneous firing activity. Horizontal bar represents 30 s of recording, the vertical bar indicates 20 mV. (B) Summary data for average spiking activity per 10 min (log root transformed). CE values were significantly lower (N=8, P<0.01) than either PW or SE values, which were not significantly different from each other (N=10, P<0.01). (C) Average number of spikes per burst (log transformed). Again, CE values were significantly lower (N=8, P<0.01) than either PW or SE values, which were not significantly different from each other (N=10, P<0.01). (D) The summary data for burst duration (log transformed). CE values were significantly lower (N=8, P<0.01) than either PW or BC values, which were not significantly different from each other (N=10, P<0.01). **P<0.01.

total number of spikes per 10 min; (2) the number of spikes per burst; and (3) the burst duration compared with PW and CE treatments (Fig. 5).

Together these data support the hypothesis that when *Lymnaea* detect a sympatric predator they alter adaptive behaviors as a result of electrophysiological changes in key neurons such as RPeD1 in a physiologically appropriate way. However, the different populations of snails do not respond in the same way to just any predator. Snails, at least as far as we can tell by assaying their behavior and the electrophysiological response of RPeD1, only perceive those predators that historically coexist with the population in question (i.e. sympatric predators).

DISCUSSION

Lab-reared *Lymnaea* (derived from a colony collected in The Netherlands) respond to the presence of crayfish (a sympatric predator) by significantly altering a number of anti-predator behaviors, including enhanced memory formation (Orr et al., 2007; Orr and Lukowiak, 2008). In those studies lab-reared snails were used exclusively indicating that predator detection was instinctual and had 'survived' lab-rearing for over 250 generations. In addition, we knew that they had never come into contact with a crayfish or CE before we experimented on them. Here we examined three geographically distinct populations of freshly collected wild *Lymnaea* and found that they also respond to predator scent by significantly altering respiratory behaviors, LTM formation and RPeD1 activity. However, we found that *Lymnaea* only responded to the scent of a sympatric predator and not to the scent of an organism that preys on them but which is not sympatric (i.e. an allopatric predator). We also, much to our surprise, found that not all Albertan snails have superior memory forming capabilities compared with Dutch snails. The distinct Jackson snail strain was more similar to the Dutch strains in that regard.

Cognitive traits such as learning and memory show individual or population wide variation (Knapp et al., 2001; Berejikian et al., 2003; Marinesco et al., 2003; Stoks et al., 2003; Hoover et al., 2006)

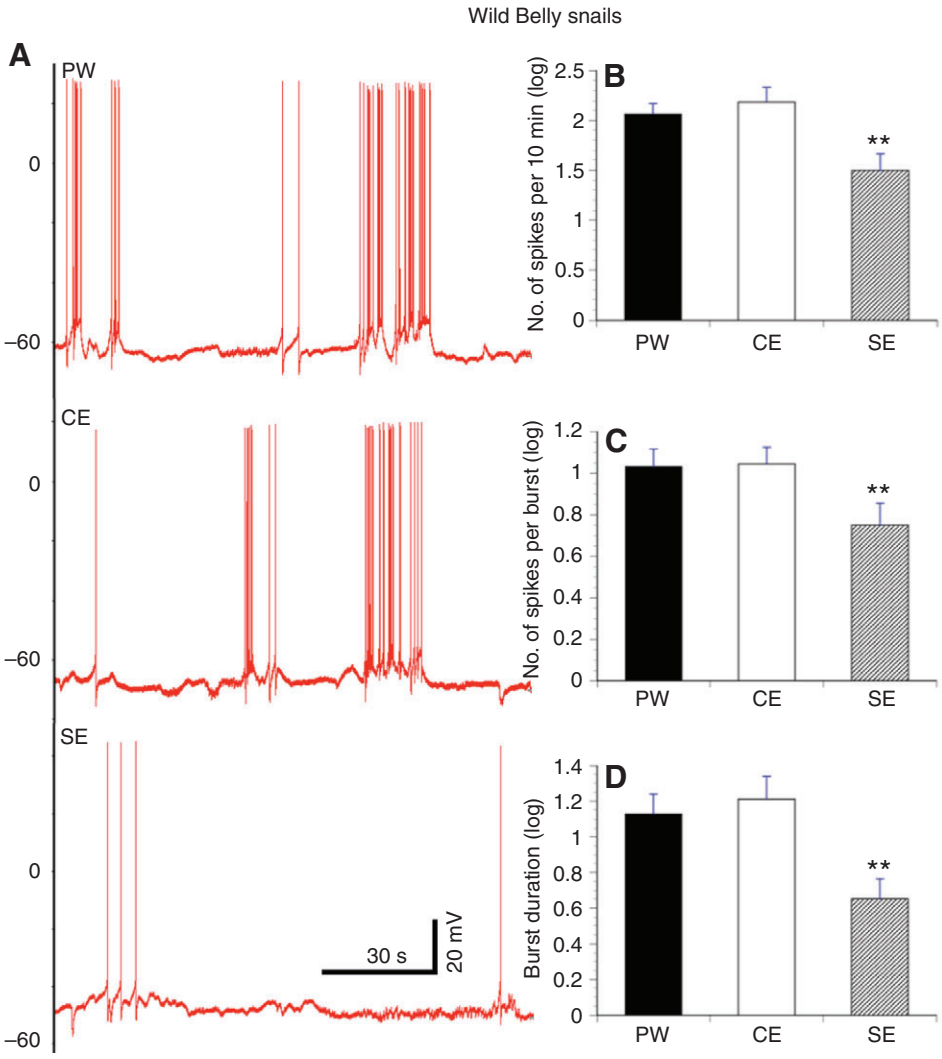

Fig. 4. (A) Representative electrophysiological recordings from RPeD1 in semi-intact preparations taken after intact Belly snails were exposed to pond water (PW; top), crayfish effluent (CE; middle) or salamander effluent (SE; bottom) water treatments. All traces demonstrate spontaneous firing activity. Horizontal bar represents 30 s of recording, the vertical bar indicates 20 mV. (B) Summary data for average spiking activity per 10 min (log root transformed). SE values were significantly lower (N=10, P<0.05) than either PW or CE values, which were not significantly different from each other (N=10, P>0.05). (C) Average number of spikes per burst (log transformed). Again, SE values were significantly lower (N=10, P<0.05) than either PW or CE values, which were not significantly different from each other (N=10, P>0.05). (D) The summary data for burst duration (log transformed). SE values were significantly lower (N=10, P<0.01) than either PW or CE values, which were not significantly different from each other (N=10, P>0.05). **P<0.01.

yet our understanding of how cognitive traits vary within and between species and specifically what the mechanisms are that drive this behavioral variation and how this variation affects a species fitness remains poorly understood. Predators impose strong selection of anti-predator behaviors in their prey and many of these behaviors are directly heritable (Vetter and Brodie, 1977; Brodie, 1992; Cousyn et al., 2001; Juliano and Gravel, 2002; O'Steen et al., 2002). Owing to these selection pressures, differential selection gradients can drive adaptive evolution of anti-predator responses within and between species resulting in a large degree of trait variation between separate populations within the same species (Stoks et al., 2003; Dalesman et al., 2006). Gaining insight into how natural trait variation is manifest between populations shaped by differential selective pressures is crucial to understanding the evolution, ecology and sensory biology of natural populations. The data obtained in our present study are consistent with those previous findings regarding within species differences in cognitive abilities. For example, Belly snails have superior memory-forming capabilities compared with Jackson snails even though they are found within a few hundred kilometers of each other (see below).

Much understanding has been gained from thorough investigations into the costs associated with predator defenses such as reduced feeding during times of vigilance, which result in reduced growth and reproduction. However, much less work has been done to understand how cognitive traits such as sensory perception, learning and memory formation/recall affect organismal fitness and how these heritable traits differ between populations experiencing differential selective pressures. This is possibly because quantifying variation in cognitive traits such as memory formation in a meaningful, biologically realistic way is difficult for several reasons: First, consistent and reliable measurement of the trait is difficult as it often involves subjective quantification; second, an organism's

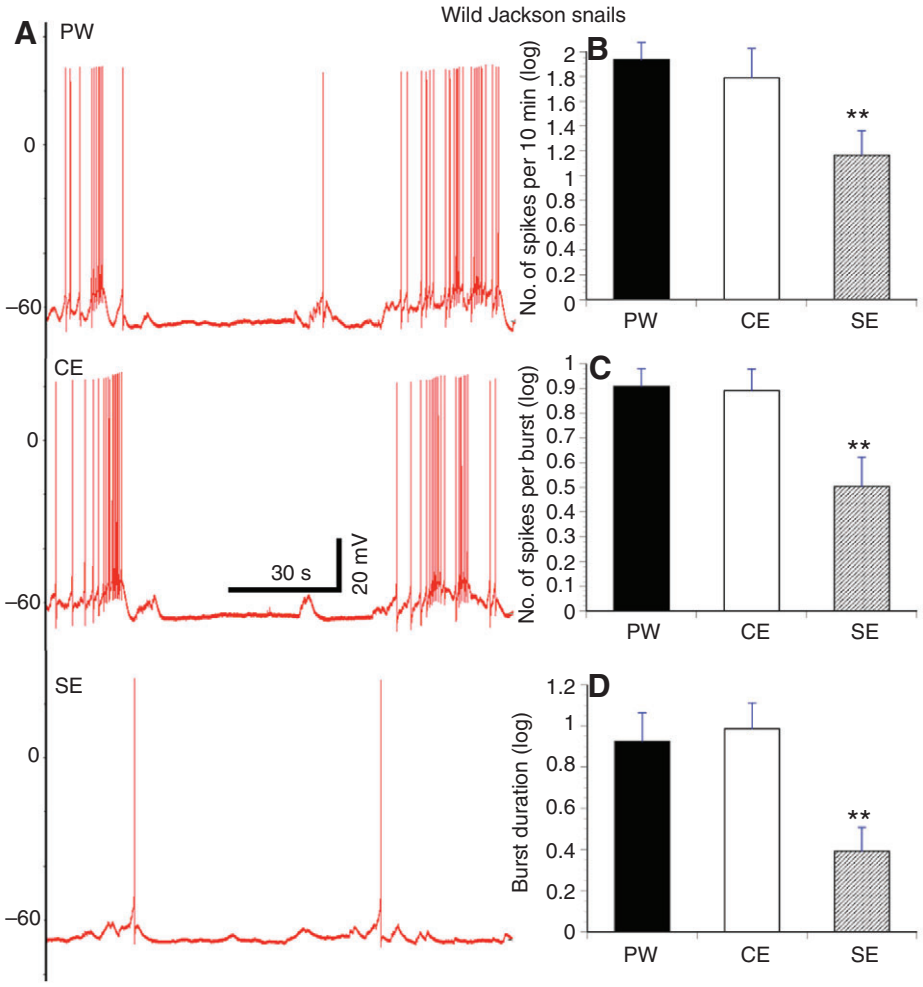

Fig. 5. (A) Representative electrophysiological recordings from RPeD1 in semi-intact preparations taken after intact Jackson snails were exposed to pond water (PW; top), crayfish effluent (CE; middle) or salamander effluent (SE; bottom) water treatments. All traces demonstrate spontaneous firing activity. Horizontal bar represents 30 s of recording, the vertical bar indicates 20 mV. (B) Summary data for average spiking activity per 10 min (log root transformed). SE values were significantly lower (N=10, P<0.01) than either PW or CE values, which were not significantly different from each other (N=8, P>0.05). (C) Average number of spikes per burst (log transformed). Again, SE values were significantly lower (N=10, P<0.01) than either PW or CE values, which were not significantly different from each other (N=8, P>0.05). (D) The summary data for burst duration (log transformed). SE values were significantly lower (N=10, P<0.01) than either PW or CE, which were not significantly different from each other (N=8, P>0.05). **P<0.01.

perceptions of the test may change throughout the procedure as habituation, sensitization or conditioning of the stimulus may occur; and third, most attempts to characterize 'cognitive skills' utilize artificial laboratory-taught tasks that may not represent natural behaviors. As such quantification of cognitive trait variation between species or populations of the same species has remained scarce.

Our experiments demonstrate natural within-species variation in both the ability to detect predators and how this inherent ability to detect specific predators affects LTM formation according to the perceived predatory threat. Our studies document this within-species variation at the behavioral, physiological and neurophysiological levels. The data allow us to draw three important conclusions. (1) Three naturally occurring, geographically separate, wild populations of *Lymnaea stagnalis* have innate, yet different capacities for predator detection. (2) Predator detection is manifest in both the whole animal defensive behaviors and the physiology of the neuronal substrates that drive these behaviors. That is, we have identified a component of the neural substrates involved in the predator-induced defense response and these underlying neural representations reflect the trait variation present in the three populations. (3) These cognitive traits are robust, quantifiable and represent natural, biologically realistic, behaviors.

Pulmonate snails use different anti-predator responses depending on predator identity (Turner et al., 1999; Dalesman et al., 2006). For example, some predators are located at the bottom of ponds (e.g. crayfish), whereas others (e.g. tiger salamanders) are located at the surface. Thus, snails should, if they wish to avoid the predator, move to the place not frequented by the predator. To effectively do so, snails not only have to detect the predator but have to make the proper decision as to where to 'hide'. Previous reports demonstrated that when *Lymnaea* are exposed to crayfish, they crawl to the surface and sometimes even crawl out of the water (Alexander and Covich, 1991; Covich et al., 1994; Chivers and Smith, 1998; McCarthy and Fisher, 2000). Consistent with those reports are our data showing that wild Dutch snails in CE increase their aerial respiratory behavior in response to crayfish scent detection. These data are similar to our previous findings in the Dutch-derived laboratory-reared snails (Orr et al., 2007). By contrast, aerial respiration in

Dutch snails was not altered in salamander effluent (SE). There are several examples in the literature demonstrating that *Lymnaea stagnalis* respond to several different sympatric predators in different, yet appropriate manners (Turner et al., 1999; Turner and Montgomery, 2003). However, here we show that the wild Dutch *Lymnaea* appear not to detect or do not 'know what to do' in the presence of the salamander predator; even though we have seen this predator catch and consume snails in the lab (K.L., unpublished observations).

In contrast to the Dutch snails, the Belly and Jackson strains of *Lymnaea* did not alter aerial respiratory behavior in CE. However, the two Albertan strains exhibited a significant decrease in aerial respiratory behavior with the SE challenge as opposed to an increase in TBT in the Dutch snails in CE. Why would these two strains of *Lymnaea* decrease aerial respiratory behavior in the SE-hypoxic challenge? Tiger salamanders prey on snails that are at the surface (K.L., unpublished observations). Thus, it would make sense for the snail when the predator is detected not to spend more time at the surface. Rather it should spend less time there in order to avoid predation. This is a different avoidance strategy than that employed by the Dutch snails when they detect a crayfish predator. Crayfish are bottom feeders so it makes sense to spend more time at the surface. Previous reports show that when pulmonate snails are presented with the odor of molluscivorous fish they demonstrate evasive maneuvers by utilizing spatial refugia in the form of hiding under cover (Turner et al., 1999; Turner and Montgomery, 2003; Dalesman et al., 2006). Similar to how Dutch snails do not respond to SE (an allopatric, i.e. non-sympatric predator), the two strains of Albertan *Lymnaea* do not alter their aerial respiratory activity in the CE-hypoxic challenge, as crayfish are a non-sympatric predator.

We hypothesize that whereas Albertan *Lymnaea* are capable of detecting the presence of crayfish and Dutch snails are capable of detecting tiger salamanders they do not associate the presence of the smell with predation. That is, detection of an allopatric predator does not elicit anti-predator behaviors, because the scent does not signal predation (i.e. there is no perceived threat). It is only when a specific scent signals threat that evasive action is taken. This situation is analogous to what researchers have discovered in studying specific stressors. It is not the stimulus *per se* that elicits a stress response, but rather it is the perception of the stimulus as stressful that elicits the stress response (Kim and Diamond, 2002). Thus the same stimulus may elicit a stress response in one individual but not another. Whether *Lymnaea* could be trained to respond to the scent of an allopatric predator is unclear and we are attempting to determine this in the laboratory.

Learning and the subsequent formation of LTM allows an organism to respond and adapt to new situations. In PW we found that Dutch and Jackson snails had similar LTM-forming capabilities. That is, neither strain has the ability to form LTM following a single 0.5 h training session. We do not yet understand why Belly snails have a significantly superior LTM forming ability (i.e. they form a 3 day LTM following a single 0.5 h training session). It is possible that some other trait has been selected for in these snails that, in spring, encounter an overflowing river as a result of the massive spring snow melt along the eastern slopes of the Rockies. We are in the process of attempting to determine why these snails posses this inherent ability to form a faster and more persistent LTM.

We previously found that laboratory-reared (~250 generations) *Lymnaea* have an inherent ability to detect crayfish predators and when learning occurs in conjunction with predator-detection LTM is dramatically augmented (Orr and Lukowiak, 2008). Furthermore, a neural correlate of the newly formed memory was demonstrated by the reduced spontaneous firing properties in RPeD1 that persist for the duration of the memory. These lab-reared snails had not been exposed to a natural predator and as a consequence these data show that these responses are innate and instinctual, as they have been maintained without selective pressures for over 50 years. Here our data demonstrate that operant conditioning of freshly collected wild Dutch and Jackson *Lymnaea* in their sympatric predator effluent (CE and SE, respectively) also results in augmented memory. However this form of enhancement is conditional upon the historical relation to the specific predator. That is, when the training is conducted in effluent from an allopatric predator LTM formation is not enhanced. These data showing enhanced LTM formation that occurs as a result of predator detection further support the hypothesis that memory formation is an anti-predator behavior. Since there is a cost to the formation of memory (e.g. Mery and Kawecki, 2005; Dukas, 1999) the enhancement of memory formation that accompanies predator detection should confer some advantage to the organism, otherwise why bother? Because we used freshly collected wild snails we cannot be certain that they have not previously encountered a predator in their natural environment. However, we do know that the behavioral phenotype of the wild Dutch snails does not appear to be different from the lab-reared snails, in regards to memory-forming capabilities and responses to CE. In a similar manner, we know that the behavioral repertoire of the F1 offspring of Belly snails is not different from freshly collected Belly snails (Orr et al., 2008). Thus, it appears that even if some of the freshly collected snails used in the present study had an encounter with a predator the changes that were induced in the snail did not persist long enough to be seen in our study.

We also previously described a neural correlate of the predator-induced stress response (Orr et al., 2007). When naïve snails are exposed to a sympatric predator, the spontaneous firing activity, bursting activity and burst duration of RPeD1 decreases in the semi-intact preparations compared with control snails (Orr et al., 2007). When we exposed the semi-intact preparations from our three wild populations of snails to their respective sympatric predators we also found significant reductions in the spontaneous firing activity, bursting activity and burst duration of RPeD1. These changes were not demonstrated when animals were exposed to regular pond water or the effluent of the allopatric predator. To our knowledge this investigation is the first evidence of within-species variation in the neurobiological response associated with predator detection in pulmonates. RPeD1 is part of a three-neuron CPG that has been shown to be both necessary and sufficient to drive the aerial respiratory behavior of *Lymnaea* (Syed and Winlow, 1991; Syed et al., 1992a). Moreover, this neuron, which initiates rhythmogenesis, is subordinate to the defensive full-body withdrawal behavior (Syed and Winlow, 1991; Inoue et al., 1996). It is therefore not surprising that the activity pattern of this neuron is altered in the manner described when a predator is detected. Furthermore, we have described that exposure to predator scent only results in short-term changes (<24 h) in the electrophysiological properties of RPeD1 (Orr and Lukowiak, 2008) and although not tested here, we would expect a similar response from these wild populations of snails.

As just mentioned, lab-reared and wild Dutch snails respond to CE by increasing aerial respiratory behavior yet at the same time spontaneous activity in RPeD1 is significantly decreased. How can we explain this apparent 'conflict'? The answer may lie in the interaction between the central and peripheral neural components of aerial respiratory behavior. Previously it has been demonstrated that there is an age-dependent change in suppressive input from the neurons located in and around the pneumostome area to CNS

neurons, such as RPeD1 (McComb et al., 2005a). That is, there is an interaction between the central and peripheral nervous systems in the mediation of aerial respiratory activity. It is possible that the 'conflict' in data is the result of an upregulation 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. We do not find it surprising that alterations occur in the peripheral nervous system activity as a result of predator detection, and may play an important role in the mediation of aerial respiratory behaviors. The interaction between the central and peripheral nervous systems of molluscs, especially as regards mediation of adaptive behaviors, is complicated, interesting and controversial (Lukowiak and Colebrook, 1988; Lukowiak and Jacklet, 1972). Further investigation into both the location and activity of these chemosensory receptors is ongoing in our laboratory.

We have yet to identify the chemoreceptive sites and neurons in *Lymnaea* that detect the karimore (a chemical messenger), and the precise nature of the chemical(s) involved are, as yet, unknown. Candidate sites include the lips and tentacles, sites associated with feeding; or they could be located in or near the osphradium, sites associated with the detection of aquatic O_2 levels. Moreover, we are also uncertain if there are detectable differences between the Dutch and Albertan snails in how these neurons respond to the respective karimores or, for that matter, to different food-associated odors. Prey species can learn to increase anti-predator behavior in response to novel predation regimes (Chivers et al., 1996; Wisenden, 2000; Berger et al., 2001; Stoks et al., 2003). However, in some cases the learned adaptation to novel predators is not adequate to prevent extirpation of the prey during predator introductions (Knapp et al., 2001; Stoks et al., 2003). Examples from other aquatic systems describing both differing responses to unique predators (Turner et al., 2000) and even the complete loss of the ability of prey to recognize potential predators when the predatory threat permanently ceases have been demonstrated (Stoks et al., 2003). Dalesman et al. (Dalesman et al., 2007) have described that populations of *Lymnaea* sampled from canals without predatory fish demonstrate reduced escape behavior when presented with predator kairomones compared with snail populations that co-occur with the predators.

We are in the process of identifying sites in the Mississippi/Missouri water shed in Montana and the Snake river watershed in eastern Washington where there are naturally occurring crayfish, tiger salamanders and *Lymnaea*. We will thus be able to determine if these *Lymnaea* respond in an appropriate manner to both predators. That is, we predict that both CE and SE will elicit anti-predator behaviors, but that SE will result in decreased aerial respiration whereas CE will significantly increase it.

We have demonstrated that wild *Lymnaea stagnalis* have an inherent ability to detect predators *via* a chemical (i.e. karimore) messenger and respond appropriately by altering both behavioral and physiological parameters. However, this ability is limited to sympatric predators; allopatric (i.e. non-sympatric) predators do not elicit anti-predator behaviors. Our data suggest that the perception of the sympatric but not the allopatric predator primes the molecular mechanisms required for LTM formation (Parvez et al., 2006) so that conditioning is more efficacious in forming LTM. Predation risk is a potent modifier of plastic traits, and learning in the presence of predators may increase an organism's fitness, but only if predictable environmental cues can be detected.

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