The Journal of Experimental Biology 214, 2528-2533

doi:10.1242/jeb.058024

RESEARCH ARTICLE

Sensory mediation of memory blocking stressors in the pond snail *Lymnaea stagnalis*

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Accepted 9 May 2011

SUMMARY

The great pond snail, *Lymnaea stagnalis*, is commonly used as a model species to study how stress affects the ability to form long-term memory (LTM); however, we still have little information about how the snail senses stressful stimuli. The osphradium is an external sensory organ that demonstrates electrophysiological responses to a variety of external chemical stimuli. We examined the role, if any, played by the osphradium in sensing two environmental stressors, crowding and low environmental calcium, both known to block LTM in intact animals. We severed the osphradial nerve, blocking external sensory input from this organ to the central nervous system, and then exposed the snails to low environmental calcium or crowding stress to assess whether these stressors continued to block LTM formation. When exposed to low environmental calcium, snails with their osphradial nerve severed responded as if they were maintained in our standard calcium environment. That is, they did not respond to low calcium as a stressor blocking LTM; therefore, the osphradium plays a crucial role in mediating how snails respond to this stressor. However, following crowding, LTM formation was blocked in both control groups and snails that had the osphradial nerve severed, indicating that sensory information from the osphradium is not required to sense crowded conditions. Together these data show that two stressors that result in the same behavioural phenotype, blocking LTM formation, do so *via* two distinct sensory pathways.

Key words: *Lymnaea stagnalis*, calcium, crowding, long-term memory, osphradium, stress.

INTRODUCTION

The ability of an animal to respond correctly to its surroundings depends on the reliability of the information it acquires. In the aquatic environment, where vision can be impaired by turbidity, chemoreception is often the primary method animals use to sense surroundings (Dodson et al., 1994; Ferrari et al., 2010a). Many aquatic species have been demonstrated to have sensitive chemoreception that can respond to a wide variety of chemical cues (for reviews, see Dodson et al., 1994; Chivers and Smith, 1998; Brönmark and Hansson, 2000; Ferrari et al., 2010b). For example, aquatic gastropods rely on chemosensory information to detect and identify risk from predators (Dalesman et al., 2006), recognise conspecific and heterospecific guild members (Dalesman et al., 2007; Dalesman and Rundle, 2010), and orientate towards volatile organic compounds produced by algae and cyanobacteria (Fink et al., 2006). There is also evidence that they can sense calcium concentration in their environment, e.g. orientation towards a high-calcium environment in choice tests (Piggott and Dussart, 1995) and rapid response to changes in calcium concentration by modulation of locomotion and respiration (Dalesman and Lukowiak, 2010).

Environmental stressors can alter the way an animal is able to learn and form memory, either enhancing or blocking memory formation depending on the nature of the stress and timing relative to the learning period (Shors, 2004). How stressful environmental stimuli are sensed by the animal will depend on the nature of the stressor. The great pond snail, *Lymnaea stagnalis*, is commonly used as a model organism to study learning and memory because of its relatively simple neuronal system and easily recordable set of behaviours that can be altered through training (reviewed in Benjamin et al., 2000; Lukowiak et al., 2003; Parvez et al., 2006; Benjamin and Kemenes, 2008). The ability of *L. stagnalis* to learn and form memories is malleable, being enhanced or reduced by a wide range of environmental stressors (reviewed in Lukowiak et al., 2008; Lukowiak et al., 2010). However, despite a large body of work demonstrating how stress alters memory, we still have little direct evidence of the way in which the stressful stimuli are being sensed by this species.

The osphradium is an external sensory organ in *L. stagnalis*, situated just above the pneumostome (respiratory orifice) on the body, within the mantle cavity (Bell et al., 2007). Because of electrophysiological responses found in neurons of the osphradium to external chemical stimulation (Wedemeyer and Schild, 1995; Kamardin et al., 2001), it is thought to be responsible for analysing many of the physiochemical properties of the water in which the snail lives. Despite this knowledge, very little is known about how sensory input from the osphradium affects the behaviour of the snail in response to changes in the environment. A recent study demonstrated that the osphradium is used to sense the presence of predator kairomones from a crayfish (Il-Han et al., 2010). The presence of crayfish kairomones during operant conditioning of aerial respiratory behaviour enhances long-term memory (LTM) formation in *L. stagnalis* (Orr and Lukowiak, 2008). By severing the osphradial nerve innervating the central nervous system (CNS), the snails no longer responded to the presence of crayfish kairomones in their environment, and responded to the training regime in the

presence of crayfish kairomones as if trained in pond water alone (Il-Han et al., 2010).

Here we assess the role of the osphradium in modulating the response to two alternate environmental stressors, low environmental calcium and crowding. Each of these stressors is known to block LTM formation in *L. stagnalis* following operant conditioning of aerial respiration. Exposure to low (20 mg l^{-1}) environmental calcium before and during training blocks LTM formation (Dalesman et al., 2011) and also prevents the active process of forgetting following training in a standard (80 mg l^{-1}) calcium environment (Knezevic et al., 2011). As the response to this stressor is relatively rapid (i.e. occurs following just 1 h exposure) and *L. stagnalis* is able to maintain calcium levels in the haemolymph in low calcium environments (Greenaway, 1971; De With et al., 1987; Grosell and Brix, 2009), even in the complete absence of calcium for at least 10 days (De With, 1977), we proposed that behavioural responses elicited by low environmental calcium are a result of sensory input from the periphery to the CNS, rather than from internal homeostatic changes. In addition, we tested another stressor, crowding for 1 h immediately prior to training, which also blocks LTM formation. The snails only respond to crowding if live snails are present, and do not respond to empty shells or chemicals released from an equal number of individuals in the water prior to training (De Caigny and Lukowiak, 2008b). Although these data support the theory that physical interaction with live snails is required to act as a stressor, we were unable to rule out the possibility that a short-lived chemical stimulus (i.e. dissipated within a few minutes of removing the snails from the water) is responsible for the stress response. By severing the osphradial nerve connecting the osphradium to the CNS, we assessed whether external environmental input *via* the osphradium is responsible for modulating the stress response – blocking LTM formation – in response to both low environmental calcium and crowding.

MATERIALS AND METHODS

Adult *Lymnaea stagnalis* (Linnaeus 1758) (spire height 25±1 mm) from a population originating from wild snails collected in the 1950s from canals in a polder located near Utrecht, the Netherlands, were used to perform all experiments. Snails were reared in the Biological Sciences building at the University of Calgary and maintained in artificial pond water (~0.25 g l^{-1} Instant Ocean®, Aquarium Systems Inc., Mentor, OH, USA) with the addition of $CaCO_3$ to maintain a calcium concentration >50 mg l^{-1} (Hermann et al., 2009). The animals were fed *ad libitum* with lettuce and Aquamax-carnivorous Grower 600 trout pellets (Purina Mills LLC, St Louis, MO, USA). Snails were transferred to the laboratory a minimum of 1 week prior to the start of experiments and maintained in oxygenated artificial pond water (0.26 g l^{-1} Instant Ocean®) with additional calcium sulphate dehydrate to provide a calcium concentration of 80 mg l^{-1}. They were kept at room temperature (20±1°C) in a 16 h:8 h light:dark schedule at a stocking density of one snail per litre and fed romaine lettuce *ad libitum*.

Surgical protocol

In addition to using animals that did not undergo any surgical procedure (intact), we also used two groups of surgically operated animals, one group in which the osphradial nerve was severed (cut) and another group that underwent the same surgical procedure but without severing the osphradial nerve (sham) to control for any effects of the surgical procedure and anaesthetic on memory formation. Animals that underwent either surgical operation were first anaesthetised using iced pond water and then injected using 2 ml of 50 mmol l^{-1} $MgCl_2$ *via* the foot into the haemocoel. The $MgCl_2$ relaxes the body of the animal, preventing withdrawal into the shell and allowing access to the area around the osphradium. Once anaesthetised, animals were placed onto a dissection dish and a small slit was made in the skin to access the osphradial nerve connecting the osphradium with the CNS. In cut animals, the osphradial nerve was then severed proximal to the osphradium; in sham animals, the small slit was made in the skin but the osphradial nerve was left unsevered. Data from right pedal dorsal 1 (RPeD1), a neuron in the CNS and part of the central pattern generator (CPG) that controls aerial respiration (Syed et al., 1990), have demonstrated an identical electrophysiological response between sham operated and naïve animals to chemical stimulation of the osphradium (M. H. Braun, unpublished data). Animals healed quickly without any intervention to close the surgical wound, and rapidly recovered from surgery, crawling around, feeding and otherwise apparently behaving as normal within an hour or so. All animals were then allowed an additional week to ensure full recovery from the surgical procedure before experiments were commenced.

Training protocol

Training to reduce aerial respiration in hypoxia was carried out in the same way for all experiments (Lukowiak et al., 1996; Lukowiak et al., 1998). Five hundred millilitres of artificial pond water, with calcium sulphate added to make a final calcium concentration of 80 or 20 mg l^{-1} depending on treatment protocol, was placed in a 1 l glass beaker. N_2 was then vigorously bubbled through the water for 20 min to make the water hypoxic (<5% [O_2]). N_2 bubbling was reduced and continued at a low level to maintain hypoxic conditions without disturbing the animals. Snails were then introduced into the beaker and allowed to acclimate for 10 min before the start of training. Training was carried out for 30 min, whereby the snail was gently 'poked' on the pneumostome each time it attempted to open it at the water's surface. The snails were then returned to their eumoxic (~100% [O_2]) aquaria for 1 h, after which they received a further 30 min training session identical to the first. Again, snails were returned to eumoxic aquaria following the second training session, and tested for LTM using a protocol identical to the first training session either 24 or 72 h following training. The number of times the snail attempted to open its pneumostome during both the first (TR1) and second (TR2) training sessions and the testing (test) session were then compared to assess whether the snails had learnt and formed LTM. The snails were considered to have demonstrated learning if the number of attempted openings was significantly lower during TR2 than TR1. If the snail demonstrated LTM, then the number of attempted pneumostome openings during the test session was both significantly lower than during TR1 and not significantly higher than during TR2.

Low calcium stress

Acute exposure to a low (20 mg l^{-1}) calcium environment for 1 week prior to and during the training procedure has been found to block the ability of intact *L. stagnalis* to form LTM (Dalesman et al., 2011; Knezevic et al., 2011). A low calcium environment experienced immediately following the training procedure has also been found to block the active process of forgetting in intact snails (Knezevic et al., 2011). Memory following the training protocol used in the present study normally lasts just 24 h; however, if snails are maintained in low calcium between training and testing, forgetting is blocked and snails still demonstrated memory at least 72 to 96 h following training. To test for the role of the osphradium in sensing the external calcium environment, we wanted to test

whether severing the osphradial nerve would alter both of these effects (LTM formation and forgetting) in a low calcium environment.

To assess whether severing the osphradial nerve alters the effect of low calcium exposure prior to training in blocking the ability to form LTM, we exposed snails from each group (intact, cut and sham) to either a low (20 mg l^{-1}) or standard (80 mg l^{-1}) calcium environment for 1 week prior to training. Snails were then trained, as outlined above, in the calcium concentration in which they had been held for the previous week, and tested in that same calcium concentration for LTM formation 24 h following TR2.

To test whether severing the osphradial nerve alters the rate at which snails forget the training procedure in either a low or standard calcium environment, we first maintained snails from each group (intact, cut or sham) for 1 week in standard calcium. All snails were then trained as outlined above in the standard calcium concentration, after which half continued to be maintained in standard calcium, whereas the other half were transferred into low environmental calcium immediately following training. Seventy-two hours after TR2, snails were then tested in standard calcium to assess whether they had LTM of the training procedure.

Crowding stress

Crowding *L. stagnalis* immediately prior to training to reduce aerial respiration in a hypoxic environment has been found to block their ability to form LTM (De Caigny and Lukowiak, 2008a; De Caigny and Lukowiak, 2008b). However, the training protocol used by De Caigny and Lukowiak differed from the training regime used here. In their study, KCl was used to pre-sensitize the snail, allowing LTM to be formed following a single 30 min training session, which in the absence of KCl normally only results in intermediate-term memory. Therefore, firstly we wanted to test whether crowding prior to training also blocks LTM in intact snails when they undergo two 30 min training sessions separated by 1 h as used here; this training procedure normally results in LTM lasting 24 h in the absence of stressful stimuli. Immediately prior to the first training session, snails were crowded for 1 h by placing 20 individuals into 100 ml of pond water in a 1 l glass beaker, and then trained as outlined above and tested for LTM 24 h following the second training session. This was repeated using both cut and sham snails to assess whether severing the osphradial nerve or the surgical procedure itself altered the snails response to crowding. Snails were maintained in our standard calcium concentration (80 mg l^{-1}) for all crowding experiments.

Statistical analyses

All data were analysed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA) using repeated-measures ANOVA. Homogeneity of variance was tested using Mauchly's test for sphericity, with the more conservative Greenhouse–Geisser *P*-values used where assumptions were not met. The within-subject factor for all analyses was the training period or test period (TR1, TR2 or test). For the experiments testing the effects of the low calcium stressor, the between-subject factors were the surgical procedure (intact, sham or cut) and the calcium concentration (low *vs* standard calcium). For the crowding experiment, the between-subject factor was the surgical procedure (intact, sham or cut). Where overall differences were found in the analyses, *post hoc* paired *t*-tests were used to assess within-subject pairwise differences; the *P*-value for significance was corrected to 0.0167 for multiple tests.

RESULTS

LTM formation in low calcium

When maintained in the standard calcium environment, all groups (intact, sham and cut) learnt and formed memory lasting 24 h (Fig. 1). However, when maintained in low calcium prior to and during the training sessions, both intact and sham snails demonstrated learning at TR2, but not LTM 24 h later, whereas the cut group demonstrated both learning and LTM at 24 h, not differing significantly from their response to training in the standard calcium environment (repeated-measures ANOVA, time tested × calcium environment × surgical procedure experienced: $F_{3.45,117.24}$=3.134, P=0.023; Fig. 1).

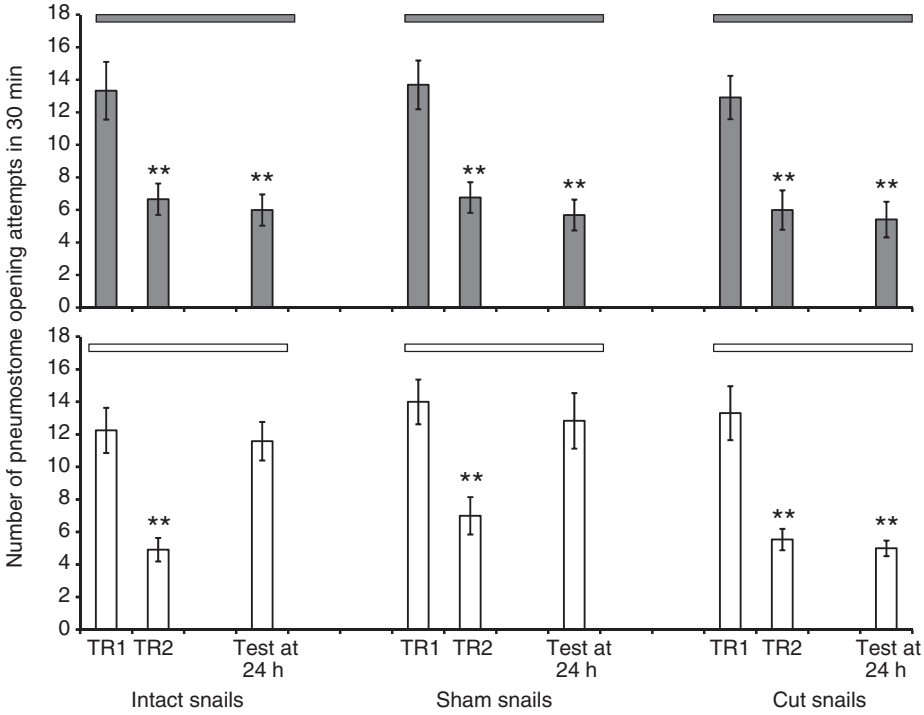

Fig. 1. Number of pneumostome opening attempts during training sessions one (TR1) and two (TR2) and the test at 24 h, following exposure to either low (20 mg l^{-1}; white bars) or standard (80 mg l^{-1}; grey bars) environmental calcium for 1 week prior to tests and throughout the training and testing period. Vertical bar colour indicates the conditions in which the snails were trained and/or tested; horizontal bars above the columns indicate the conditions in which snails were maintained in their eumoxic aquaria. Data are means ± s.e.m. **Differs significantly from TR1 within the training group (paired *t*-test, P<0.01).

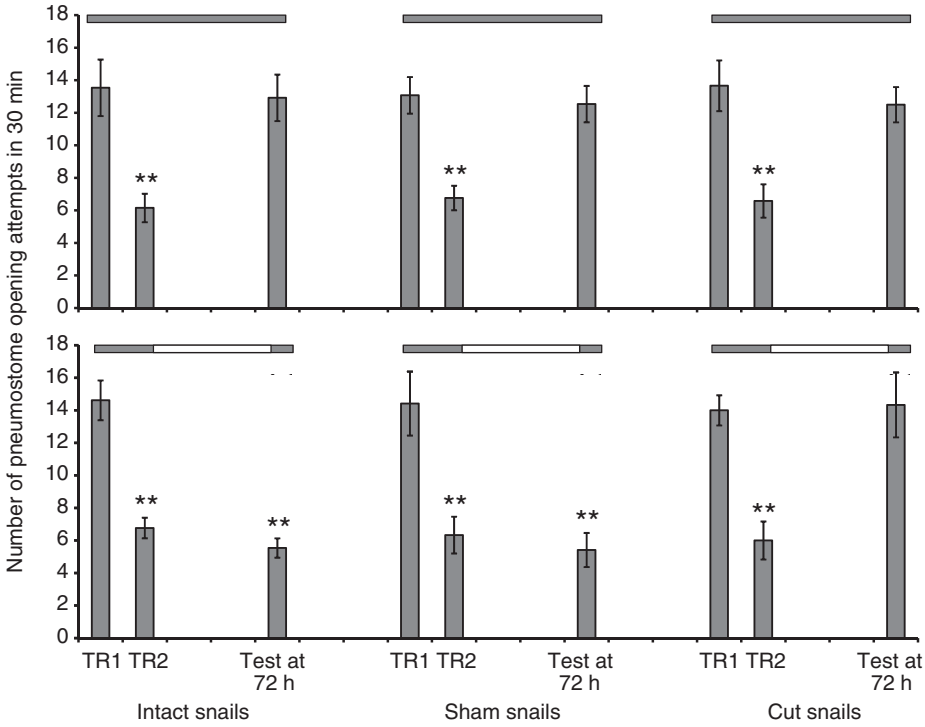

Fig. 2. Number of pneumostome opening attempts during TR1 and TR2 and the test at 72 h, following training in standard calcium for all snails, then exposure to either low (20 mg l^{-1}; white bars) or standard (80 mg l^{-1}; grey bars) environmental calcium in eumoxic aquaria between the end of TR2 and test session 72 h later. Vertical bar colour indicates the conditions in which the snails were trained and/or tested; horizontal bars above the columns indicate the conditions in which snails were maintained in their eumoxic aquaria. Data are means ± s.e.m. **Differs significantly from TR1 within the training group (paired *t*-test, $P<0.01$).

Forgetting in low calcium

If snails trained in standard calcium levels were placed into standard environmental calcium in their eumoxic aquaria between TR2 and testing, all snail groups (intact, sham and cut) demonstrated learning during TR2, but did not show LTM at 72 h following training (Fig. 2). However, when snails were exposed to low environmental calcium in their eumoxic aquaria between TR2 and the test, all groups demonstrated learning at TR2, and both the intact and sham operated animals demonstrated LTM at 72 h, whereas the cut individuals that had the osphradial nerve severed did not demonstrate LTM at 72 h, demonstrating no difference from when they had been held in standard calcium throughout, i.e. they forgot (repeated-measures ANOVA, time tested × calcium environment × surgical procedure experienced: $F_{4,138}=3.862$, $P=0.005$; Fig. 2).

Crowding

When held in our standard environmental calcium concentration and crowded for 1 h prior to TR1, all snail groups, irrespective of surgical procedure, demonstrated learning during TR2 but not LTM when tested 24 h later (repeated-measures ANOVA, main effect of training period: $F_{2,66}=55.603$, $P<0.001$; Fig. 3).

DISCUSSION

The osphradium is thought to be a primary sensory organ for physiochemical information in *L. stagnalis* (Wedemeyer and Schild, 1995; Kamardin et al., 2001). As such, it may play an important part in modulating the response to environmental stressors through the sensory information it transmits to the CNS *via* the osphradial nerve. Here, we tested whether severing the osphradial nerve altered the way in which *L. stagnalis* responds to two environmental stressors, crowding and low environmental calcium, both known to block LTM formation in this species (De Caigny and Lukowiak, 2008b; Dalesman et al., 2011; Knezevic et al., 2011).

Lymnaea stagnalis that had their osphradial nerve severed were equally capable of learning and forming LTM lasting 24 h as our intact and sham operated snails in standard calcium (80 mg l^{-1}) conditions, confirming previous data demonstrating that severing the osphradial nerve does not prevent *L. stagnalis* from performing aerial respiration in hypoxia, or learning and remembering to reduce this behaviour in response to training (Il-Han et al., 2010). When intact and sham operated snails were exposed to low environmental calcium for 1 week before and during training, these groups demonstrated learning (i.e. the number of pneumostome opening attempts decreased between the first and second training sessions), but they did not demonstrate LTM 24 h following training. This result confirms previous data demonstrating that low environmental calcium blocks LTM formation (Dalesman et al., 2011; Knezevic et al., 2011). However, when osphradially cut snails were exposed to a low calcium environment prior to training, it did not prevent them from forming LTM at 24 h. This demonstrated that the response to low calcium blocking LTM formation is modulated *via* external sensory input from the osphradium; when the connection between the osphradium and the CNS is severed, the snails no longer respond to this stressor. Stressors are able to alter LTM formation in *L. stagnalis*, but only if the stressor is perceived by the snail (Il-Han et al., 2010). It appears that osphradial input to the CNS is necessary for stressors such as low calcium and predator kairomones to alter LTM formation, blocking and enhancing it, respectively.

To confirm that the ability to form LTM in snails with their osphradial nerve severed was the result of not detecting and/or not responding to low calcium availability as a stressor, we also assessed the effect of low environmental calcium on the ability of snails whose osphradial nerve had been severed to forget. Intact snails, trained in standard calcium but maintained in low calcium between training and testing, retain memory of the training procedure for at least 96 h, i.e. they do not forget during this time (Knezevic et al., 2011). In the present study, when snails were maintained in standard calcium following training, all three groups of snails failed to demonstrate LTM 72 h following training. This result is in agreement with previous findings, where the training method used in this study, two 30 min training sessions separated by 1 h, has been found in previous work to result in memory lasting 24 h but not 72 h (Sangha et al.,

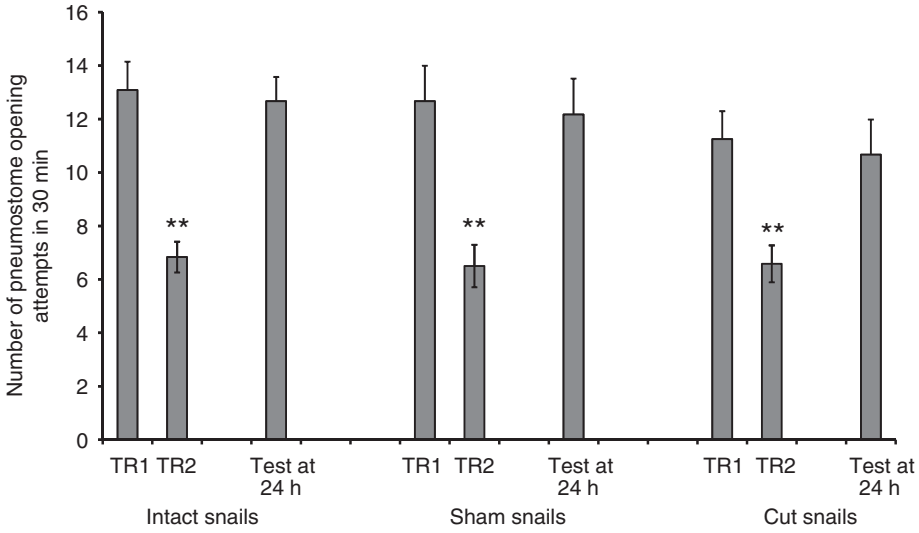

Fig. 3. Number of pneumostome opening attempts during TR1 and TR2 and the test at 24 h, following crowding for 1 h immediately prior to TR1. Data are means ± s.e.m. **Differs significantly from TR1 within the training group (paired *t*-test, $P<0.01$).

2003; Knezevic et al., 2011). However, when exposed to low environmental calcium following training, both the intact and sham operated snails demonstrated memory 72 h later. These data indicate that exposure to low environmental calcium had effectively blocked the active process of forgetting. In contrast, the snails that had the osphradial nerve severed did not demonstrate LTM at 72 h when exposed to low environmental calcium following training. We interpret these data to indicate that snails whose osphradial nerve has been severed could not detect and/or signal to the CNS that they were in a low calcium environment. These data provide further evidence that the osphradium is used to sense calcium concentration in the environment, conveying this information to the CNS. Therefore, without osphradial input, the snail appears unaware of the external calcium environment over an acute period (≤1 week), and does not react to low calcium availability as a stressor.

Previous work demonstrating that *L. stagnalis* responds rapidly to changes in the environmental calcium concentration (Dalesman and Lukowiak, 2010; Dalesman et al., 2011), along with data indicating that this species is able to orientate towards a high calcium environment (Piggott and Dussart, 1995), strongly suggested that this species is capable of sensing and responding directly to the external calcium concentration. Here we provide the first direct evidence that this is indeed the case. *Lymnaea stagnalis* is a calciphile, obtaining at least 80% of its calcium requirements directly from the water (Van Der Borght and Van Puymbroek, 1966). Its distribution is limited by environmental calcium availability, with populations requiring a minimum of 20 mg l^{-1} environmental calcium (Boycott, 1936; Young, 1975), and, at low levels of environmental calcium, growth and reproduction decline (McKillop and Harrison, 1972). Above 50 mg l^{-1} environmental calcium, uptake is mainly passive *via* an electrochemical gradient between the external environment and haemolymph; below this level, the energy required for calcium uptake will increase as the external calcium concentration declines (Greenaway, 1971). In addition, *L. stagnalis* embryos require an external source of calcium for successful development (Ebanks et al., 2010a; Ebanks et al., 2010b). Hence, the ability to sense calcium resource availability, allowing the snail to rapidly reduce energy allocation to activities such as reproduction when they are in suboptimal environmental conditions, would be of benefit to this species.

Crowding *L. stagnalis* for 1 h before a training procedure, which normally results in LTM lasting 24 h in the absence of stress, resulted in all three groups tested demonstrating learning (i.e. they significantly reduced the number of pneumostome opening attempts between TR1 and TR2), but none of the groups demonstrated LTM 24 h later. This data is in agreement with that of De Caigny and Lukowiak (De Caigny and Lukowiak, 2008b), who showed that, following an alternate training procedure, where snails are exposed to KCl to sensitise them to training, crowding prior to training to reduce aerial respiration acts as a stressor that blocks LTM formation. In contrast to the response seen in low environmental calcium in the present study, snails with their osphradial nerve severed did not differ from either intact or sham operated individuals, all responding equally to crowding as a stressor blocking LTM formation. We therefore conclude that the osphradium is not used in sensing the stimulus associated with the crowding stress, playing little role, if any, in signalling crowded conditions to the CNS. It is possible that a chemical stimulus may still play an important role in the response to the crowding stress, and is sensed elsewhere such as *via* the lips or tentacles. It seems most likely that it is the multiple and frequent physical contact between individuals under crowded conditions that stresses the snails, particularly as no response was found to chemicals alone from crowded individuals in previous work (De Caigny and Lukowiak, 2008b).

One reason why the osphradium may play a role in sensing predators (Il-Han et al., 2010), but not live conspecifics, may be the necessity to detect predator threat at a distance in order to allow the snail time to respond, as *L. stagnalis* is considerably less motile than the majority of the species that predate on aquatic snails. Crowded conditions will not have such immediate fitness consequences as being predated, and so the snail can afford to only detect these conditions at close range. *Lymnaea stagnalis* has been found to respond to chemical cues from injured conspecifics, modulating their antipredator behaviour as juveniles (Dalesman et al., 2006). It is possible that the osphradium may play a role in sensing conspecifics if they are injured, as this can indicate predation threat, though this remains to be investigated.

Aerial respiration in *L. stagnalis* is driven by a three-neuron CPG whose sufficiency and necessity has been experimentally demonstrated (Syed et al., 1990; Lukowiak, 1991; Syed et al., 1992); however, the activity of this CPG is also altered by peripheral sensory input. For example, information from the periphery, including the osphradium, can directly alter activity of one of these neurons in the CPG, RPeD1 (McComb et al., 2003; Bell et al., 2008). We have previously found that the electrical activity in RPeD1 significantly declines in a standard calcium environment following training, but in the low calcium environment this response is

significantly reduced (Dalesman et al., 2011). The data presented here demonstrate that sensory input from the osphradium indicating calcium concentration in the environment will be modulating this response in the CPG. As yet we have no evidence of the sensory mechanism controlling the way *L. stagnalis* responds to crowding, though it may be that mechanical stimuli play an important role in this stress response. For example, physical contact with the mantle cavity elicits an excitatory response in a mechanosensory neuron in the CNS, right parietal dorsal 3, which then generates action potentials in right pedal dorsal 11 (Inoue et al., 1996b), this in turn then alters activity in RPeD1 (Inoue et al., 1996a), part of the CPG that controls aerial respiration, the presence of which is necessary for the snail to form LTM to reduce aerial respiration (Scheibenstock et al., 2002; Sangha et al., 2004). Further work is required to elucidate the sensory input and neurophysiological response to crowding. Together, these data demonstrate that the response to two environmental stressors that block LTM formation following training to reduce aerial respiration, but not the ability to demonstrate learning, are modulated *via* two distinct sensory pathways, but ultimately result in an identical behavioural phenotype.

ACKNOWLEDGEMENTS

We would like to thank two anonymous reviewers for their insightful comments that helped improve this manuscript. Funding for this work was provided by an Alberta Heritage Foundation for Medical Research fellowship from Alberta Innovates – Health Solutions to S.D.; the Markin Undergraduate Student Research Program (USRP) in Health and Wellness supported V.K. and the Canadian Institutes of Health Research supports K.L. and the laboratory.

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