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RESEARCH ARTICLE

Shell damage leads to enhanced memory formation in *Lymnaea*

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ABSTRACT

Ecologically relevant stressors alter the ability of the pond snail, *Lymnaea stagnalis*, to form long-term memory (LTM). Here, we show that an environmentally relevant stressor, shell damage, has a dramatic effect on the enhancement of LTM formation. Damage in the form of a shell clip 24 h before operant conditioning training resulted in long-term memory (LTM) formation following a single 0.5 h training session (TS). Typically, in these snails, two 0.5 h TSs with a 1 h interval between the sessions are required to cause LTM formation. We show here that even with a 72 h interval between shell clip and training, memory enhancement still occurred. The stress associated with shell clip could be mitigated by an ongoing high-Ca^{2+} pond water environment, an injection of propranolol and a DNA methylation blocker. However, use of an anaesthetic ($MgCl_2$) during the clip or intermittent exposure to the high-Ca^{2+} pond water environment did not mitigate the stress associated with the shell clip. Shell clip was also sufficient to cause juvenile snails, which neither learn nor form memory, to gain the capacity to form LTM. Together, the experiments demonstrate that shell clipping is an environmentally relevant stressor that can cause enhancement of LTM formation.

KEY WORDS: Enhancement of memory formation, Shell clip, *Lymnaea*, Propranolol, Emotional memory

INTRODUCTION

One of the main research thrusts of the Lukowiak lab has been to elucidate how environmentally relevant stressors impact cognition (i.e. learning, memory formation and memory recall; Lukowiak et al., 2003, 2008, 2010, 2014a). Environmentally relevant stressors studied include: predator detection, levels of environmental calcium and other ions in pond water, crowding, thermal stress, suspended nanoparticles and various combinations of those stressors (Tan and Lukowiak, 2018).

The initial stressor studied was predator detection (Orr et al., 2007). In that study predator-experienced snails (e.g. the inbred W-strain) innately responded to crayfish effluent (CE) by increasing aerial respiration, exploratory/searching behaviour and enhanced withdrawal in response to a shadow stimulus. Other behaviours were decreased, including their righting response and basal cutaneous oxygen consumption. It was then shown that if snails were trained in CE, enhanced long-term memory (LTM) formation occurred (Orr and Lukowiak, 2008). However, the enhanced CE-induced memory formation is an activity-dependent process (i.e. operant conditioning training) that only occurs in the presence of CE or within a short period of time (tens of minutes) of CE exposure (Orr and Lukowiak, 2008). The detection of crayfish kairomones (i.e. CE) was mediated by neurons in a sense organ, the osphradium, and involved both the neurotransmitter serotonin and an epigenetic effect (Il-Han et al., 2010; Lukowiak et al., 2014b; Forest et al., 2016). Since then, other stressors have been shown to alter (i.e. enhance or suppress) LTM formation following operant conditioning of aerial respiration (Lukowiak, 2016). Finally, we have shown that emotional memories created with specific combinations of stressors are susceptible to alteration (e.g. blocking of consolidation and reconsolidation) by the drug propranolol (Hughes et al., 2016; Shymansky et al., 2018).

As mentioned above, CE elicits an increase in aerial respiration activity and a decrease in cutaneous respiration. The net effect of this change in respiration is to cause snails to spend more time at the water's surface. This also serves to make it more difficult for the crayfish predator living on the substrate to capture the snail (i.e. surfacing is an anti-predator behaviour). In observing how crayfish capture and devour snails, we were struck by how the crayfish actually does this. The crayfish does not crack open the shell with its large chelipeds (i.e. claws); rather, it uses the first or second smaller pereiopods to chip away the shell at its margin (see Fig. 1A,B,E) and then attempt to extract snails from the shell (see also Alexander and Covich, 1991; DeWitt, 1998). This suggested to us that a damaged shell might also be a stressor that could alter LTM formation. We showed this in a cohort of freshly collected 'wild' snails that are subject to predation (Orr et al., 2009a,b; Hughes et al., 2017) but have not yet determined whether our inbred laboratory snails (i.e. the W-strain) behave in a similar manner (Fig. 1C).

Damage to shells caused by unsuccessful predation is repaired by regeneration (Kunigelis and Saleuddin, 1983). The degree of shell repair is dependent on the extent to which the shell has been damaged and the location of the damage (Wagge, 1951; Wong and Saleuddin, 1972; Kunigelis and Saleuddin, 1983). Injury to the shell initiates a physiological response to repair the damaged area and this repair typically takes place within 1 week (Fig. 1D). Kunigelis and Saleuddin (1983) suggested that sensory receptors in the mantle epithelium under the shell detect and define the extent of shell injury and transmit this information to neurosecretory cells in the central ganglia. This input enables snails to concentrate or localize the process of shell repair to the injured site. We hypothesize that this sensory input to the CNS alters how memory is formed following shell damage. The growth of snails and, if necessary, shell repair following an accident or attempted predation is dependent on the levels of Ca^{2+} in pond water because of their high Ca^{2+} requirements for shell formation (De Schamphelaere et al., 2008).

Ca^{2+} pond water levels are a significant limiting factor affecting the growth and survival of *Lymnaea stagnalis* (Dalesman and Lukowiak, 2010). Further, *L. stagnalis* are unable to satisfy their calcium requirements through their food sources and are thus considered to be calciphiles (Greenaway, 1971). Therefore, Ca^{2+} must be actively (i.e. expenditure of energy) taken up from the pond water in which they reside (Ebanks et al., 2010). We have found that the levels of Ca^{2+} in pond water also alter the ability to learn and form LTM (Dalesman et al., 2011; Knezevic et al., 2011, 2016;

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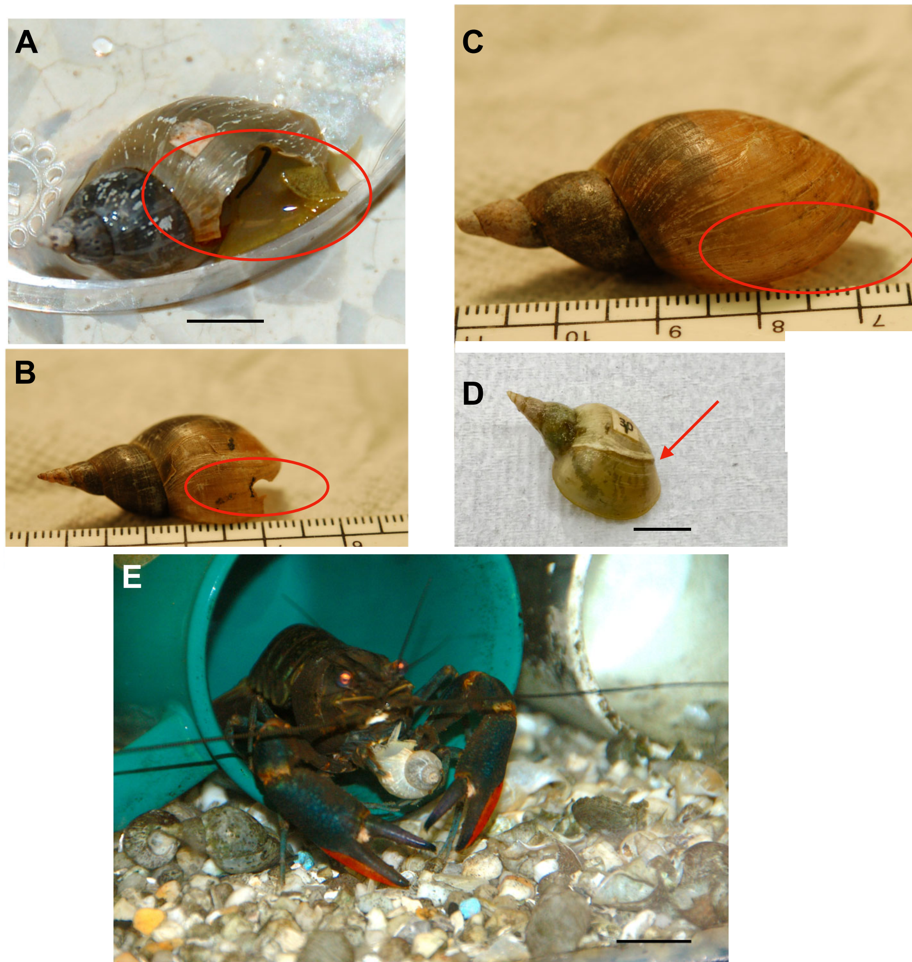

Fig. 1. Damage to *Lymnaea stagnalis* shells. (A) A live W-strain *L. stagnalis* that had been attacked by a crayfish in our laboratory. This snail did not survive. (B) A Whitesand Lake (WSL; ∼250 km east of Saskatoon, SK, Canada) snail shell showing presumed crayfish damage. This shell was found at the side of the pond. (C) A WSL snail whose shell was clipped as described in Materials and Methods. In this snail, the clip was approximately 1.5 cm. (D) A W-strain snail that had its shell clipped 2 weeks previously. The arrow points to the growth line. This snail completely recovered from the clip. (E) A crayfish purchased at a local pet store attacking a W-strain snail. This snail did not survive. Scale bars in A, D and E: 1 cm. A ruler (measurements in cm) can be seen in B and C. Notice that the WSL snails are larger than the W-strain snails raised in the lab.

Knezevic and Lukowiak et al., 2014). In our experience, *L. stagnalis* require at least 50 mg l^{-1} of Ca^{2+} in their environment to flourish, learn and remember. Interestingly, crayfish kairomones only elicit shell thickening when levels of environmental Ca^{2+} exceed 80 mg l^{-1} (Rundle et al., 2004).

Here, we hypothesized that: (1) shell damage (i.e. clipping of the shell) will result in enhanced LTM formation in both adult and juvenile W-strain snails; (2) the stress associated with shell damage is long lasting, possibly due to an epigenetic effect; and (3) the enhancing effects of shell damage can be mitigated by stress-reducing interventions such as propranolol and a high-Ca^{2+} pond water environment.

MATERIALS AND METHODS

Animals

Lymnaea stagnalis (Linnaeus 1758) used in this study are an inbred laboratory strain (i.e. the W-strain) reared in our facility in the Department of Biological Sciences at the University of Calgary. W-strain snails respond innately to crayfish kairomones; thus, they are classified as predator experienced (Orr et al., 2007; Ferrari et al., 2010). However, it has to be noted that the snails used in the experiments reported here have never previously been exposed to a crayfish kairomones. These snails were originally derived from the stocks maintained at the Vrije University in Amsterdam. The ancestors of these snails were obtained from canals in a polder located near the city of Utrecht in the early 1950s. These polders contain crayfish (K.L., personal observation). The snails used here were not exposed to any naturally occurring predator or predator kairomones. Snails were maintained in artificial pond water (deionized water+80 mg l^{-1} $CaSO_4$+0.26 g l^{-1} Instant Ocean) at room temperature (∼20°C), fed romaine lettuce *ad libitum* and experienced a Calgary summer time light:dark cycle of 16 h:8 h.

Aerial respiration

Lymnaea stagnalis are bimodal breathers, which means that they obtain oxygen necessary for life via cutaneous and aerial respiration. In eumoxic conditions (6 ml O_2 l^{-1}), snails mainly obtain oxygen through cutaneous respiration, meaning dissolved pond water oxygen diffuses directly through the skin. However, in hypoxic conditions (<0.1 ml O_2 l^{-1}), snails perform gas exchange with the atmosphere via a lung. In order to perform aerial respiration, a snail comes to the surface and opens a structure called the pneumostome, which is a respiratory orifice that opens and closes via the contraction and relaxation of respiratory muscles to allow gas exchange to occur.

Operant conditioning

Each snail was labelled 24 h prior to the 0.5 h training session (TS). Snails were placed in a 1 litre beaker filled with 500 ml of pond water made hypoxic by bubbling with N2 gas for 20 min prior to the TS (Lukowiak et al., 1996). Animals were allowed to acclimate for 10 min in the beaker prior to the initiation of the TS. During the 0.5 h TS, a tactile stimulus ('poke') was applied to the edge of the pneumostome each time a snail attempted to open it. This results in the closing of the pneumostome without causing the snail to retract

into its shell. The number of pokes was recorded for each snail. This same procedure was performed for the memory test (MT).

In the W-strain of snails, normally two 0.5 h TS with a 1 h interval between sessions is necessary to cause LTM formation. Thus, these snails have been classified as 'average' (Dalesman et al., 2011). However, to test for enhanced LTM formation, only a single 0.5 h TS is used and LTM is tested for 24 h later. We operationally define LTM as being present if the number of attempted openings in the MT is significantly lower than that in the TS (Dalesman and Lukowiak, 2012).

Shell clipping

The shell was damaged at the shell margin. Snails were removed from their home aquarium and, using forceps, a 10×3 mm strip was clipped along the pneumostome side of the snail shell (Fig. 1C). During this procedure, snails withdraw into their shells and squirt out their haemolymph through the renal pore. Thus, this is considered to be a stressful situation. Snails were then returned to their home aquarium. Normal behaviour was observed 1 h later; however, snails were given 24 h to recover and were then trained.

Calcium exposure

The usual Ca^{2+} level in our pond water is ∼60 mg l^{-1}. In the high-Ca^{2+} condition, snails were maintained in pond water with a Ca^{2+} level of 100 mg l^{-1}.

Drug exposure

(±)-Propranolol hydrochloride (TLC) powder was obtained from Sigma-Aldrich (St Louis, MO, USA). The concentration of propranolol was chosen based on studies previously done in the Lukowiak lab, and is consistent with the published literature (Hughes et al., 2016).

Before injection, snails were anaesthetized by placing them in an ice bath for 15 min. Snails were injected into their foot with 0.1 ml of 50 mmol l^{-1} propranolol in *Lymnaea* saline (composition in mmol l^{-1}: 51.3 NaCl, 1.7 KCl, 4.1 $CaCl_2$, 1.5 $MgCl_2$, 5.0 Hepes, pH 7.9). Snails were placed in eumoxic home aquaria for 1 h after injection. Control group snails were anaesthetized according to the same procedure as the drug-treated snails before injection of 0.1 ml of *Lymnaea* saline.

The DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (5-AZA; Sigma-Aldrich) was injected as above at a dose of 87 µmol l^{-1} (see Lukowiak et al., 2014a,b).

For $MgCl_2$ anaesthesia, we injected 2 ml of cold isotonic $MgCl_2$ (50 mmol l^{-1}) into the haemocoel through the foot. All snails recovered from this injection within a 30–40 min period.

Data analysis

For the effect of shell clipping on homeostatic breathing behaviour, a one-way ANOVA was used to determine whether the number of breaths or the total breathing time was different following shell damage. Paired-sample *t*-tests were used to determine whether LTM was present in snails that were trained with a single 0.5 h TS and tested for memory 24 h later. The number of attempted pneumostome openings in the MT had to be significantly lower than that in the TS for LTM to be present. All tests defined $P<0.05$ as significant. Statistics were performed using GraphPad Prism (v.6.00 for Mac OS X, GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

In both our laboratory setting (i.e. crayfish and snails in the same aquarium) and ponds containing both *L. stagnalis* and the northern crayfish (*Orconectes virilis*) in eastern Saskatchewan, Canada, we have found evidence of shell damage (Fig. 1A; for the laboratory setting; Fig. 1B, inferred in the natural setting as a result of crayfish predation of *L. stagnalis*). An example of a shell clip experimentally produced (see Materials and Methods) is shown in Fig. 1C, whilst a picture showing shell repair (2 weeks after the clip) in the laboratory setting (normal pond water) is presented in Fig. 1D. A crayfish (*Cherax quadicarinatus*, purchased at a local pet store) breaking a snail's shell is shown in Fig. 1E. Although not shown here, we have seen evidence of shell repair in freshly collected *L. stagnalis* in both Saskatchewan and the Somerset Levels in the UK (Dalesman et al., 2011).

We hypothesized that following shell clipping, homeostatic aerial respiratory behaviour would be increased. To determine this, we measured both the total breathing time (Fig. 2A) and the number of breaths taken (Fig. 2B) in a 0.5 h session in hypoxic pond water. We measured these two parameters 24 h before and 1 and 24 h after clipping the shell. An ANOVA performed on each of these datasets showed that neither parameter was statistically altered following shell clipping. That is, clipping the shell did not significantly alter the hypoxia-induced increase in aerial respiratory behaviour. Thus, our first hypothesis was negated.

Previously, Hughes et al. (2017) found in freshly collected TC2 *L. stagnalis* (i.e. snails that may have been exposed to a predator) that, following induced shell damage (i.e. shell clipping), LTM formation was enhanced. TC2 snails exhibit the 'average' ability to form memory as do the W-strain snails used here. As an 'average' snail, the W-strain snails used here require two 0.5 h training sessions separated by a 1 h interval in order to form LTM (Orr et al., 2009a,b). In a naive cohort of W-strain *L. stagnalis* (n=20; Fig. 3), we clipped the shell and then 24 h later subjected the snails to a single 0.5 h TS. When memory was tested 24 h later (MT), the number of attempted pneumostome openings was significantly lower than that in the TS; that is, memory was present. Thus, shell damage enhanced LTM formation. We present these data in two ways in Fig. 3 to better show the effect that shell clipping had on LTM formation.

We next asked whether after a delay of 72 h between shell clipping and training, memory enhancement still occurred (Fig. 4). This interval was previously used when we examined the effect of heat shock on memory enhancement in W-strain snails (Teskey et al., 2012; Sunada et al., 2016). We found that with a delay of 72 h between shell clipping and snails receiving a single 0.5 h TS, LTM was observed up to 72 h later. Thus, shell clipping has a long-lasting enhancing effect on LTM formation.

Juvenile W-strain *L. stagnalis* are unable to form LTM, but juveniles of 'smart' snails (i.e. the strains possessing enhanced memory formation) have that capability (McComb et al., 2005; Shymansky et al., 2017). However, enhanced LTM formation occurs in juvenile W-strain *L. stagnalis* when they detect a crayfish predator (Orr et al., 2010; Forest et al., 2016). Thus, we hypothesized that shell clipping in juvenile W-strain snails would allow juveniles to form LTM with a single 0.5 h TS. We clipped the shell of these juvenile snails and then trained them with a single 0.5 h TS 24 h later and found that LTM was present (MT; Fig. 5). Thus, shell clipping enhanced the ability of juvenile W-strain *L. stagnalis* to form LTM.

Because shell clipping was such a powerful enhancer of LTM formation, we hypothesized that it is the nociceptive component of shell clipping that underlies its enhancing effect on LTM formation. To test this, we first performed a control experiment injecting *Lymnaea* saline into the snail (see Materials and Methods) 1 h

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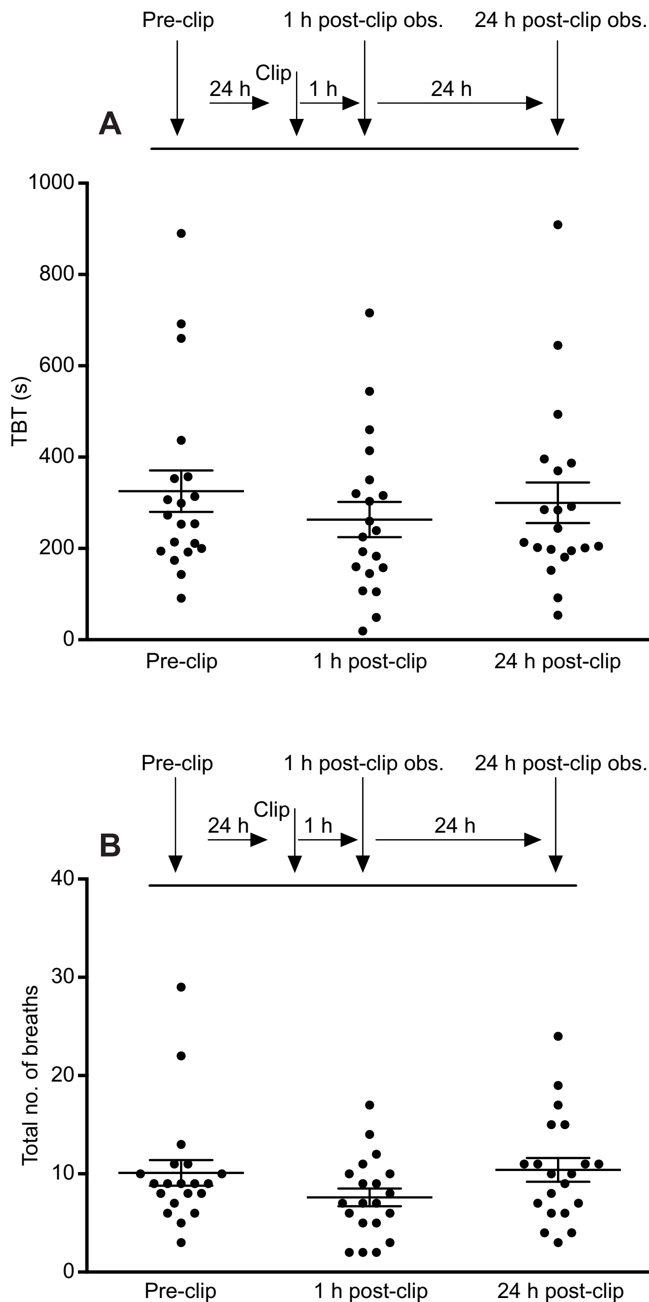

Fig. 2. Homeostatic breathing behaviour and shell clipping. (A) Total breathing time (TBT) was calculated (mean and s.e.m., n=20) over a 0.5 h period in hypoxic pond water in three separate sessions. TBT was first calculated before any shell clipping was performed (Pre-clip). Twenty-four hours later, the shell was clipped and 1 h later TBT was again calculated (1 h post-clip). A second post-clip observation session was then performed 24 h later (24 h post-clip). We then performed an ANOVA on these datasets. This analysis ($F_{19,38}$=1.095, P=0.3926) showed that TBT was not statistically different (i.e. clipping did not alter it) in each of the three sessions. Thus, shell clipping did not alter TBT. (B) As in A, except we counted the total number of breaths taken. Again, as in A, there was no difference in the number of breaths across sessions (ANOVA: $F_{19,38}$=1.213, P=0.2978).

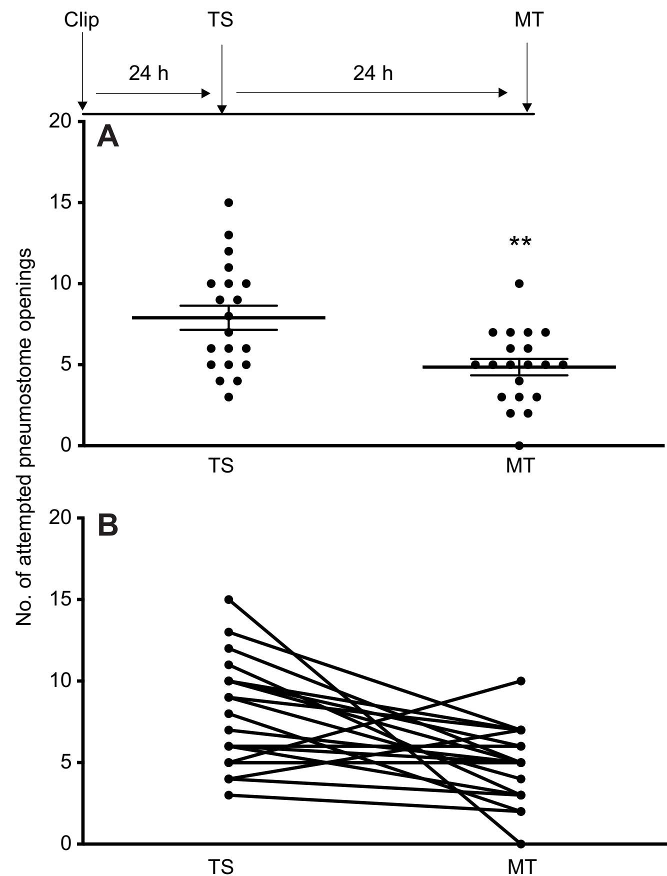

Fig. 3. Shell damage enhances long-term memory (LTM) formation in W-strain snails. (A) Twenty W-strain snails had their shell clipped and then 24 h later they received a single 0.5 h training session (TS). Twenty-four hours after this, they received a memory test (MT). The number of attempted pneumostome openings in the TS and MT is plotted. There were significantly fewer attempted openings in the MT than in the TS (paired t-test; t=3.165, d.f.=19, P=0.0051). Thus, LTM was present and the shell clipping experience enhanced LTM formation. (B) The data in A replotted to show the change that occurred in each snail between TS and MT.

before shell clipping. We then trained these snails (n=11) with a single 0.5 h TS. When we tested for LTM 24 h later (MT) we found that the number of attempted pneumostome openings in the MT was significantly lower than that in the TS, thus LTM formed (Fig. 6A). We concluded that the injection of saline, which possibly is a stressful stimulus, did not alter the enhancing effect that shell clipping has on LTM formation. In a second cohort of naive snails (n=29), we injected isotonic, cold $MgCl_2$ into the snails 10 min before shell clipping. Cold isotonic $MgCl_2$ completely blocks synaptic transmission in *L. stagnalis* (Scheibenstock et al., 2002) and causes the snail to go completely limp. In this state, the snail is totally unresponsive to tactile stimuli and surgical dissection (Sangha et al., 2003a,b; Sunada et al., 2017). In these snails, we damaged the shell (i.e. clipped it) 10 min after the injection of $MgCl_2$ and then trained the snails 24 h later with a single 0.5 h TS; LTM was found to be present 24 h later (MT) (Fig. 6B; t=4.506, d.f.=28, P=0.0001). That is, there was a significant decrease in the number of attempted pneumostome openings in the MT compared with the TS. Thus, clipping the shell when the snail could not sense the insult still caused LTM enhancement.

Viewed through the lens of a stressful stimulus altering memory formation (Lukowiak et al., 2014a; Ito et al., 2015a,b), we hypothesized that if we decreased the 'emotional stress' associated with the shell clipping procedure, then shell clipping would not result in enhancement of LTM formation. We previously found that the synthetic beta-blocker propranolol disrupts consolidation and reconsolidation of emotional memory in *L. stagnalis* (Hughes et al.,

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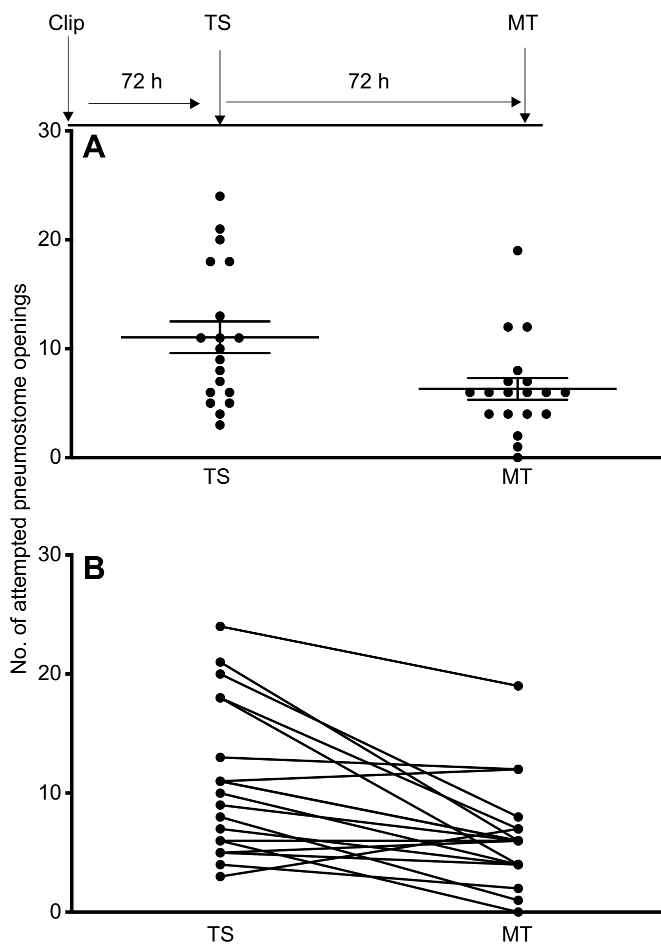

Fig. 4. Shell damage enhances LTM formation in W-strain snails when training occurs 72 h after shell clipping. A cohort of 20 snails had their shells clipped 72 h before training. They then received a single 0.5 h TS. Memory was tested 72 h later (MT). There were significantly fewer attempted pneumostome openings in the MT than in the TS (paired *t*-test; *t*=3.927, d.f.=19, *P*=0.0010). Thus, even with a 72 h interval between shell clipping and training, enhanced LTM formation still occurred. (B) The data in A replotted to show the change that occurred in each snail between the TS and MT.

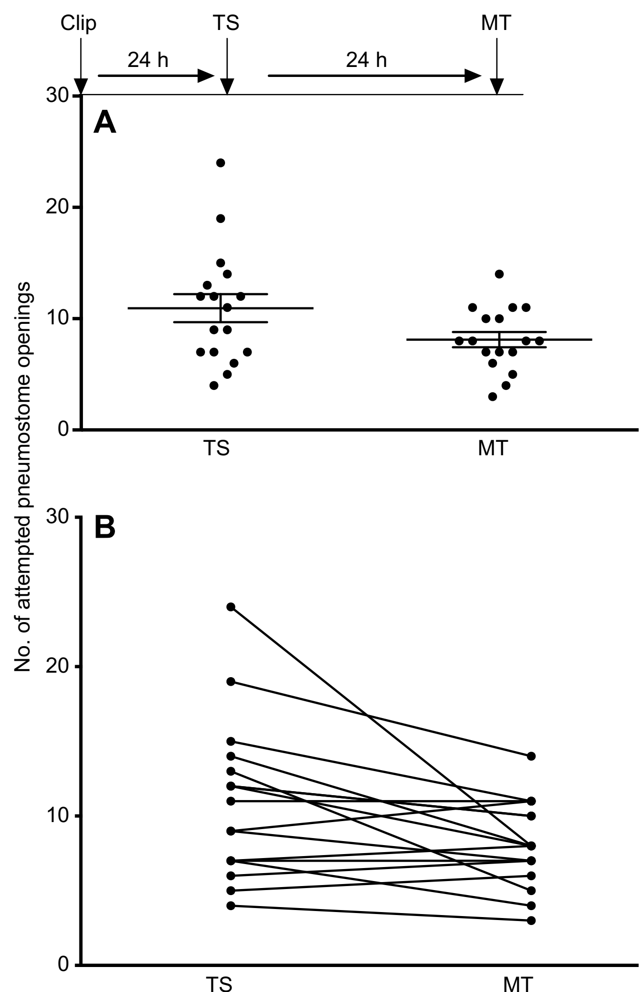

Fig. 5. Shell damage enhances formation of LTM in juvenile W-strain snails. A cohort of 17 juveniles had their shell clipped and then 1 h later were trained with a single 0.5 h TS with a MT 24 h later. There were significantly fewer attempted pneumostome openings in the MT than in the TS (paired *t*-test; *t*=2.660, d.f.=16, *P*=0.0171). Thus, even in these juvenile snails, which typically do not learn and form LTM, shell clipping enhanced LTM formation. (B) The data in A replotted to show the change that occurred in each snail between the TS and MT.

2016; Shymansky et al., 2018). Thus, we injected propranolol (see Materials and Methods) into a naive cohort of snails (*n*=17) and then damaged their shells 1 h later (Fig. 7A). We then trained these snails with a single 0.5 h TS and tested for LTM formation (MT) 24 h later. We found that LTM had not formed; that is, the number of attempted pneumostome openings in the MT was not statistically different from the number in the TS. Thus, propranolol injected before shell clipping obstructs the enhancing effect of the clip. These data are consistent with our hypothesis that decreasing the stress associated with shell clipping would mitigate the enhancing effect of shell clipping on LTM formation.

It is known that the concentration of Ca^{2+} in pond water has significant effects on the growth and viability of *L. stagnalis* (Dalesman and Lukowiak, 2010). Having shown that shell damage is a stressful event that is mitigated by propranolol, and that blockage of nociceptive input was not sufficient to occlude the enhancement of LTM formation caused by shell clipping, we hypothesized that the effects of shell damage on memory formation would be mitigated by high-Ca^{2+} pond water. In a series of experiments, we altered the pond water calcium environment at various times before, during and after shell clipping.

In the first of these experiments, we placed naive snails (*n*=18) in high-Ca^{2+} pond water (see Materials and Methods) 24 h before we clipped the shell. The snails then remained in the high-Ca^{2+} pond water throughout the rest of the experiment; they were trained with a single 0.5 h TS and their memory was tested 24 h later (MT). In this cohort of snails, LTM was not observed (Fig. 7B). That is, the number of attempted pneumostome openings in the TS and MT was not significantly different. Thus, the high-Ca^{2+} pond water environment before, during and after shell clipping, training and memory test sessions blunts the memory-enhancing effect of shell damage.

We placed another naive cohort (*n*=25) of snails in the high-Ca^{2+} pond water environment for 1 h prior to shell clipping (Fig. 8A). Immediately following clipping, they were transferred into normal pond water and trained 24 h later (TS) then tested for LTM after 24 h. We found that LTM was present; that is, shell clipping still had an enhancing effect on LTM formation even if snails experienced the high-Ca^{2+} pond water environment for 1 h right before clipping.

It was possible that snails did not spend enough time in the high-Ca^{2+} pond water environment before shell clipping. Therefore, we

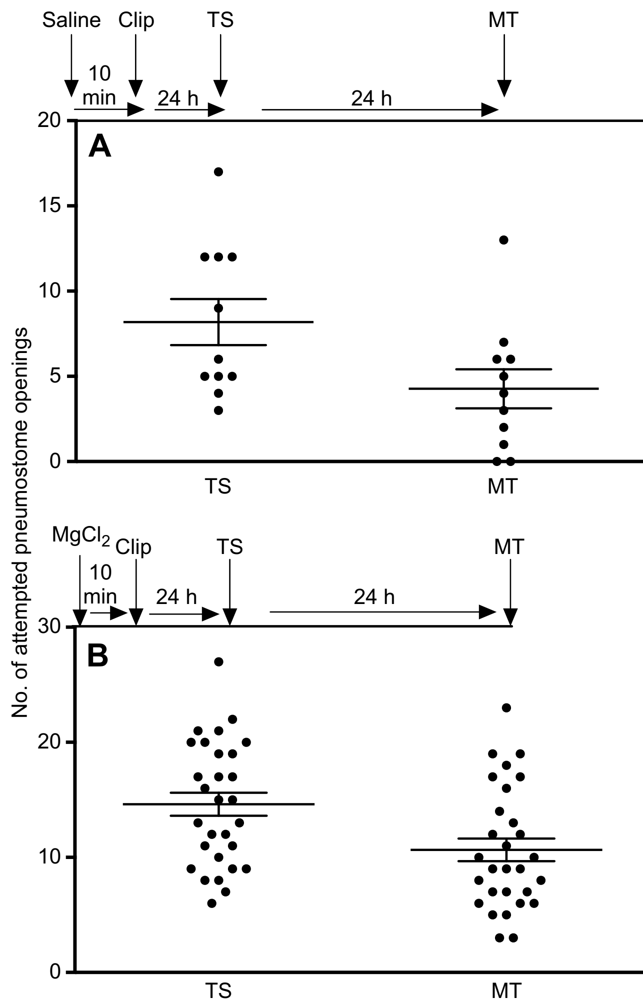

Fig. 6. Effect of $MgCl_2$ injection and shell damage on LTM formation in W-strain snails. (A) Snails (*n*=11) were injected with saline and 10 min later the shell was clipped. Training (TS) commenced 24 h after shell clipping, with memory (MT) tested 24 h later. There were significantly fewer attempted pneumostome openings in the MT than in the TS (paired *t*-test: *t*=2.333, d.f.=10, *P*=0.041), indicating that LTM formed. Thus, saline injection did not interfere with the enhancing effect of shell clipping. (B) $MgCl_2$ was injected into the snails (*n*=29) and then 10 min later the shell was clipped. Training (TS) commenced 24 h after shell clipping, with memory (MT) tested 24 h later. There were significantly fewer attempted pneumostome openings in the MT than in the TS (paired *t*-test: *t*=4.506, d.f.=28, *P*=0.0001), indicating that LTM formed. Thus, $MgCl_2$ injection did not interfere with the enhancing effect of shell clipping.

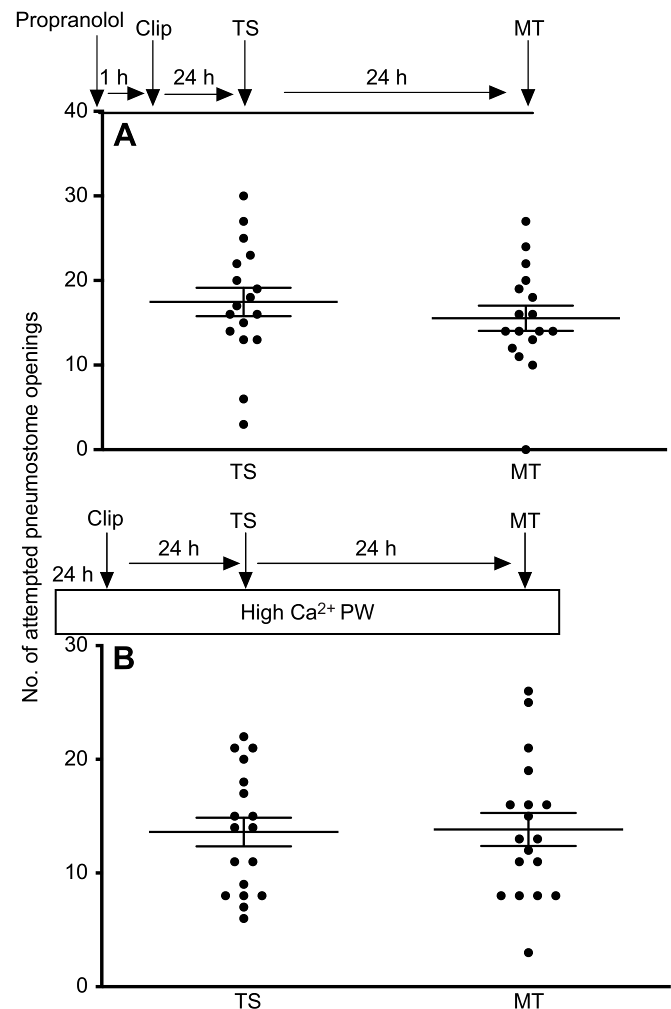

Fig. 7. Propranolol and high-Ca^{2+} pond water mitigate the effect of stress associated with shell damage on LTM formation. (A) Snails (*n*=17) were injected with propranolol 1 h before shell clipping. Twenty-four hours later, the snails were trained (TS), and memory (MT) was tested 24 h later. The number of attempted pneumostome openings in the MT was not statistically lower than that in the TS (paired *t*-test: *t*=1.460, d.f.=16, *P*=0.1637), indicating that clipping in the presence of propranolol did not enhance LTM formation. (B) A high-Ca^{2+} pond water (PW) environment before, during and after shell clipping mitigates the stress associated with shell clipping. Twenty-four hours before shell clipping, snails (*n*=18) were moved to a high-Ca^{2+} pond water environment. The snails then had their shells clipped. Twenty-four hours later they were trained (TS), and memory (MT) was tested 24 h later. The number of attempted pneumostome openings in the MT and TS was not statistically different (paired *t*-test: *t*=0.1173, d.f.=17, *P*=0.9080), indicating that LTM had not formed.

placed a cohort (*n*=17; Fig. 8B) of naive snails in the high-Ca^{2+} pond water environment for 24 h before shell clipping. Following clipping, they were then placed into normal pond water for training and memory testing. In this cohort of snails, memory formation was still observed. That is, the number of attempted openings in the MT was significantly lower than that in the TS. Thus, pre-exposure to the high-Ca^{2+} pond water environment did not occlude the enhancing effect of shell clipping on LTM formation.

In *L. stagnalis*, following operant conditioning training, it is still possible to modify LTM formation by altering the pond water environment immediately after training (i.e. during the consolidation period; see Fernell et al., 2016). We therefore tested whether exposing snails to the high-Ca^{2+} pond water environment immediately after clipping would alter the enhancing effect produced by shell clipping. We found, however (Fig. 9A,B), that neither a 1 h (*n*=10) nor a 24 h (*n*=20) exposure to the high-Ca^{2+} pond water environment just after shell clipping blocked the enhancing effect on LTM formation. Thus, experiencing the high-Ca^{2+} pond water environment only following shell clipping was not sufficient to block the enhancing effect produced by clipping.

Finally, because the enhancing effect of shell clipping on LTM formation persisted for at least 72 h before training (Fig. 4), we investigated whether an epigenetic effect was at play. We hypothesized that similar to the situation with predator detection (Lukowiak et al., 2014a,b; Forest et al., 2016), blocking a DNA methylation process would blunt the enhancing effect of shell clipping. To this end, a cohort (*n*=11) of naive snails was first

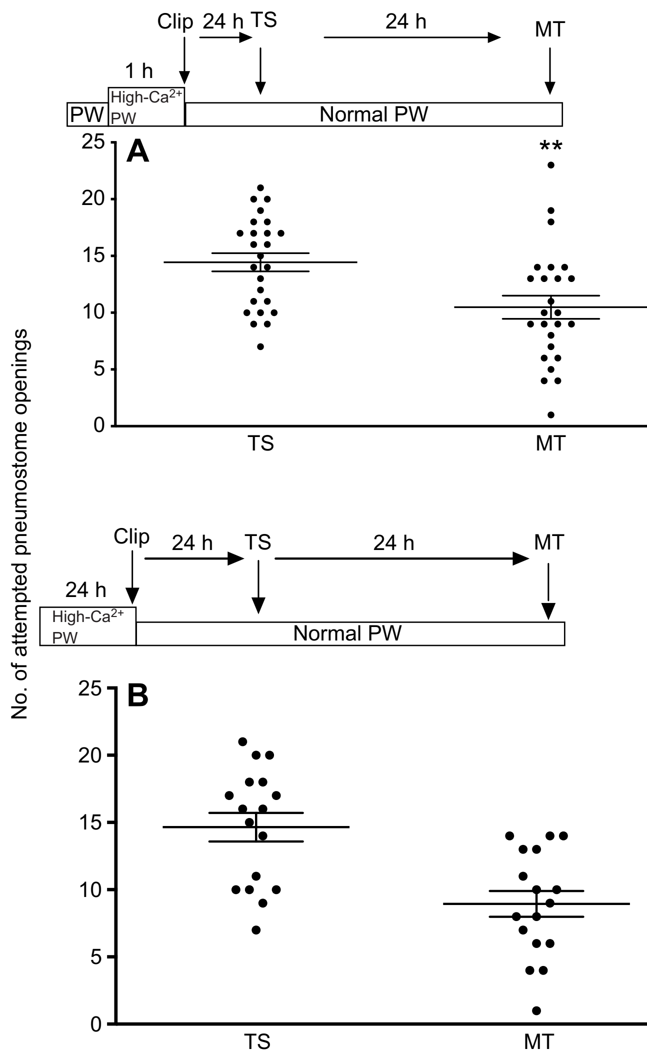

Fig. 8. A high-Ca^{2+} pond water environment before shell clipping does not mitigate the effect of stress associated with shell damage on LTM formation. (A) Snails (n=25) were placed in a high-Ca^{2+} pond water environment for 1 h just prior to shell clipping. Following shell clipping, they were placed back into normal pond water; 24 h later, they were trained (TS) and LTM was tested (MT) 24 h afterwards. The number of attempted pneumostome openings in the MT was significantly lower than that in the TS (paired t-test: t=3.268, d.f.=24, P=0.0033). Thus, LTM was present. **P<0.01. (B) As in A, except snails (n=17) were maintained for 24 h before shell clipping in a high-Ca^{2+} pond water environment. Again, there was a significant difference between the number of attempted pneumostome openings in the MT versus TS (paired t-test: t=5.608, d.f.=16, P<0.0001). Thus, experiencing a high-Ca^{2+} pond water environment just before shell clipping does not alter the memory enhancement caused by shell damage.

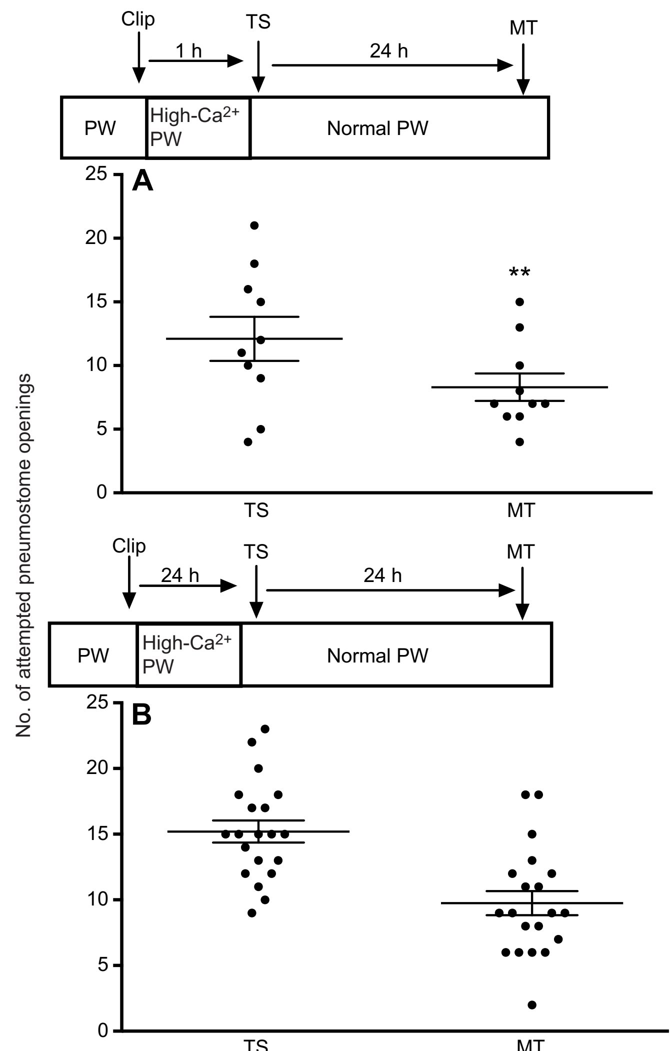

Fig. 9. A high-Ca^{2+} pond water environment just after shell clipping does not mitigate the effect of stress associated with shell damage on LTM formation. (A) Snails (n=10) in normal pond water had their shells clipped. They were then immediately placed in a high-Ca^{2+} pond water environment for 1 h, and subsequently returned to normal pond water. They were then trained (TS) in normal pond water and 24 h later they were tested for LTM (MT). The number of attempted pneumostome openings in the MT was significantly lower than that in the TS (paired t-test: t=2.514, d.f.=9, P=0.0331). Thus, LTM had formed. (B) As in A, except snails (n=20) were maintained for 24 h in the high-Ca^{2+} pond water environment following shell clipping. They were then trained (TS) and had a memory test (MT) 24 h later. The number of attempted pneumostome openings in the MT was significantly lower than that in the TS (paired t-test: t=5.346, d.f.=19, P<0.0001). Thus, LTM had formed.

injected with 5-AZA (see Materials and Methods) and then 1 h later the shells were damaged (Fig. 10). These snails were then trained 24 h later with a single 0.5 h TS. When we tested for LTM we found that there was no significant difference in the number of attempted openings between the MT and TS (paired t-test). Thus, blocking DNA methylation negated the enhancing effect of shell clipping on LTM formation.

DISCUSSION

We show here that: (1) inflicting damage to the shell (i.e. clipping) of W-strain snails enhanced their ability to form LTM; (2) enhancement occurred even with a 3 day interval between clipping and training; (3) as 5-AZA injection before clipping obstructed memory enhancement, an epigenetic effect (i.e. DNA methylation) may play a role in the enhancement process; (4) a continuous high-Ca^{2+} pond water environment (i.e. before, during and after clipping) blocked memory enhancement, presumably because this specific environment mitigates the shell repair costs (i.e. lessens the perception of a stressful stimulus); (5) likewise, utilizing a stress-reduction strategy (propranolol injection) occluded enhancement of LTM formation; (6) anaesthetizing snails with $MgCl_2$ before shell clipping did not alter the enhancement of LTM formation, indicating that snails were aware of their damaged shell after the anaesthetizing effect wore off and during shell repair; and (7) shell clipping is effective in enhancing LTM formation in juvenile W-strain snails.

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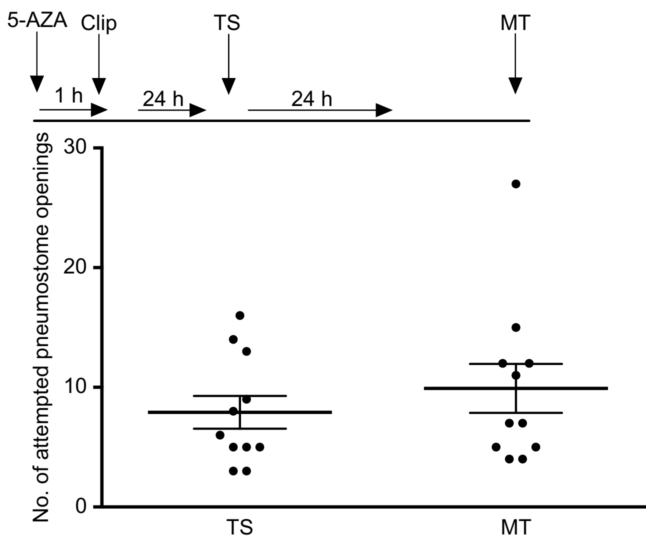

Fig. 10. A DNA methylation process may underlie the enhancing effect on LTM formation of shell damage. 5-AZA, a DNA methylation blocker, was injected into snails (n=11) 1 h before shell clipping. A TS was performed 24 h later, with LTM tested (MT) 24 h after the TS. The number of attempted pneumostome openings in the MT was not significantly lower than that in the TS (paired t-test: t=0.4591, d.f.=10, P=0.4591). Thus, LTM was not formed.

The W-strain snails used here have previously been shown to be predator experienced (i.e. they innately respond to CE) and to initiate anti-predator behaviours, including enhanced LTM formation, when they detect crayfish kairomones (Orr et al., 2007; Orr and Lukowiak, 2008, 2010; Swinton et al., 2019). However, the enhancing effect of CE on LTM formation is transitory, as operant conditioning training must occur within a 0.5 h period after CE is detected or snails must be trained in CE (Orr and Lukowiak, 2008; Swinton et al., 2019). Here, we showed that training up to 3 days following shell clipping still led to memory enhancement. Moreover, the memory formed following shell clipping persisted for at least 72 h. Thus, shell damage has a much more lasting effect on enhancing *L. stagnalis* memory formation than does CE. There are a number of possible explanations for this. First, shell injury elicits a recovery process entailing sensory information sent to CNS neurons from sensory receptors in the mantle area under the damaged shell (Kunigelis and Saleuddin, 1983). This sensory input is maintained until the shell has been repaired, which usually occurs in about a week. We hypothesized that it is this maintained sensory input that underlies the longevity of the enhancing effect of clipping. Second, as shells are highly effective defensive structures, designed (hopefully) to withstand an attack by a predator, such as crayfish, involving shell breakage (Covich, 2010), a damaged shell is a very stressful state that requires resolution. That is, while the shell is undergoing the majority of its repair, the snail continues to experience stress and should have heightened defensive behaviours. Thirdly, insulin has been shown to enhance LTM formation in *L. stagnalis* (Murakami et al., 2013a,b; Hatakeyama et al., 2013; Mita et al., 2014; Totani et al., 2019). It is known that molluscan insulin-related peptides (MIPs) are synthesized by about 150 neurons (the light green cells) in the cerebral ganglia of *L. stagnalis* and these are released both centrally in the CNS and in the periphery to regulate shell growth (Geraerts, 1976). We hypothesize that MIP synthesis and release are necessary for shell repair and thus MIP release will be up-regulated until shell repair is complete. A by-product of MIP up-regulation and release to repair shell damage would be enhanced LTM formation. Future experiments will test this hypothesis.

Somewhat unexpectedly, we found that shell clipping did not alter homeostatic aerial respiratory behaviours 1 and 24 h after the clip. That is, neither total breathing time nor the number of breaths was altered following shell damage. We had expected an increase in these parameters following shell clipping as we anticipated there would be an elevation in respiratory need to support the metabolic demands underlying shell repair. This was not the case.

A snail that is attacked but not killed by a crayfish most likely will exhibit shell damage from its encounter with the predator. We know this from our observations both in the lab and of snails in natural populations that commonly display evidence of shell repair of damage that most likely was caused by a crayfish predator (see Krist, 2002). It makes ecological sense that detection of CE would only cause behavioural changes for a relatively short period, only for as long as the predator is present and 'interacting' with the snail, otherwise the snail would almost certainly be continuously stressed. Damage to the shell from such an encounter should have a longer lasting behavioural effect as it takes days to repair the shell. Snails are in a more vulnerable stressed state with a damaged shell. Here, we did not attempt to use different degrees of shell damage to determine whether the duration of the enhancing effect on LTM formation was related to the degree of damage.

We previously showed that the concentration of Ca^{2+} in pond water has direct effects on learning and subsequent memory formation (Dalesman et al., 2011, 2013; Knezevic et al., 2011, 2016; Swinton et al., 2018). A low Ca^{2+} environment blocked learning and memory, but just a brief (1 h) exposure to a high-Ca^{2+} environment reversed this block (Dalesman et al., 2013). Here, we showed that the high-Ca^{2+} pond water environment throughout the entire experiment had a mitigating effect on memory enhancement caused by shell damage. That is, there was no enhancement of memory formation. It is well known that the environmental Ca^{2+} pond water concentration is of the upmost importance for survival in *L. stagnalis* as it affects habitat, shell growth and repair (Boycott, 1936). *Lymnaea stagnalis* rely on calcium in pond water for growth of their shell and are therefore highly dependent on the concentration of calcium in pond water for survival. Environmental Ca^{2+} availability also alters important *L. stagnalis* behaviours. For example, the speed of locomotion was significantly reduced in snails exposed to low environmental Ca^{2+} (20 mg l^{-1}) compared with that in snails exposed to a higher (80 mg l^{-1}) Ca^{2+} environment (Dalesman and Lukowiak, 2010). This is thought to be due to increased metabolic demands on *L. stagnalis*, as more energy is required for Ca^{2+} uptake from pond water. Further, in a low-Ca^{2+} environment there was a significant increase in oxygen consumption, consistent with the increased metabolic demands of Ca^{2+} uptake (Dalesman and Lukowiak, 2010). As shown by Rundle et al. (2004), the continued presence of crayfish induced significant shell thickening (i.e. an inducible morphological defence) but only when pond water Ca^{2+} was greater than 90 mg l^{-1-1}. Here, when snails were in 100 mg l^{-1} Ca^{2+} pond water before, during and after shell clipping, LTM enhancement was not observed. We inferred from this finding, based on the Yerkes–Dotson/Hebb law (YD/H curve; Hebb, 1955; Ito et al., 2015a,b), that snails in a high-Ca^{2+} pond water environment are not as stressed as when they are in a normal or low-Ca^{2+} pond water environment. That is, if the environment they inhabit when the shell is clipped and during recovery is perceived by the snail to be beneficial to shell repair, then stress associated with clipping is reduced. If snails are not stressed, enhancement of LTM formation does not occur. That is, it is not the stimulus itself that is the stressor; rather, it is how the organism

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perceives the stimulus that determines whether the animal becomes stressed (Kim and Diamond, 2002). We have not as yet determined whether shell healing is faster in the high-Ca^{2+} environment, but as shown in the Rundle et al. (2004) study, the inducible shell morphological changes only occur at higher levels of Ca^{2+}. In future experiments, we will determine whether healing occurs at a faster rate in high-Ca^{2+} pond water.

We attempted to determine whether it was sufficient to experience a high-Ca^{2+} pond water environment just before or just after shell clipping, to mitigate the stress associated with shell damage. These experiments mirrored those performed to test how low-Ca^{2+} pond water altered LTM formation, where it was shown that even a brief exposure to modified Ca^{2+} environments affected memory formation (Dalesman and Lukowiak, 2012; Dalesman et al., 2013; Knezevic et al., 2011, 2016). In those experiments, decreasing the Ca^{2+} pond water concentration just prior to or just after operant conditioning training (i.e. the consolidation period) was sufficient to alter LTM formation. However, unlike that situation, we found that snails had to continuously experience the high-Ca^{2+} pond water concentration throughout the entire process of shell clipping, training and testing for LTM formation in order to occlude the memory-enhancing effects of shell clipping. If the snails were not in the continuous high-Ca^{2+} pond water environment, LTM formation was still enhanced. As snails use osphradial input from sensory neurons located in the mantle, we suspect that this input, signalling the high-Ca^{2+} concentration, altered neuronal activity necessary for shell repair. At lower Ca^{2+} pond water levels, this signal is perceived as stressful until shell repair is complete.

As alluded to above, when investigating how shell clipping in the high-Ca^{2+} pond water environment did not result in enhancement of LTM formation because in the high-Ca^{2+} pond water environment there was less perceived stress, we showed that an injection of propranolol, but not saline, before shell clipping also blocked LTM enhancement. Our interpretation of these data is that propranolol decreased the stress arising at the time of shell clipping. Thus, the level of stress experienced by the snail (i.e. the YD/H curve) did not cross the stress threshold necessary to cause LTM enhancement. Previously, we have shown that propranolol applied at the correct time can disrupt the consolidation or reconsolidation of memory, but only if it is acting on an emotional memory (Hughes et al., 2016; Shymansky et al., 2018). As the shell is so important to the snail (i.e. necessary for life), we propose that inflicted damage to the shell is perceived by the snail as an emotional/traumatic experience. Thus, we postulate that both propranolol and the high-Ca^{2+} environment reduced the stress/emotional level elicited by shell clipping. The YD/H curve demonstrates how the perception of a stressor by the organism alters memory formation. That is, as the organism's perception of stress changes, the ability to form memory changes (Kim and Diamond, 2002).

The data from our $MgCl_2$ injection experiment might at first appear to be inconsistent with the high-Ca^{2+} pond water and propranolol injection shell clipping data. Our $MgCl_2$ injection data showed that anaesthetizing the snail during the clipping procedure did not obstruct the clipping-induced LTM enhancement. The $MgCl_2$ injection blocks synaptic communication throughout the snail and this procedure has been used as an anaesthetic for molluscs, such as *Aplysia*, since the 1920s (Bethe, 1930). In *L. stagnalis*, we have successfully used it in soma-ablation experiments (Scheibenstock et al., 2002; Sangha et al., 2003a,b, 2005). The effects of $MgCl_2$ are transitory, lasting up to 1 h (Burton et al., 1987; Scheibenstock et al., 2002). During this 'effective time', all chemical synaptic transmission in the CNS is blocked. Thus, the snail will not receive sensory information resulting from the clipping procedure. However, when the effect of the injection wears off, as evidenced by the snail moving around, the snail would then receive sensory information from the mantle area 'informing' the snail that the shell requires repair. We hypothesized that it is this on-going sensory information, possibly altering MIP levels, from the mantle area under the damaged shell, to CNS neurons, that is sufficient to cause enhancement of LTM formation. The sensory information causing shell repair is sufficient to push the snail along the YD/H curve above the stress threshold required to cause LTM enhancement. Consequently, it is not sensing the clipping procedure per se that caused enhancement of LTM formation, but rather the sensory input from the mantle area that is necessary for shell repair that is causal for memory enhancement. Presumably, both the propranolol injection and experiencing the high-Ca^{2+} pond water environment mitigated the effects of the stress associated with shell repair.

This study also further extends previous findings relating to a DNA methylation effect on LTM enhancement. Here, we showed that LTM enhancement by shell clipping is blocked by the DNA methyltransferase inhibitor 5-AZA. That is, if 5-AZA is injected prior to clipping and subsequent operant conditioning training, enhancement of LTM is not observed. These findings are consistent with previous findings concerning DNA methylation-dependent enhancement of LTM formation brought about by both CE and a thermal stressor (Lukowiak et al., 2014b; Sunada et al., 2016; Rothwell and Lukowiak, 2017). Our working hypothesis is that the shell damage stressor enhanced LTM formation via DNA methylation changes, possibly mediated by MIPs. We have previously shown that there is at least a 24 h period in which 5-AZA is capable of blocking DNA methylation (Rothwell and Lukowiak, 2017). Accordingly, we hypothesized that the epigenetic changes possibly underling LTM enhancement were brought about by the continued sensory input from the mantle area under the clipped shell. We did not attempt to determine whether an injection of 5-AZA at some later point following shell clipping would block enhancement of LTM formation. These experiments will be undertaken in the future. It is possible that the propranolol injection experiments before shell clipping that occluded enhancement of LTM formation do so by blocking a DNA methylation event initiated by the traumatic shell clip stimulus.

Juvenile W-strain *L. stagnalis* do not have the capability of forming LTM following operant conditioning of aerial respiratory behaviour (McComb et al., 2005; Forest et al., 2016). Previously, we showed that juvenile W-strain snails trained in CE gained the capacity to form LTM (Orr et al., 2010; Sunada et al., 2010; Forest et al., 2016). Based on those data it was concluded that training juvenile *L. stagnalis* in CE altered their nervous system such that associative learning and LTM formation occurred. Here, we showed a similar enhancement in juveniles following shell clipping. Interestingly, Forest et al. (2016) found that when juveniles trained in CE matured into adults some 4 weeks later, they exhibited the 'smart' phenotype. We have not yet performed such experiments following shell clipping. From an evolutionary perspective, it follows that an altered central state, such as an enhanced ability to form memory after a predator encounter, is preserved across the organism's lifetime. It should be noted that in our laboratory we have not observed crayfish, even small ones, attempting to devour a recently hatched or juvenile snail. Thus, it may be an infrequent occurrence that an average juvenile W-strain snail is turned into a smart snail as a result of shell damage due to a predatory encounter.

Finally, these studies add further to the notion of emotional memory (e.g. Shymansky et al., 2018) in invertebrate species. This is a topic that can easily generate debate. However, as Darwin (1872) pointed out, invertebrates possess emotion. More recently, many authors (e.g. Damasio, 2010; LeDoux, 2012) writing about emotion in mammals, state that emotion can be present in simple organisms (i.e. invertebrates). This is not necessarily to say that snails, etc., have 'feelings'. However, several studies (e.g. Fossat et al., 2014; Perry et al., 2016; Baciadonna and Perry, 2017) suggest that invertebrates do in fact exhibit not only negative affect, but also positive emotion-like states. We suggest here that shell clipping elicits emotion (e.g. fear) in the snail, which, as we have shown here, significantly alters LTM formation.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: K.L.; Validation: K.L.; Formal analysis: K.L.; Investigation: E.S., C.S., K.L.; Data curation: E.S., C.S.; Writing - original draft: E.S.; Writing - review & editing: K.L.; Supervision: K.L.; Funding acquisition: K.L.

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