

## Predator detection enables juvenile *Lymnaea* to form long-term memory

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### SUMMARY

**Learning and memory provide the flexibility an organism requires to respond to changing social and ecological conditions. Juvenile *Lymnaea* have previously been shown to have a diminished capacity to form long-term memory (LTM) following operant conditioning of aerial respiratory behavior. Juvenile *Lymnaea*, however, can form LTM following classical conditioning of appetitive behaviors. Here, we demonstrate that laboratory-reared juvenile *Lymnaea* have the ability to detect the presence of a sympatric predator (i.e. crayfish) and respond to the predator by altering their aerial respiratory behavior. In addition to increasing their total breathing time, predator detection confers on juvenile *Lymnaea* an enhanced capability to form LTM following operant conditioning of aerial respiratory behavior. That is, these juveniles now have the ability to form long-lasting memory. These data support the hypothesis that biologically relevant levels of stress associated with predator detection induce behavioral phenotypic alterations (i.e. enhanced LTM formation) in juveniles, which may increase their fitness. These data also support the notion that learning and memory formation in conjunction with predator detection is a form of inducible defense.**

**Key words:** *Lymnaea stagnalis*, long-term memory, juvenile, predator, crayfish, operant conditioning, environmental stressor.

### INTRODUCTION

In adult *Lymnaea* aerial respiratory behavior can be operantly conditioned by applying a tactile stimulus to the pneumostome area (the respiratory orifice) as the snail attempts to open it to breathe aerially. After repeated contingent pneumostome stimulation snails demonstrate learning and memory formation by opening their pneumostomes fewer times. This form of associative learning can then be consolidated into long-term memory (LTM) (Lukowiak et al., 1996; Lukowiak et al., 2003a; Lukowiak et al., 2003b; Lowe and Spencer, 2006; Khan and Spencer, 2009). Neural correlates of LTM have been found in RPeD1 (Spencer et al., 1999; Spencer et al., 2002; McComb et al., 2005b), one of the three central pattern generator neurons (Syed et al., 1990) that drive this behavior. Moreover, this neuron is a necessary site for the processes of memory consolidation, reconsolidation, extinction and forgetting (Scheibenstock et al., 2002; Sangha et al., 2003a; Sangha et al., 2003b; Sangha et al., 2003c; Sangha et al., 2003d; Sangha et al., 2005). However, the story in juvenile *Lymnaea* is different. In juveniles, RPeD1 spontaneous activity is significantly higher than in adults (McComb et al., 2003) and this may partially explain why juvenile *Lymnaea* learn significantly less well than adults not to perform aerial respiration following operant conditioning of aerial respiratory behavior and do not form LTM (McComb et al., 2005a). This leads us to ask: could predator detection in juvenile *Lymnaea* alter their ability to form LTM?

Predator detection elicits so-called inducible defenses which allow an organism to rapidly develop defensive morphological and behavioral phenotypes to ward off predation (Dodson, 1988; Larsson and Dodson, 1993). These defensive strategies include alteration of behaviors to enable an adaptive strategy best suited to the organism and the environmental demand. These cue-induced responses may allow the organism to reap the benefits of the defense only when advantageous to the organism without the cost associated with the full-time maintenance of the defensive strategy.

Our model system has been used to show how the stress associated with the detection of a naturally occurring predator alters cognitive behaviors in adult snails. Crayfish are a sympatric predator of *Lymnaea* in The Netherlands where the snails used in this study were originally collected in the 1950s. Our laboratory-bred snails demonstrate defensive behavioral strategies when crayfish scent is detected (Orr et al., 2007; Orr et al., 2009); as do freshly collected snails from other locations (Chivers and Smith, 1998; Covich et al., 1994; Orr et al., 2008; Rundle and Bronmark, 2001). Aerial respiratory behavior in *Lymnaea*, which is driven by a three-neuron network, the sufficiency and necessity of which has been experimentally verified (Syed et al., 1990; Syed et al., 1992), is also significantly altered when a snail detects a predator (Orr et al., 2007; Orr et al., 2009). As stated above, this respiratory behavior can be operantly conditioned to induce LTM (Lukowiak et al., 1998; Lukowiak et al., 2000); the duration of which is dependent, in part, on the training procedure used. Thus, in adult laboratory-reared and wild Netherlands snails, a single 30 min training session results in intermediate-term memory (ITM) that persists for up to 3 h; while two 30-min training sessions with a 1-h rest interval results in long-term memory (LTM) that persists for 24 h (Sangha et al., 2003a; Lukowiak et al., 2000; Lukowiak et al., 2003b; Parvez et al., 2005; Parvez et al., 2006a; Orr et al., 2008). Finally we report that when adult laboratory-bred *Lymnaea* are faced with the ‘smell’ of a crayfish predator, memory formation is significantly enhanced and the correlates of this augmented LTM can be observed concurrently in both the whole animal behavior and in the electrophysiological characteristics of RPeD1 (Orr and Lukowiak, 2008).

A hypothesis we have developed (Orr et al., 2008; Orr et al., 2009) is that enhanced LTM formation as a result of predator detection is an anti-predator response. Thus, it is possible that predator detection in juvenile *Lymnaea* will enable these snails to form LTM. Here we demonstrate that when juvenile *Lymnaea* are faced with a biologically relevant stressor, the ‘smell’ of a crayfish predator, they gain the ability to form LTM.

## MATERIALS AND METHODS

### Animals

*Lymnaea stagnalis* L. were reared in our snail facility at the University of Calgary from a strain of *Lymnaea* originally obtained from Vrije Universiteit in Amsterdam. The ancestors of these snails were collected from ditches in a polder located in the province of Utrecht in the early 1950s (Orr et al., 2008; Orr et al., 2009). Cohorts of 10–14 juvenile snails (McComb et al., 2005a) with a shell length of 12–17 mm (mean 14 mm), as measured from the apex of the spiral to the distal prominence of the apertural opening, were labeled and maintained within home aquaria at room temperature ( $\approx 20^\circ\text{C}$ ) until training.

### Brief description of aerial respiratory behavior

*Lymnaea* are bimodal breathers obtaining oxygen through either cutaneous respiration (i.e. directly through their skin) or through aerial respiration *via* a rudimentary lung [i.e. gas exchange with the atmosphere; Lukowiak et al. (Lukowiak et al., 1996)]. To perform aerial respiration, the snail must surface and open its pneumostome (the respiratory orifice) while contracting and relaxing the appropriate respiratory muscles (Taylor and Lukowiak, 2000). Thus, training snails not to perform aerial respiration is not harmful. For a more detailed description of aerial respiratory behavior the reader is directed to two recent reviews (Lukowiak et al., 2003b; Lukowiak et al., 2008).

### Exposure to crayfish effluent

Crayfish of the genus *Cambrus* were housed in a 1101 aquarium and maintained on a diet of snails and lettuce. We trained snails (see below) using 500 ml of water taken from the crayfish aquaria. We termed this water CE [crayfish effluent; see Orr et al. (Orr et al., 2007; Orr and Lukowiak, 2008)]. During the CE procedures, snails are not in the presence of crayfish, only water taken from aquaria containing crayfish.

### Breathing observations

To determine if exposure to CE altered aerial respiratory behavior, snails were first placed in 500 ml of room-temperature hypoxic pond water ( $P_{\text{O}_2} < 931 \text{ Pa}$ ) and then after a 24-h rest interval, placed in 500 ml of hypoxic ( $P_{\text{O}_2} < 931 \text{ Pa}$ ) CE. The water is made hypoxic by bubbling  $\text{N}_2$  gas through the water for 20 min prior to introducing the snails. This is standard technique for measuring the aerial respiratory behavior and operant conditioning of this behavior in *Lymnaea* (Haney and Lukowiak, 2001; Lukowiak et al., 2003a; Lukowiak and Syed, 1999; Orr et al., 2007; Parvez et al., 2006a). 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.

### Operant conditioning

Snails were removed from home aquaria and placed into a 1-l beaker containing 500 ml of hypoxic pond water at an ambient temperature of approximately  $20^\circ\text{C}$ . The animals were given a 10-min acclimatization period prior to the 30-min training session. By subjecting snails to a hypoxic challenge, the animals increase their rate of aerial respiration (Lukowiak et al., 1996). The animals were operantly conditioned by applying a gentle tactile stimulus with a sharpened wooden applicator to their pneumostome as it begins to open (i.e. an attempted pneumostome opening). 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. This pneumostome closer response is a graded 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 the yoked control experiments. Yoked controls (see below) were performed for all behavioral experiments. All behavioral experiments were done ‘blind’ where the person performing the training paradigm was unaware of the status of the cohort being tested (i.e. in CE or pond water).

Two training paradigms were used: (1) the one-session training procedure and (2) the two-session training procedure [similar to that of Orr and Lukowiak (Orr and Lukowiak, 2008)]. The one-session procedure consisted of a single 0.5-h training session (TS) in hypoxia after which the snails were returned to their home eumoxic (i.e. normal  $\text{O}_2$  levels;  $P_{\text{O}_2} > 9975 \text{ Pa}$ ) aquaria. The snails were then tested for memory (MT; i.e. a ‘savings-test’) using a similar test to that of the training. The time of the memory test was at the indicated times after the TS. The two-session procedure consisted of two 0.5-h training sessions separated by a 1-h interval. The snails were placed into a eumoxic holding tank for the 1-h interval between TS1 and TS2 and after TS2. The memory test was performed at the indicated times after TS2.

### Yoked control experiments

Snails that received yoked training were treated in an identical manner to that outlined in the Operant conditioning procedure section except that in the ‘training period’, the stimuli were not contingent upon pneumostome opening. That is, the yoked control snails received the same number of stimuli in the exact same temporal pattern as the operantly conditioned snails but the stimuli were not contingent upon the opening of their pneumostome (Lukowiak et al., 2000; Lukowiak et al., 2003a; Lukowiak et al., 2008). The yoked snails did receive a contingent stimulus to the pneumostome during the savings test session (e.g. 24 h yoke).

### Operational definition of learning and memory

As previously described (Lukowiak et al., 2000; Martens et al., 2007) for one-session training, memory is defined as a significant reduction in the number of pneumostome openings after training. That is, the number of attempted pneumostome openings in the memory test session must be significantly less than TS1. In the case of two-session training, TS2 must be significantly lower than TS1 and the number of attempted openings in the memory test session must be significantly less than in TS1 but must also not be significantly greater than TS2.

### Statistics

We analyzed water treatment effects on snail breathing behavior data with paired sample *t*-tests. All between-groups tests (i.e. between various TS1 or MT treatment) were analyzed using one-way ANOVA after homogeneity of variance tests were passed. All statistics were performed using SPSS, version 11.0.4 for Macintosh.

## RESULTS

### Predator detection and the respiratory behavior

We have demonstrated that adult *Lymnaea* both detect and respond to the scent of a crayfish predator (i.e. CE exposure) with multiple predator-evoked vigilance behaviors such as increasing their shadow elicited full-body withdrawal response, exploratory/search phase and their aerial respiratory behavior (Orr et al., 2007). Here we utilized the robust increase in aerial respiratory behavior during predator detection as a means to determine if juvenile snails can also detect

the scent of this predator. We found that like adults, juvenile *Lymnaea* alter their aerial respiratory behavior in the presence of CE (Fig. 1). During a 0.5-hour observation period in each context (pond water vs CE), significant increases in both the total breathing time and the average breathing time per opening were observed in the snails exposed to CE (Fig. 1B,C). Interestingly, the total number of pneumostome openings was not different between each treatment. These results indicate that although juvenile snails do not open their respiratory orifice more often while exposed to CE, they do increase significantly their total breathing time.

#### Operant conditioning of juvenile snails in the presence of predator scent

Using the *Lymnaea* model system, we have utilized different training paradigms to evoke different forms and durations of memory. We

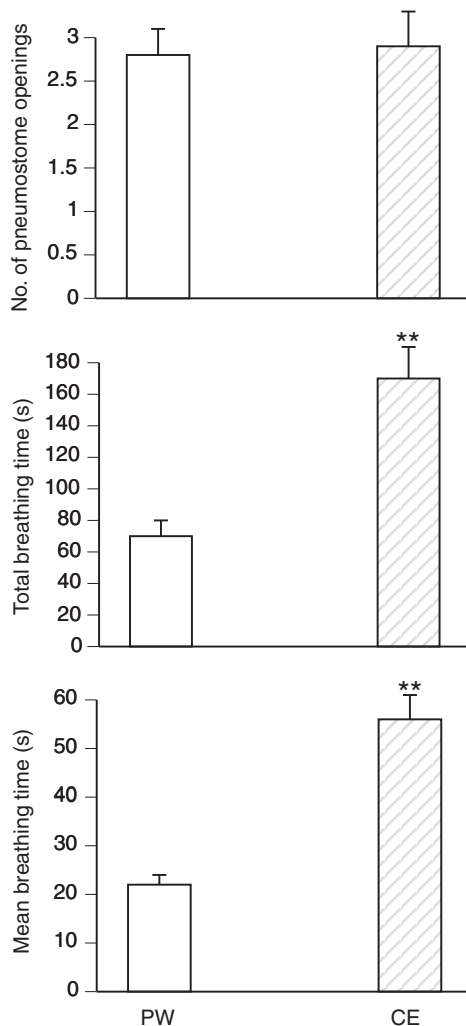


Fig. 1. Aerial respiratory behavior and predator detection. The mean ( $\pm$ s.e.m.) number of pneumostome openings (top panel); total breathing time (middle panel); and average breathing time per pneumostome opening (lower panel) of juvenile *Lymnaea* in pond water (PW) and crayfish effluent (CE). (Top) Number of pneumostome openings in PW=2.83 $\pm$ 0.3, compared with CE=2.92 $\pm$ 0.4 s.e.m. Treatments were not significantly different ( $P > 0.05$ ,  $N=37$ , paired  $t$ -test). (Middle) The total breathing time in PW was 72.41 $\pm$ 8.18, and in CE, 163.86 $\pm$ 22.05 (measured in seconds). The total breathing time in CE was significantly higher (\*\* $P < 0.001$ ,  $N=37$ ) than in PW. (Bottom) The average breathing time in CE was significantly higher (56.22 $\pm$ 6.22) than in PW (22 $\pm$ 2.08,  $N=37$ , \*\* $P < 0.001$ ). Values are means  $\pm$  s.e.m.

have demonstrated that adult *Lymnaea*, when trained with the two-session training procedure (i.e. two 0.5-h sessions with a 1-h interval between sessions) in pond water form an LTM that persists for at least 24 h but not 48 h (Lukowiak et al., 2000; Parvez et al., 2006a; Parvez et al., 2006b; Lukowiak et al., 2007; Rosenegger et al., 2008). We recently reported that exposure to CE during the two-session operant conditioning procedure results in a dramatic (at least an eightfold) increase in memory duration in adult snails (Orr and Lukowiak, 2008). We have also demonstrated that juvenile *Lymnaea* when trained with the two-session training procedure in pond water fail to form LTM (McComb et al., 2005a). We therefore asked the question: does exposure to CE during the two-session operant conditioning procedure alter the ability of juvenile snails to form LTM? To answer this question, we first selected a naïve cohort of juvenile snails and subjected them to the two-session operant conditioning procedure in pond water and tested them for memory 24 h later. In pond water, learning was observed (i.e. the number of attempted openings in TS2 was significantly fewer than in TS1) but no memory was present 24 h later, confirming the earlier McComb et al. (McComb et al., 2005a) study (i.e. the number of attempted pneumostome openings in the memory test session was not significantly less than TS1; Fig. 2, top panel). A yoked control group also demonstrated no LTM as the number of attempted pneumostome openings in the yoked memory test was not significantly different from TS1 or for that matter from the 24-h memory test of snails that received operant conditioning training (Fig. 2). We next trained a new, naïve cohort of juvenile snails in CE and tested for memory 24 h later (Fig. 2, bottom panel). We found that LTM was present. That is, the number of attempted pneumostome openings in the memory test was significantly lower than both TS1 and TS2 (Fig. 2, bottom panel). Yoked control snails 'trained' in CE, by contrast, did not demonstrate memory at 24 h (Fig. 2, bottom panel).

Having demonstrated that juvenile snails trained in CE now had the capability of forming LTM we next sought to determine if CE exposure enhanced the duration of memory (Fig. 3). To this end, we trained new cohorts of snails with the two-session training procedure in CE and tested memory at either 48 or 72 h. We also ran concurrent yoked groups for these time periods. We found that juvenile snails demonstrated LTM for up to 48 h but not 72 h when trained with the two-session procedure in CE (Fig. 3). As with the 24-hour yoked control snails, yoked control snails at 48 and 72 h did not demonstrate memory (Fig. 3).

In adult snails, we have found that the one-session 0.5-h training procedure in pond water results in intermediate-term memory (ITM) that persists for 3 but not 24 h. However, when the single training procedure is used in CE a 48 h memory results (Orr and Lukowiak, 2008). We, therefore, were interested to determine in juvenile snails the duration of memory resulting from a single 0.5-h session training procedure in both pond water and CE. A novel cohort was trained and tested in pond water 24 h later, and not surprisingly, memory was not demonstrated (Fig. 4, top). The yoked group also did not demonstrate a change in behavior from the naïve state (Fig. 4, top). By contrast, juveniles when trained similarly in CE demonstrated LTM at 24 but not 48 h (Fig. 4); whereas the 24 h yoke control group also did not demonstrate LTM (Fig. 4, bottom). These data demonstrate that in juvenile *Lymnaea* exposure to CE during operant conditioning training results in an enhanced ability to form LTM. Furthermore, just as in adult snails, the strength of the LTM formed in juvenile snails can be differentially and specifically induced using different training procedures. However, the LTM formed in juvenile snails exposed to CE is of a shorter duration than the enhanced duration of LTM seen in adults (Orr and Lukowiak, 2008).

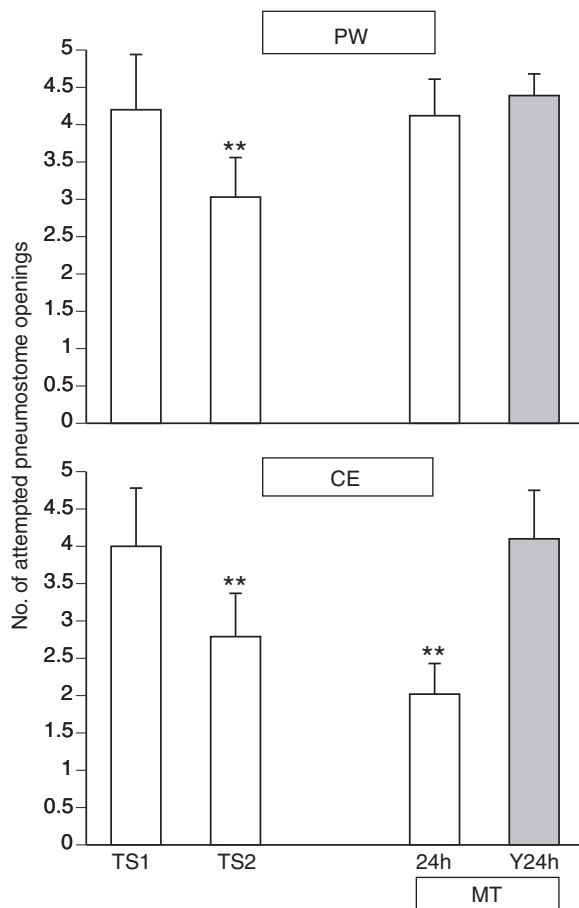


Fig. 2. Crayfish effluent (CE) enables juvenile *Lymnaea* to form long-term memory (LTM). (Top panel) In pond water (PW) the two-session training procedure resulted in learning but not LTM. That is, there was a significant reduction in the number of attempted pneumostome openings in training session 2 (TS2) compared with TS1 (TS1,  $4.20 \pm 0.74$ ; TS2  $3.03 \pm 0.53$ ;  $N=40$ ,  $**P < 0.01$ ) but the 24-hour memory test was not significantly different from TS1 (24-h memory test,  $4.12 \pm 0.49$ ;  $N=40$ ,  $P > 0.05$ ). Yoked controls ( $N=40$ ) did not demonstrate memory, as the 24-h yoked memory test was not significantly different from TS1 (Y24h,  $4.39 \pm 0.29$ ;  $N=40$ ,  $P > 0.05$ , gray bar). (Bottom panel) In CE, the two-session training paradigm resulted in both learning and LTM. That is, TS2 ( $2.79 \pm 0.58$ ) was significantly less ( $**P < 0.01$ ) than TS1 ( $4.00 \pm 0.78$ ,  $N=40$ ). Moreover, the number of attempted openings in the 24h MT ( $2.02 \pm 0.41$ ,  $N=25$ ) was significantly less than TS1 ( $**P < 0.01$ ) whereas in the Y24h MT ( $N=25$ ) the number of attempted openings ( $4.1 \pm 0.65$ ) was not significantly different from TS1 ( $**P > 0.05$ ). Values are means  $\pm$  s.e.m.

## DISCUSSION

We demonstrated here that laboratory-reared juvenile *Lymnaea* have the ability to detect the presence of a crayfish predator; and that detection of the predator significantly alters their cognitive abilities. Juvenile *Lymnaea* do not have the capacity to form LTM following operant conditioning of aerial respiration, however, upon detection of a sympatric predator (e.g. crayfish) they gain the competence to form LTM.

The data presented here confirm the earlier findings (McComb et al., 2005a) that juvenile *Lymnaea* do not have the capability to form LTM following operant conditioning of aerial respiratory behavior. This should not be taken to mean that juvenile *Lymnaea* are incapable of learning or forming LTM. Juvenile *Lymnaea*, similar to other animals, have the capacity to learn and form LTM on tasks not involving the withholding of a behavior. Thus, for

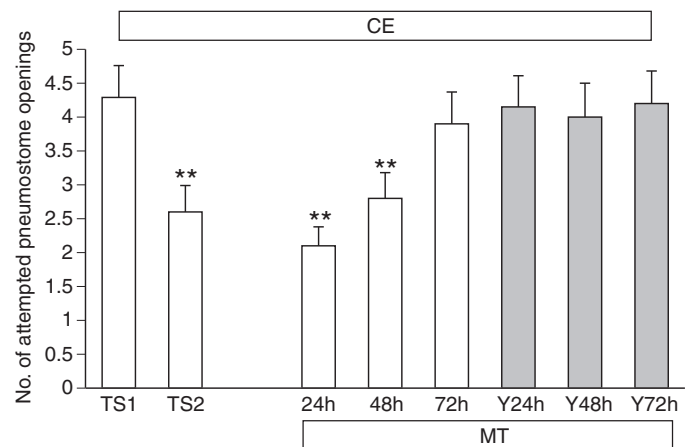


Fig. 3. Training juveniles in crayfish effluent (CE) resulted in a long-term memory (LTM) that persisted for 48h. A naive cohort of juvenile snails ( $N=93$ ) was subjected to the two-trial training procedure and LTM was tested (MT sessions) once in randomly selected snails at 24, 48, and 72h, respectively. Juvenile snails demonstrated LTM 24 and 48h after TS2 but not 72h later. That is, the snails demonstrated reduced pneumostome openings during TS2 ( $2.6 \pm 0.39$ ,  $N=93$ ,  $**P < 0.01$ ), 24-hour memory test ( $2.1 \pm 0.28$ ,  $N=25$ ,  $**P < 0.01$ ) and the 48-h memory test ( $2.8 \pm 0.38$ ,  $N=38$ ,  $**P < 0.01$ ) compared to the TS1 ( $4.29 \pm 0.47$ ). However, the number of attempted pneumostome openings in the 72h MT ( $3.9 \pm 0.47$ ,  $N=30$ ,  $P > 0.05$ ) was not significantly less than TS1; thus, LTM was not present. As well, the 24-, 48- and 72-h yoked controls ( $N=25$ , 38, 30, respectively) were also not significantly different from the TS1 ( $P > 0.05$  in each comparison).

example, juvenile *Lymnaea* learn and form memory for appetitive food behaviors (Yamanaka et al., 2000). Juveniles of all species typically perseverate on tasks where they have to withhold a response, including habituation (Lukowiak, 1980), suppression of attack responses (Dickel et al., 1997) passive avoidance (Blozovski and Cudennec, 1980; Mattingly and Zolman, 1980), spatial discrimination (Bronstein and Spear, 1972) and the classical conditioning of *Lymnaea*'s whole-body withdrawal response (Ono et al., 2002).

However, here we found that if we trained juvenile *Lymnaea* in the presence of CE, they became competent in forming robust LTM. Thus, juvenile *Lymnaea*, as part of the response to predator detection, acquire the ability to form LTM. From this it follows that the neuronal network and the molecular changes in the neurons necessary for LTM formation following operant conditioning of aerial respiratory behavior in juveniles can be modified by this ecologically relevant stressor. For LTM consolidation to occur, both altered gene activity and new protein synthesis must occur in neurons such as RPeD1 that are sites for LTM formation following operant conditioning training (Sangha et al., 2003a; Scheibenstock et al., 2002). Future experiments will be performed to show that this indeed occurs. We will also attempt to determine the length of time that the juveniles must be exposed to CE for the necessary changes in neuronal circuitry to occur that allow for LTM formation.

The electrophysiological phenotype of RPeD1 in naïve juvenile snails recorded from in semi-intact preparations demonstrates that this neuron is more excitable than in adult snails (Klaassen et al., 1998; McComb et al., 2003). This difference in the electrophysiological properties is not seen when examining isolated ganglia from both juvenile and adult snails. These data suggest that the peripheral input from the pneumostome area and osphradial ganglion exert a suppressive regulatory control over the aerial respiratory central pattern generator which includes RPeD1 (Inoue

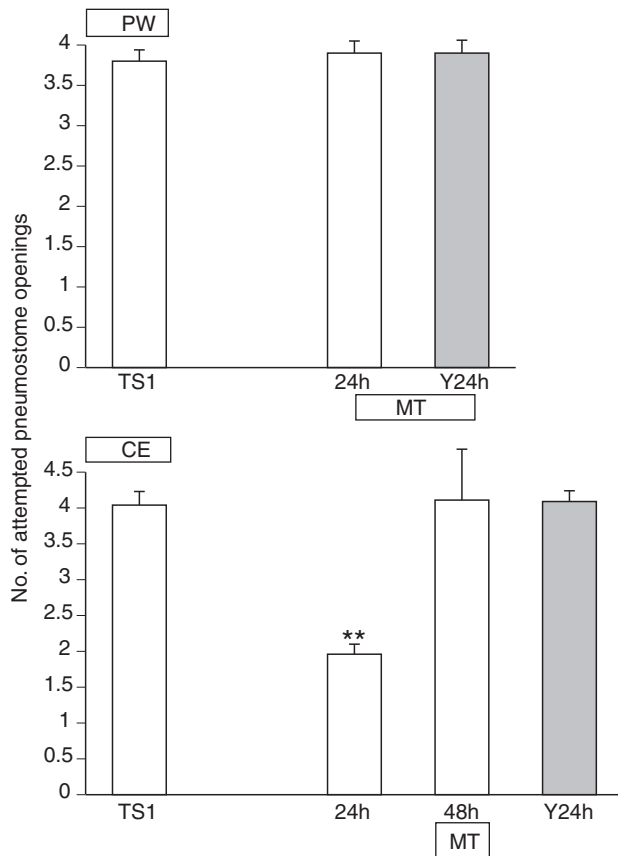


Fig. 4. Long-term memory (LTM), crayfish effluent (CE) and the one-session operant conditioning procedure in juveniles. When we trained juvenile snails ( $N=30$ ) using the less robust one-session training procedure in pond water (PW), they did not demonstrate memory after 24 h (top, white bars) or in the yoked control ( $N=30$ ; top, gray bar). That is, the number of attempted pneumostome openings in memory training (MT;  $3.9 \pm 0.15$ ) was not significantly less than the number of attempted openings in TS1 ( $3.8 \pm 0.14$ ;  $P > 0.05$ ). In addition the yoked control snails also did not demonstrate LTM ( $3.9 \pm 0.16$ ). However when given one-session training in CE, the juveniles ( $N=108$ ) demonstrated memory for at least 24 h. That is, the snails exhibited significantly reduced attempted pneumostome openings in the 24-h memory test ( $N=40$ ,  $1.96 \pm 0.14$ ) compared with TS1 ( $4.04 \pm 0.19$ ,  $**P < 0.01$ ). Juvenile snails did not demonstrate memory in the 48 h MT ( $N=68$ ;  $4.11 \pm 0.71$ ) or during the 24 h yoked control ( $N=40$ ,  $4.09 \pm 0.15$ ). Values are means  $\pm$  s.e.m.

et al., 2001; McComb et al., 2003). At this point we are not certain how the 'smell' of the predator alters RPeD1 activity, if in fact it does in juvenile *Lymnaea*. These experiments are presently being performed. We also believe that RPeD1 activity in juveniles will be suppressed in CE and that the osphradium plays a significant role in both CE detection and signaling to CNS that results in the alteration of RPeD1 activity.

We have found in adult snails that exposure to CE induces a transient reduction (<24 hours) in the spontaneous firing rate of RPeD1 and that the combination of operant conditioning in the presence of CE is required to prolong this reduction of neural activity for the duration of the memory (Orr et al., 2007; Orr and Lukowiak, 2008). Our current working hypothesis is that in juvenile snails, exposure to CE counteracts the increased excitability of RPeD1 and forces the network into a more 'adult-like' configuration, thereby allowing operant conditioning to result in LTM formation. That is, the concurrent combination of suppressive input from peripheral

centers during predator exposure and the operant conditioning procedure enhances the juvenile snail's ability to form robust LTM. Further investigations into this hypothesis are currently underway.

The term allelochemical is used to describe a chemical that conveys information that mediates an interaction between two organisms (Burks and Lodge, 2002). The use of kairomones (an allelochemical that conveys information between different species) has been described in many situations including alarm signaling (Chivers and Smith, 1998; Jacobsen and Stabell, 1999), planktonic interactions (Larsson and Dodson, 1993) and predator-prey interactions (Abrams, 1994; Jacobsen and Stabell, 2004; Kats and Dill, 1998; Dalesman et al., 2006). In vertebrate models, the utilization of kairomones to study the relationship between stress and memory have traditionally included the responses of mice and rats to the presence of cat or fox doors (Kim and Diamond, 2002; Maren, 2005; Takahashi et al., 2005). These investigations, despite their closer relevance to clinical (i.e. the human condition) situations, present the added complexity of dealing with complex vertebrate neuroanatomy where any noted changes in the physiological state of the animal due to predator detection can only be correlated with changes in behavior.

In aquatic environments chemical cues are important signals between and among species (Burks and Lodge, 2002). Predator scent has been shown to effect behaviors such as feeding and locomotion resulting in changes in growth and reproduction rates of gastropods in both fresh and marine systems (Burks and Lodge, 2002; Chivers and Smith, 1998; Covich et al., 1994; Palmer, 1990). Gastropods are also known to use chemical cues to assess and reduce the risk of predation (Covich et al., 1994; Jacobsen and Stabell, 2004) by increasing anti-predator behaviors such as crawling out of the water or increasing the use of a refuge during a predator presence. Numerous studies involving the response of various freshwater snails to the presence of crayfish predators have demonstrated the potency of the kairomone involved (Burks and Lodge, 2002; Chivers and Smith, 1998; Covich et al., 1994; Jacobsen and Stabell, 1999; Jacobsen and Stabell, 2004; Kats and Dill, 1998; Rigby and Jokela, 2000; Ryer et al., 2004), however, the precise chemical(s) has yet to be identified.

Juvenile *Lymnaea* were found to be capable of detecting the presence of a crayfish predator *via* a kairomone and responding with a functionally appropriate response. That is, the juvenile snails significantly increase their total aerial respiratory behavior in CE. This finding is consistent with the observation that adult snails also increase their respiratory behavior or even crawl out of the water when shell-crushing predators are detected (Alexander and Covich, 1991; Levri, 1998; Orr et al., 2007). We did, however, find a difference in the respiratory response between the juveniles and adults. We observed that the juvenile snails did not increase their total number of pneumostome openings during the breathing observations in CE as the adults do (Orr et al., 2007). Instead the juveniles increased their average duration of the pneumostome opening. The functional significance for this is as of yet unknown. A possible explanation for this finding, as pointed out by Vogel (Vogel, 1983) is that juvenile snails living in an aquatic environment possess a low Reynolds number and an increased relative viscosity; thus, they may have to use relatively more energy than an adult to break the surface tension in order to open the pneumostome. To overcome this energy constraint they open their pneumostome for a longer time rather than more often (Daniels et al., 1999). Nonetheless, our data provides support for the hypothesis that in juvenile *Lymnaea*, the sensory network has reached a stage of development whereby they can both detect and respond

appropriately to a natural predator threat. Finally, we have to differentiate between our results regarding aerial breathing behavior in the absence of the presentation of a tactile stimulus to the pneumostome (i.e. the data shown in Fig. 1) with the data obtained when snails were operantly conditioned (e.g. Fig. 2). CE exposure causes a significant increase in aerial respiration; whereas training in CE results in a significant decrease in the number of attempted pneumostome openings (i.e. memory). Typically, snails attempt to open their pneumostome more often during operant conditioning training than they do during breathing observation sessions. The reason for this is that in hypoxic conditions where we are only observing breathing behavior (i.e. no tactile stimulus to the pneumostome area) the snails are able to satisfy their O<sub>2</sub> requirements; whereas because they close their pneumostome when they receive a tactile stimulus during operant conditioning training they do not satisfy their O<sub>2</sub> requirement, thus they attempt in the training session to open the pneumostome more often. However, if they learn not to open their pneumostome to avoid the tactile stimulus, and remember this in the memory test session there will be fewer attempts to open the pneumostome. Thus, in CE snails breathe aurally more often, but with operant conditioning training they learn and remember better, thus fewer attempted openings are recorded.

The new data presented here provide support for our hypothesis that enhanced memory formation as a result of predator detection is a form of an inducible defense that, at least in *Lymnaea*, is manifest at a very young age. It is unclear how predator detection alters the nervous system of juvenile *Lymnaea* so that they gain the ability to form LTM following our operant conditioning procedure. However, this model system presents a unique opportunity to study chemically mediated predator-prey interactions at the behavioral, neuronal and biochemical levels. Through this model system, the tools are at hand to fully investigate the neurobiological mechanisms of behavioral defense induction from the naturally occurring populations to the molecules involved in these defenses. In doing so we can gain a better understanding of the specific mechanisms involved in these processes and how they may relate to both natural and laboratory-reared populations.

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