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RESEARCH ARTICLE

Strain-specific differences of the effects of stress on memory in *Lymnaea*

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ABSTRACT

Stress alters the ability to form, recall and maintain memory according to the Yerkes–Dodson/Hebb (YDH) law. The effects of environmentally relevant stressors, such as low environmental calcium and crowding, on learning and memory have previously been described in a laboratory-reared 'average' strain of *Lymnaea stagnalis* (i.e. the Dutch strain) as well as two strains of freshly collected *L. stagnalis* with enhanced memory formation abilities (i.e. 'smart' snails). Here, we use *L. stagnalis* to study the effects of other environmentally relevant stressors on memory formation in two other strains of freshly collected snails, one 'smart' and one 'average'. The stressors we examined are thermal, resource restriction combined with food odour, predator detection and, for the first time, tissue injury (shell damage). We show that the same stressor has significantly different effects on memory formation depending on whether snails are 'smart' or 'average'. Specifically, our data suggest that a stressor or a combination of stressors act to enhance memory in 'average' snails but obstruct memory formation in 'smart' snails. These results are consistent with the YDH law and our hypothesis that 'smart' snails are more easily stressed than 'average' snails.

KEY WORDS: *Lymnaea*, Learning and memory, Smart, Average, Strain-specific learning abilities, Environmentally relevant stressors

INTRODUCTION

An animal's ability to learn and remember throughout its lifetime enables it to effectively respond to a changing environment, improving fitness. Environmental stressors have a profound, yet sometimes unpredictable, modulatory effect on learning and memory formation such that depending on the nature of the stressor and when it is encountered relative to a period of learning, it may block or enhance learning and memory formation (Lukowiak et al., 2014a,b). In its natural habitat, the pond snail, *Lymnaea stagnalis*, encounters many stressors in its lifetime that have previously been shown to alter learning and memory (Lukowiak et al., 2014a). As a species that lives in shallow, often stagnant bodies of water, *L. stagnalis* encounter broad fluctuations in temperature, depending on prevailing weather conditions (Teskey et al., 2012). They also experience changes in threats of predation, and sub-optimal conditions such as resource restriction, including lack of food availability. A snail may also experience tissue injury from encounters with predators, resulting in adaptations to promote repair and prevent future injury (Alexander and Covich, 1991; Crowl and Covich, 1990; Sih et al., 1998; Dalesman et al., 2006). Populations within a species may vary greatly in their responses to environmental stress, reflecting adaptation to local conditions. Because stress affects cognition, this variation in response between geographically distinct strains may result in differential learning capacity and ability to form memory in response to the same stressor.

Aerial respiratory behaviour in *L. stagnalis* can be operantly conditioned such that the animal learns not to perform the behaviour. This operant conditioning procedure has been used extensively in studies of learning and memory since 1996 (Lukowiak et al., 1996). In the inbred laboratory-reared strain (referred to as the Dutch strain), which can be considered the worldwide standard, two 0.5 h training sessions with a 1 h interval between the training sessions are necessary to form a long-term memory (LTM) that persists for at least 24 h. A single 0.5 h training session is sufficient to form intermediate-term memory (ITM) that persists for 1–3 h, but not LTM (Lukowiak et al., 2000). We term this learning and memory forming ability 'average'. We have since encountered freshly collected *L. stagnalis* that exhibit enhanced memory-forming capabilities and have termed these snails as 'smart' (Orr et al., 2009a,b; Dalesman et al., 2011c). In 'smart' snails, a single 0.5 h training session is sufficient to result in LTM persisting at least 24 h.

The Dutch strain was originally collected from a polder near Utrecht, the Netherlands, in the 1950s, and has been laboratory-reared since then. This strain was brought to the University of Calgary in the late 1980s. Most of the work on *L. stagnalis* has been carried out on individuals reared from the Dutch strain. However, *L. stagnalis* present a unique opportunity in studies of learning and memory because strains of the same species from different geographic locations vary in cognitive ability. The majority of wild strains of *L. stagnalis* we have sampled exhibit learning and memory-forming capabilities identical to those of the Dutch strain. However, some populations of snails, freshly collected from certain ponds, are able to form a memory with only a single 0.5 h training session that persists for at least 24 h (i.e. 'smart' snails; Dalesman et al., 2011a,b,c). There is a growing body of evidence suggesting that populations of *L. stagnalis* differ in their LTM-forming capabilities as well as their responses to environmental stimuli (Dalesman and Lukowiak, 2012). Cognitive ability, as well as responses to environmental stress, are conserved in the wild as well as in successive generations reared in the laboratory, indicating a genetic or epigenetic basis to these abilities and responses (Orr et al., 2009a,b; Dalesman et al., 2011c).

Our current, yet previously unsubstantiated, working hypothesis is that 'smart' snails are more easily stressed than 'average' snails (Lukowiak et al., 2014a). According to the Yerkes–Dodson/Hebb (YDH) law (Ito et al., 2015b), a good level of stress is a level the individual can cope with, but is sufficient to keep the individual's

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List of symbols and abbreviations

5-HT	serotonin
CE	crayfish effluent
HSP	heat shock protein
ITM	immediate-term memory
LTM	long-term memory
MT	memory test
TC2	Trans Canada 2
TS1/TS2	training session 1/2
WBWR	whole-body withdrawal response
WSL	Whitesand Lake
YDH	Yerkes–Dodson/Hebb (law)

attention. Good stress encourages memory formation (Sandi and Pinelo-Nava, 2007). At high levels of stress, the individual finds it difficult to cope with the stressor and maintain homeostasis, resulting in poor memory (Shors, 2006; Lukowiak et al., 2014a; Ito et al., 2015b). Essentially, too much stress overwhelms the individual (i.e. they can no longer 'cope'), and they are unable to learn or remember. If 'smart' snails are more easily stressed than 'average' snails, it therefore seems reasonable to hypothesize that a particular stressor or combination of stressors in 'smart' versus 'average' snails could have markedly different effects on the ability to form memory (Mery, 2006, 2013).

Here, we selected two geographically distinct populations of 'wild' snails, one 'average' and one 'smart,' and compared their responses to the same stressors. Between the 'average' and 'smart' populations, we assessed and compared learning and memory responses to training in the context of the same environmentally relevant stressors. Guided by previous work, we studied the effects of thermal stress, resource restriction (lack of food availability) and detection of predators. Then, for the first time, we investigated the effect of tissue injury (shell damage) as an environmentally relevant stressor.

MATERIALS AND METHODS

Animal collection and maintenance

Lymnaea stagnalis snails were freshly collected from ponds in Alberta and Saskatchewan. The snails were kept in the laboratory at 20–22°C with an approximate light and dark cycle of 16 h:8 h (i.e. summer hours). Aerated artificial pond water was used (0.26 g l^{-1} Instant Ocean, Spectrum Brands, USA) with our standard calcium conditions (80 mg l^{-1} [Ca^{2+}]) (Dalesman and Lukowiak, 2010; Dalesman et al., 2011a,b,c). Romaine lettuce was provided *ad libitum*.

Snails from two different populations were used. One of the populations, Trans Canada 2 (TC2), which contains 'average' snails, has been described elsewhere (Braun et al., 2012; Lukowiak et al., 2014a). This pond is approximately 50 km west of Calgary, parallel to the Trans Canada Highway (51°05′26.4″N, 114°32′15.8″ W). We chose this population over the Dutch strain to mitigate any potential effect of 'freshly collecting' on learning and memory. The second, 'smart' cohort of snails was collected from Whitesand Lake (WSL; 51°46′12.45″N, 103°21′14.16″W), approximately 250 km east of Saskatoon, Saskatchewan. Thus, the TC2 ('average') and WSL ('smart') populations are separated by approximately 900 km. There has likely been no recent genetic interaction between them.

Operant conditioning

The standard operant conditioning procedure requires two 0.5 h training sessions spaced 1 h apart to form a 24 h LTM in 'average' adult snails (Lukowiak et al., 2000). We define LTM as significantly fewer attempted pneumostome openings during the second training session (TS2) and the 24-h memory test (MT) compared with the first training session (TS1). The number of attempted pneumostome openings in the MT cannot be significantly greater than the number in TS2. In 'smart' snails, however, a single 0.5 h training session results in LTM 24 h later. In 'smart' snails, we define LTM as significantly fewer attempted pneumostome openings during the 24 h MT.

During a typical training session, snails are carefully transferred from their home aquarium into a 1 litre beaker containing 500 ml of hypoxic pond water at room temperature (~20°C). The hypoxic water is made by bubbling N_2 gas through the water for 20 min before the transferring of snails. Snails are then given a 10-min acclimatization period before a 0.5 h training session. A training session consists of applying a tactile stimulus with a sharpened wooden applicator to the pneumostome as it begins to open (i.e. an attempted pneumostome opening). The stimulus is gentle enough that the snails do not perform a full body withdrawal response but strong enough to cause the snails to close their pneumostome. Snails are returned to their home aquarium following the training session and may receive a second training session, identical to the first, 1 h after returning to their home aquarium. The snails are tested for LTM 24 h after their last training session using another 30-min training session in hypoxia with the procedure described above. This session is termed the MT.

Stressors

Exposure to stressors before and/or during training can alter memory in *L. stagnalis* (Lukowiak et al., 2014a). Individually, or in combination, stressors can obstruct or enhance memory formation. Enhancement of memory has occurred if, when the stressor is applied, 'average' snails are able to form LTM following a single 0.5 h training session. Without application of stressors, 'average' snails require two 0.5 h training sessions, spaced 1 h apart, to form LTM that persists for at least 24 h. Memory has been obstructed in 'smart' snails if, when the stressor(s) has been applied, snails are unable to form LTM following a single 0.5 h training session. Without stressors, 'smart' snails form LTM that persists at last 24 h with one training session. The following stressors were used in this study: thermal, resource restriction, carrot odour, predator detection, KCl bath and tissue injury.

Thermal

Lymnaea stagnalis are exposed to a broad temperature range depending on the weather conditions. For example, snails may experience temperatures close to freezing during an autumn night and temperatures close to 30°C during a warm summer day (Brown, 1979; K.L., personal observations). Thus, in *L. stagnalis*, temperature acts as an environmentally relevant stressor that affects memory formation (Teskey et al., 2012; Foster et al., 2015; Sunada et al., 2016).

Here we exposed snails to 30°C for 1 h in a heated tank of pond water. This tank acted as a water bath for a 1 litre beaker filled with 500 ml of pond water in which the snails were placed. After 1 h at 30°C, the snails were trained using the standard operant conditioning procedure, in room-temperature pond water (~20°C). This procedure is consistent with previous thermal stress protocols used in operant conditioning of *L. stagnalis* (Foster et al., 2015; Sunada et al., 2016).

Resource restriction

For *L. stagnalis*, food deprivation can act as an environmentally relevant stressor as restrictions of food can lead to stunting of growth and reproduction (Ito et al., 2015b). 'Average' snails that have been food-deprived for 5 days form LTM normally (Haney and

Journal of Experimental Biology

Journal of Experimental Biology (2017) 220, 891-899 doi:10.1242/jeb.149161

Lukowiak, 2001). However, what food deprivation does to a 'smart' snail is unknown. We predicted here that for freshly collected 'average' and 'smart' snails, food deprivation would not alter their respective abilities to learn and form memory. To minimize suffering of the animals, and guided by pilot data in our laboratory, snails were food-deprived for 3 days instead of 5 days.

Carrot odour experiments

We predicted here that for freshly collected 'average' and 'smart' snails, food deprivation plus the smell of carrot before training would act as a stressor. It is possible to expose snails to carrot odour without feeding them carrot. This was done through an apparatus that bubbles eumoxic air through blended carrots placed in a sealed flask, while simultaneously diverting the carrot-scented air from the sealed flask into a beaker containing pond water and the snails. Snails were exposed to 0.5 h of carrot scent immediately before training in hypoxic pond water. They were not trained in the presence of the carrot odour; training in carrot odour produces a context-specific memory (Haney and Lukowiak, 2001). That is, a memory formed in the presence of carrot odour can only be recalled in the presence of carrot odour. We chose our modified protocol to be consistent with the other stressors used in this study, none of which, when applied, result in context-specific memories.

Predator detection

Crayfish are a natural predator of *L. stagnalis*. In our laboratory, crayfish *Orconectes virilis* were housed in a 70 litre aquarium and fed a diet of snails and lettuce. We call the water in the tank crayfish effluent (CE) (Orr et al., 2007). Training snails in hypoxic CE as opposed to pond water causes an enhancement of LTM formation in 'average' snails (Orr and Lukowiak, 2008; Orr et al., 2010; Sunada et al., 2010; Lukowiak et al., 2014a,b). Here, we tested the effect of CE on memory formation in 'smart' snails obtained from WSL. Training in CE does not block memory formation in 'smart' snails obtained from Chilton Moor (51.19°N, 2.88°W), a drainage ditch located in the Somerset Levels, UK (Dalesman et al., 2011a,b,c). To our knowledge, the effect of CE on memory formation has never before been determined in a 'smart' population of snails obtained from a North American pond known to be naturally inhabited by crayfish.

CE is an environmentally relevant stressor only for snails that come from a pond inhabited by crayfish, such as WSL 'smart' snails. TC2 'average' snails do not respond to CE as crayfish are not present in Alberta (Orr et al., 2009a,b). CE works through the serotonin (5-HT) predator detection pathway; thus, even though TC2 snails do not respond to CE, they still have natural predators that are possibly detected through the 5-HT pathway (Il-Han et al., 2010). Thus, we gave the TC2 snails an injection of 0.1 ml of 10.63 µg ml^{-1} 5-HT 1 h before training as a substitute for CE.

KCl bath

KCl exposure is noxious to *L. stagnalis*, eliciting the whole-body withdrawal response (WBWR). A 30 s exposure to 25 mmol l^{-1} KCl immediately before a training session in pond water causes significant enhancement of memory formation in 'average' snails (Martens et al., 2007). Here, for the first time, we tested the effect of KCl on memory formation in 'smart' snails. We also tested the combined effect of CE and KCl on memory formation.

Tissue injury

We also determined whether direct damage to the snail's shell had any effect on learning and memory. 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. During this procedure, snails withdraw into their shells and squirt out their hemolymph through the renal pore (i.e. the WBWR). Thus, this is considered to be a stressful situation. Snails are then returned to their home aquarium. Normal behaviour is observed 1 h later; however, snails are given 24 h to recover and are then trained.

Data analysis

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 openings in the MT had to be significantly less than in TS1 for LTM to be present. A one-way ANOVA was used to determine whether LTM was present in snails that received two 0.5 h training sessions (TS1 and TS2) with a 1 h interval between sessions and tested for memory 24 h after TS2. If a significant difference was found, *post hoc* paired *t*-tests with Bonferroni corrections were run to compare TS versus MT to determine which group learned and formed memory. All tests defined $P<0.05$ as significant. Statistics were performed using GraphPad Prism (v. 6.00e for Mac OS X, GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

Memory retention at 24 h in control conditions

Although operant conditioning data have been published using TC2 strain (i.e. 'average') snails (Braun et al., 2012), we decided to repeat those experiments and test a new strain of snails, WSL, under control conditions.

A separate cohort of snails from each pond (TC2 and WSL) received a 0.5 h training session under control conditions. At the 24 h test for LTM, WSL snails showed significantly fewer attempted pneumostome openings than in the training session (Fig. 1A; $t=3.889$, $P=0.0019$, $N=14$). Thus, they can be termed 'smart'. The number of attempted pneumostome openings in the MT in the TC2 snail cohort was not significantly different from in the training session (Fig. 1B; $t=0.8321$, $P=0.4269$, $N=10$). Thus, the TC2 snails did not meet criterion for being termed 'smart'.

A second cohort of TC2 snails then received two 0.5 h training sessions under control conditions, spaced 1 h apart, followed by a 24 h test for LTM. The number of attempted pneumostome openings in TS2 was significantly less than in TS1, and the number of attempted pneumostome openings in the 24 h MT was significantly less than in TS1 and not significantly different from TS2 (Fig. 1C; $F_{11,22}=4.447$, $P=0.0014$, $N=12$). These data are consistent with previous results indicating that TC2 snails are 'average'.

Thermal stress

In previous studies, it was shown that a 1 h exposure to 30°C pond water 1 h before training results in enhancement of memory in 'average' Dutch snails (Teskey et al., 2012; Sunada et al., 2016). Thus, a single 0.5 h training session is now sufficient for LTM memory formation. Here, we repeated this experiment on 'average' freshly collected snails and observed similar results (Fig. 2A; $t=4.833$, $P<0.001$, $N=23$). We next used an identical protocol on a group of 'smart' snails. The snails did not show memory at the 24 h MT (Fig. 2B; $t=0.8275$, $P=0.4272$, $N=11$). Thus, while thermal stress before training enhanced memory in the 'average' snails, it obstructed LTM formation in 'smart' snails.

Resource restriction

Memory formation is metabolically expensive; thus, we previously hypothesized that food-depriving 'average' snails may block LTM

Journal of Experimental Biology (2017) 220, 891-899 doi:10.1242/jeb.149161

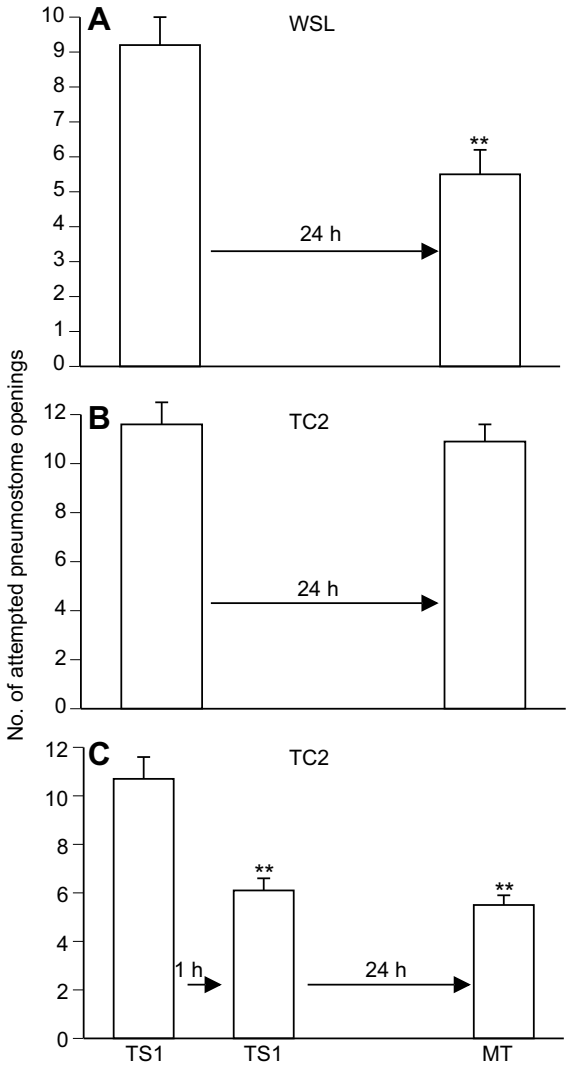

Fig. 1. 'Smart' and 'average' snails and memory formation. Whitesand Lake (WSL) adult snails (*Lymnaea stagnalis*) are classified as 'smart' and Trans Canada 2 (TC2) adults as 'average'. (A) WSL adult snails received a single 0.5 h training session (TS1) and memory was tested 24 h later (MT). In these snails, MT was significantly less than in TS1, showing that LTM was present. These snails therefore met the criterion of a 'smart' snail. (B) As in A, except snails from another pond (TC2) were used. In these snails, LTM was not formed. These snails were classified as 'average'. (C) TC2 snails are capable of forming LTM if they receive two 0.5 h training sessions (TS1 and TS2) with a 1 h interval. LTM was tested (MT) 24 h later. Data are means+s.e.m. $^{**}P<0.01$.

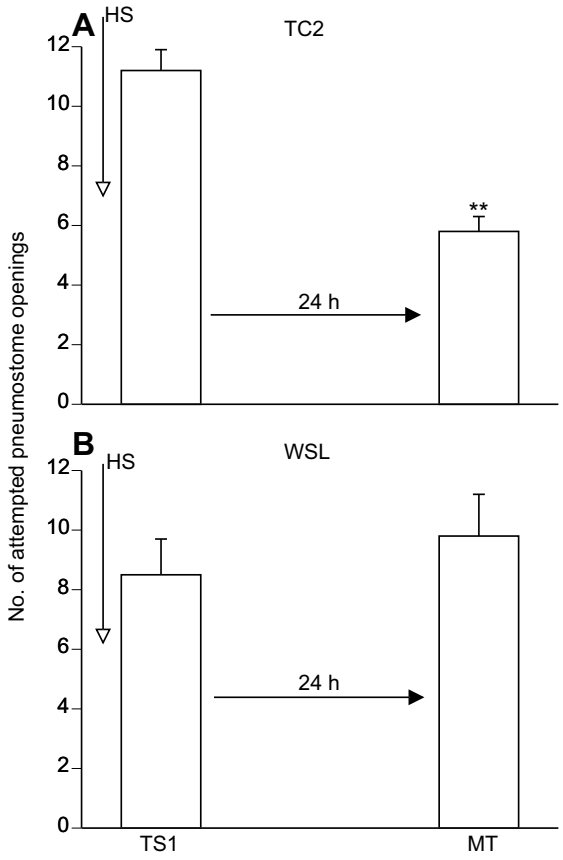

Fig. 2. 'Average' and 'smart' snails are differently affected by the thermal stressor. (A) In TC2 snails, experiencing the heat stressor (HS) 1 h before training results in enhancement of memory formation as a single 0.5 h TS results in LTM 24 h later. (B) In the WSL 'smart' snails, experiencing the heat stressor 1 h before training obstructs LTM formation. A single 0.5 h training session no longer results in LTM. Data are means+s.e.m. $^{**}P<0.01$.

formation to conserve energy (Haney and Lukowiak, 2001). However, contrary to our initial hypothesis, food-deprived *L. stagnalis* had no problem forming LTM (Haney and Lukowiak, 2001). Here, we tested 3-day food deprivation as a stressor in 'smart' snails. Snails received one training session after 3 days of food deprivation; they showed memory at the 24 h MT. That is, the number of attempted pneumostome openings in the MT was significantly less than in TS1 (Fig. 3A; t=2.338, P=0.0415, N=11). Thus, 3-day food deprivation did not obstruct LTM formation in 'smart' snails. Next, we confirmed the results of the Haney and Lukowiak (2001) study. 'Average' freshly collected snails were food-deprived for 3 days, and then received one 0.5 h training session. They did not show memory at the 24 h MT; the number of attempted pneumostome openings in the MT was statistically similar to the training session (Fig. 3B; t=1.053, P=0.3172, N=11). Thus, 3-day food deprivation did not act as a memory-enhancing stressor in 'average' freshly collected snails.

In 'average' snails, when a food source is detected, but cannot be accessed (i.e. the carrot odour experiment), the food odour acts as a stressor when the snails are food-deprived (Haney and Lukowiak, 2001). Food detection in an 'average' non-food-deprived snail does not act as a stressor (Haney and Lukowiak, 2001). 'Smart' snails were food-deprived for 3 days, then exposed to carrot odour without being able to access the food. Immediately after carrot odour exposure the snails received a 0.5 h training session. When tested for LTM 24 h later, snails did not show memory; the number of attempted pneumostome openings was not significantly different between TS1 and MT (Fig. 4A; t=0.2778, P=0.7859, N=13). That is, the combination of food deprivation and carrot odour obstructed LTM formation in 'smart' snails. When non-food-deprived 'smart' snails were exposed to carrot odour immediately before training, they showed memory in the 24 h MT; the number of attempted pneumostome openings in the MT was significantly less than in TS1 (Fig. 4B; t=3.345, P=0.0074, N=11). The identical protocol of food deprivation and exposure to carrot odour was tested in TC2 'average' snails given the single 0.5 h training procedure. These snails showed memory at the 24 h MT; the number of attempted pneumostome openings was significantly fewer in MT than in TS1 (Fig. 4C, t=6.148, P<0.0001, N=14). Therefore, food deprivation combined with carrot odour obstructs LTM formation in a 'smart' snail, but enhances LTM formation in a freshly collected 'average'

Journal of Experimental Biology

Journal of Experimental Biology (2017) 220, 891-899 doi:10.1242/jeb.149161

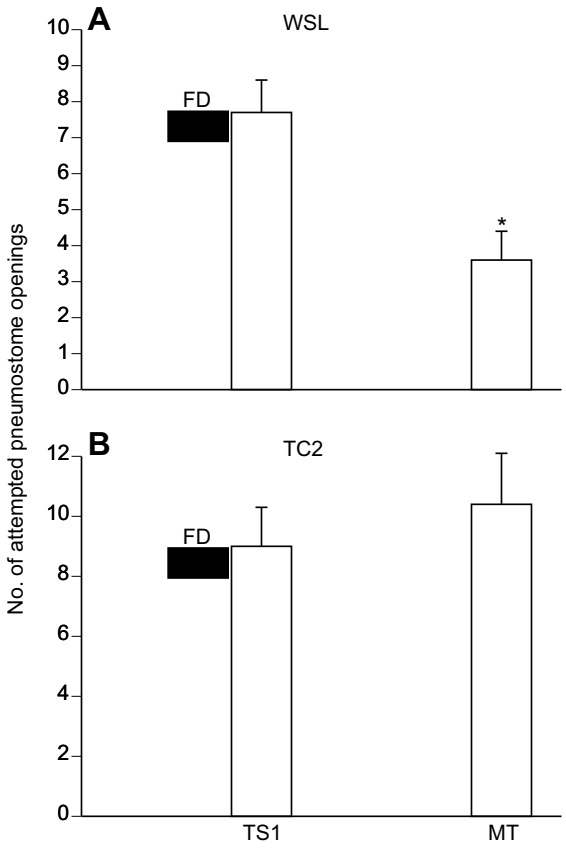

Fig. 3. Food deprivation does not alter the ability of 'smart' or 'average' snails to form memory. All snails were food-deprived (FD) for 3 days before receiving the single 0.5 h training session (TS1). (A) WSL snails still exhibited the 'smart' snail phenotype, as LTM was shown 24 h after the single training session. (B) TC2 snails did not show enhanced memory-forming ability following 3 days of food deprivation, as the single 0.5 h training session did not result in LTM 24 h later. Data are means+s.e.m. *P<0.05.

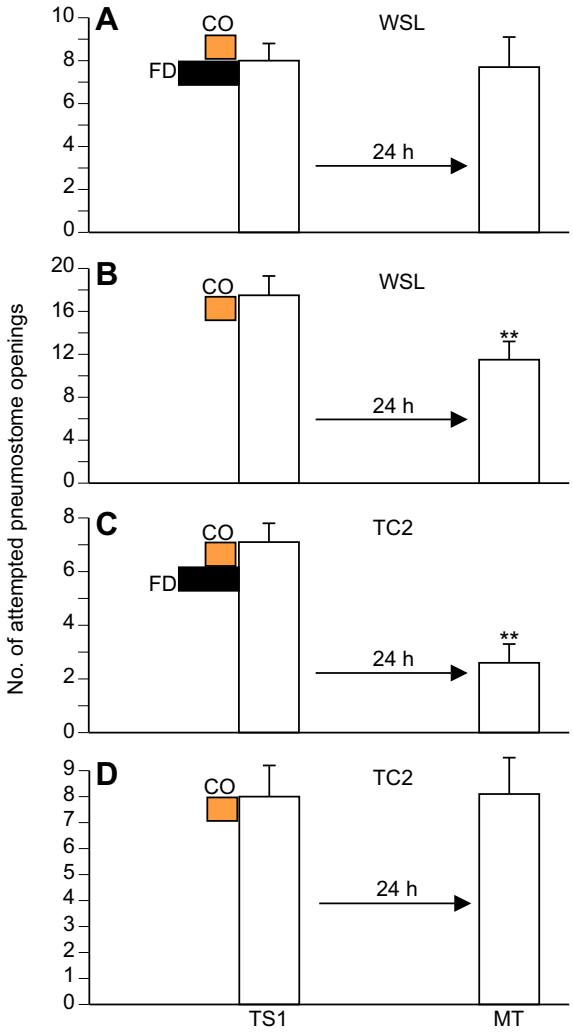

Fig. 4. Food deprivation and exposure to carrot odour separately and combined in 'smart' versus 'average' snails trained with the single 0.5 h training session (TS1) with memory (MT) tested 24 h later. (A) In WSL snails (i.e. 'smart'), the combined stressors, 3 days food deprivation (FD) plus smell of unattainable carrot (CO) result in an obstruction of LTM. (B) In WSL snails, CO by itself does not obstruct LTM formation in WSL snails. (C) In TC2 snails ('average'), the combination of the two stressors (FD+CO) results in an enhancement of memory formation. (D) In TC2 snails, exposure to only CO does not result in enhancement of memory formation. Data are means+s.e.m. **P<0.01.

snail. When not food-deprived, 'average' TC2 snails were exposed to carrot odour immediately before training, they did not show memory at the 24 h MT; the number of attempted pneumostome openings was not significantly different in the MT compared with the training session (Fig. 4D; t=0.08422, P=0.9347, N=10). Thus, food detection in 'average' snails that are not food-deprived does not act as a memory-enhancing stressor.

Predator detection

Lymnaea stagnalis detect predator kairomones via the osphradium through a serotonergic pathway (Il-Han et al., 2010). Crayfish, a natural predator of *L. stagnalis*, release kairomones in the water in which they are housed. 'Average' Dutch snails trained in this water, which we term CE, form LTM with only one training session. Thus, training in CE enhances memory in these 'average' snails. Here, we trained 'smart' snails in CE and tested memory 24 h later. The number of attempted pneumostome openings was significantly less than in the training session (Fig. 5A; t=2.568, P=0.0280, N=11). Thus, training in CE did not obstruct memory formation in 'smart' snails.

It is important to note that CE is an environmentally relevant stressor only to snails with crayfish as a historical predator. Thus, Dutch and WSL snails respond to this predator. Because crayfish are not present in ponds in Alberta, they are not a sympatric predator (Orr et al., 2009a,b), thus TC2 snails do not respond to CE. However, CE is detected through the 5-HT predator detection pathway, and an injection of 5-HT before training causes the same enhancing effect on memory as exposure to CE in an 'average' snail (Il-Han et al., 2010; Lukowiak et al., 2014a,b). Therefore, as a substitute for CE in TC2s, we used a 5-HT injection. TC2 snails injected with 5-HT 1 h before training showed enhanced memory; they had significantly fewer attempted pneumostome openings in the MT than in the training session (Fig. 5B; t=4.500, P=0.0015, N=10).

When a snail faces imminent predation by a crayfish, as a last-resort defense mechanism, it fully withdraws into its shell. This has been termed the WBWR. We have observed this behaviour in the laboratory as the crayfish takes hold of the snail to eat it. Exposing snails for 30 s to 25 mmol l^{-1} KCl elicits a similar WBWR. Exposure to KCl immediately before training 'average' Dutch snails results in enhanced memory formation. In our study, we observed

Journal of Experimental Biology (2017) 220, 891-899 doi:10.1242/jeb.149161

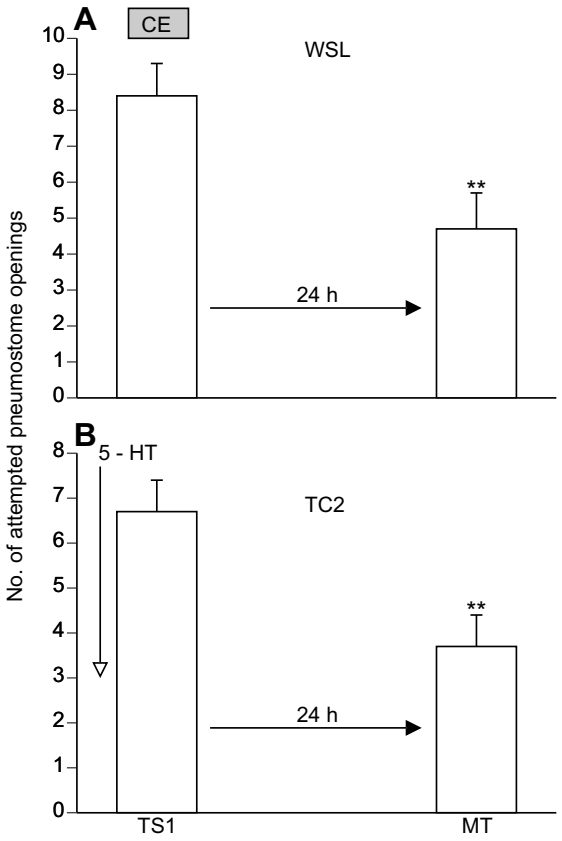

Fig. 5. Predator detection or a proxy of predator detection in 'smart' versus 'average' snails. (A) WSL snails respond to CE. Training WSL snails in CE still results in the single 0.5 h TS1 resulting in LTM. (B) TC2 snails do not respond to CE, thus a proxy of predator was used, a 5-HT (serotonin) injection. 5-HT was injected 1 h before training. The injection resulted in TC2 having enhanced memory-forming ability. Data are means+s.e.m. **P*<0.05; ***P*<0.01.

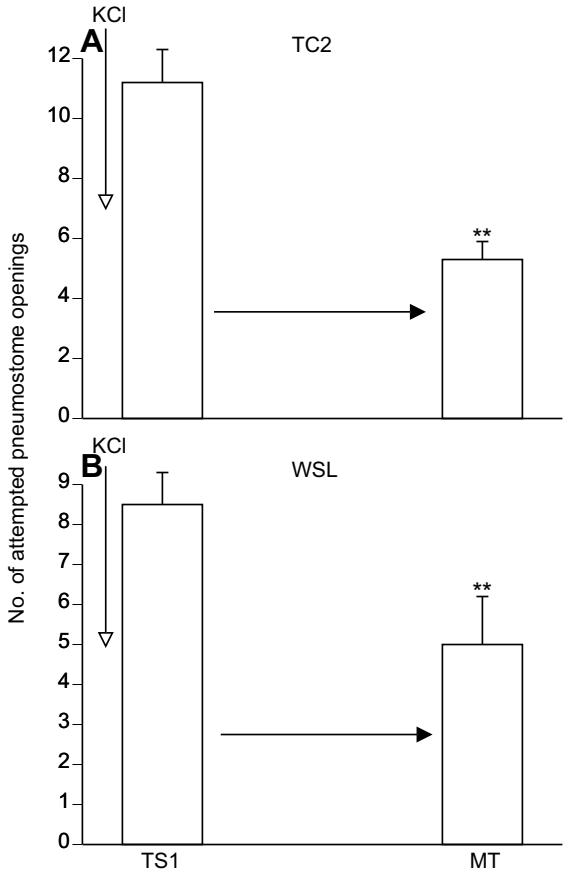

Fig. 6. A KCl bath results in the whole-body withdrawal response (WBWR). The KCL bath enhances LTM formation in 'average' snails and does not block LTM formation in 'smart' snails. (A) TC2 snails exposed to the KCl bath have enhanced memory-forming ability. (B) The KCl bath does not obstruct LTM formation in the WSL 'smart' snails. Data are means+s.e.m. **P*<0.05; ***P*<0.01.

consistent results; TC2 snails exposed for 30 s to 25 mmol l^{-1} KCl immediately before training showed significantly fewer attempted pneumostome openings in the 24 h memory test compared with the training session (Fig. 6A; t=4.742, P=0.0004, N=14). We then followed an identical protocol using 'smart' snails. Snails showed memory in the 24 h MT; the number of attempted pneumostome openings was significantly less than in the training session (Fig. 6B; t=2.764, P=0.0220, N=10). Thus, this protocol did not obstruct memory formation in 'smart' snails.

In 'average' Dutch snails, exposure to KCl immediately before training in CE enhances memory; one training session is sufficient to form a memory that persists for at least 24 h (Hughes et al., 2016). However, this memory is qualitatively different (as evidenced by its susceptibility to disruption) than a memory formed after training in CE alone or training under control conditions immediately after exposure to KCl (Hughes et al., 2016). Guided by these data, we injected TC2 snails with 5-HT as a proxy for predator detection, waited 1 h, then exposed the snails for 30 s to 25 mmol l^{-1} KCl immediately before training. Consistent with previous results on 'average' Dutch snails, the TC2 'average' snails showed enhanced memory, i.e. significantly fewer attempted pneumostome openings in the MT compared with the training session (Fig. 7A; t=5.292, P=0.0003, N=12). We next exposed 'smart' snails for 30 s to 25 mmol l^{-1} KCl, then immediately trained the cohort in CE. At the 24 h MT, the snails did not have a significantly different number of attempted pneumostome openings than in the training session (Fig. 7B; t=0.07992, P=0.9380, N=10). That is, the combination of exposure to KCl and training in CE obstructed memory formation in the 'smart' snails.

Tissue injury – shell damage

Here, for the first time, we investigated the effect of shell damage as an environmentally relevant stressor on memory formation. In separate cohorts of TC2 and WSL snails, shells were clipped and then snails were returned to their home aquarium for 24 h. After the 24 h recovery period, the two separate cohorts received one 0.5 h training session, followed by a 24 h MT. At the 24 h MT, the WSL (i.e. 'smart') cohort did not show a significantly different number of attempted pneumostome openings compared with the training session (Fig. 8A; t=0.7830, P=0.4537, N=10). Thus, shell clipping obstructed the ability to form memory in these 'smart' snails. The TC2 cohort showed significantly fewer attempted pneumostome openings in the 24 h MT compared with the training session (Fig. 8B; t=3.919, P=0.0024 N=12). That is, shell clipping enhanced memory in the TC2 snails.

DISCUSSION

Thus far, our work has not found differences in baseline behavioural traits between strains of snails with differing learning and memory-formation abilities (e.g. locomotion and aerial respiratory behaviour) (Orr et al., 2009a,b; Dalesman et al., 2011a,b,c; Braun et al., 2012;

Journal of Experimental Biology (2017) 220, 891-899 doi:10.1242/jeb.149161

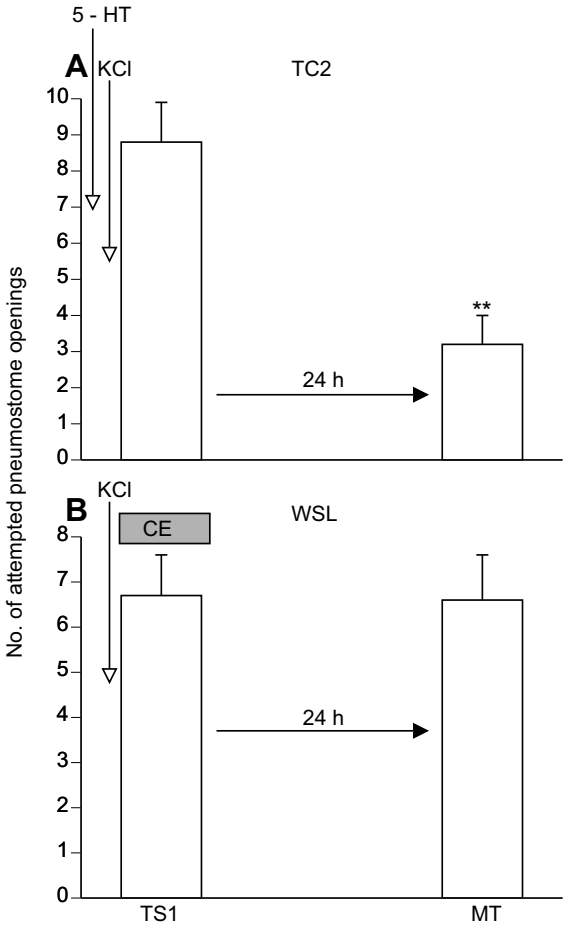

Fig. 7. A combination of stressors enhances memory formation in 'average' snails but obstructs LTM formation in 'smart' snails. (A) TC2 snails were injected with 5-HT (as a proxy for predator detection) and then exposed to the KCl bath. In these snails, this combination of stressors caused an enhancement of memory formation. (B) In the WSL 'smart' snails, the combination of the KCL bath followed by training in CE (i.e. predator detection) obstructs memory formation. Data are means+s.e.m. $^{**}P<0.01$.

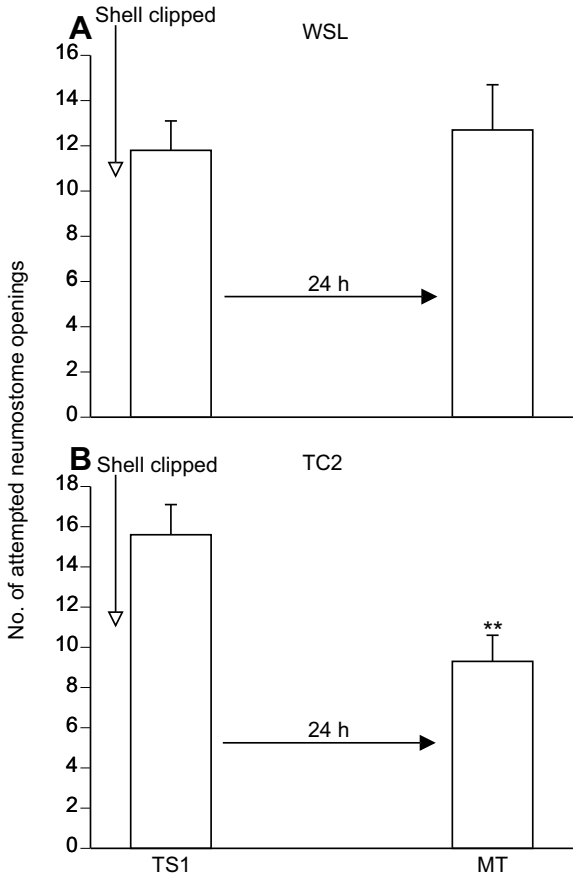

Fig. 8. Tissue injury (clipped shell) differentially affects 'smart' versus 'average' snails. (A) WSL 'smart' snails had their shell clipped 24 h before receiving the 0.5 h TS1 session. LTM formation was obstructed. (B) In TC2 snails, clipping the shell 24 h before TS1 resulted in enhanced memory formation. Data are means+s.e.m. $^{**}P<0.01$.

Dalesman and Lukowiak, 2012). However, the populations may differ in response to environmental stimuli, co-varying with cognitive ability, and offer insights into the evolution of memory formation in *L. stagnalis*. Here, we assessed ability to form memories in two freshly collected strains of *L. stagnalis* (one 'smart' and one 'average'), and the effects of a variety of stressors on memory formation in these two strains. We view the obtained results in light of the YDH curve, hypothesizing that 'smart' snails are unable to cope with stressors or combinations of stressors as well as 'average' snails. This differential coping ability thus affects their respective abilities to form memory when exposed to certain stressors.

We initially confirmed strain-specific differences in memory-formation abilities between populations from the two geographically distinct ponds that are approximately 900 km apart. Snails freshly collected from WSL exhibited the 'smart' snail phenotype; one training session was sufficient for LTM formation. Snails freshly collected from TC2 did not form LTM after one training session; these snails required two 0.5 h training sessions, spaced apart by 1 h, to form LTM. Thus, consistent with previous data (Braun et al., 2012), TC2 snails were termed 'average'.

We found that certain stressors or combinations of stressors enhanced memory formation in 'average' snails, but obstructed memory formation in 'smart' snails (Table 1). The YDH curve illustrates the effect of stress on memory, stating that at different stress levels, the ability to form memory changes. The YDH law is represented as an inverted U function: when levels of stress are too low or too high, the ability to form memory is impaired. This inverted U function is a figure adapted from Donald Hebb's 1955 presidential address to the American Psychological Association (Hebb, 1955; see also Ito et al., 2015a,b). It was Hebb who hypothesized that too much or too little stress is not optimal for memory formation. The YDH curve provides a conceptual framework for the interpretation of our results. Perhaps, under control conditions, 'average' snails are left of centre on the inverted

Table 1. Stressors and memory formation

Stressor	'Average'	'Smart'
Thermal	✓	×
FD	—	—
CO	—	—
FD+CO	✓	×
CE or 5-HT	✓	—
KCl	✓	—
KCl+(CE or 5-HT)	✓	×
Shell clipped	✓	×

FD, food deprivation; CO, carrot odour; 5-HT, serotonin; CE, crayfish effluent; –, no difference from control; ×, memory is obstructed; ✓, memory is enhanced.

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U, whereas 'smart' snails are close to centre. Thus, 'average' snails require exposure to stress for optimal memory formation. In contrast, 'smart' snails, without any external stress applied, are in an optimal state for memory formation under control conditions. That is, 'smart' snails are at a higher basal level of stress under control conditions than 'average' snails. Alternatively, the difference in the ability to form memory under control conditions between 'smart' and 'average' snails reflects a difference in perception between the strains. Perhaps 'smart' snails are more sensitive perceivers of stress, such that the same stressor or combination of stressors pushes a 'smart' snail further to the right on the YDH curve than it does an average snail. Strain differences in stress perception affecting memory have been reported in other species, such as rats (e.g. maze bright versus maize dull rats; see Innis, 1992; Andrews, 1996).

We tested a variety of stressors, some individually and some in combination, on the ability to form memory in the two strains of *L. stagnalis* (Table 1). Although not all of the pathways of stress detection for the stressors we studied have been characterized, the stressors used here are not detected through the same sensory pathway (Dalesman and Lukowiak, 2012). The finding that a variety of stressors are detected differently but act similarly to enhance memory in 'average' snails, but obstruct memory formation in 'smart' snails, may point to a final common pathway by which stress modulates memory formation.

DNA methylation, as well as activation of heat shock proteins (HSPs), requires both transcriptional and translational activity. Both are required for the enhancement of memory by thermal stress in 'average' snails (Sunada et al., 2016). If 'average' snails experience heat stress before training and either activation of HSPs or DNA methylation are blocked, the snails are unable to form memory. The obstruction of memory formation in 'smart' snails by heat stress could be indicative that after a threshold of 'too much stress' is reached, it is the inhibition of certain epigenetic processes, such as DNA methylation or activation of HSPs, that prevents memory formation. If there is a final common pathway by which stress modulates memory formation, perhaps it is the inhibition of certain epigenetic processes required for enhancement of memory formation in 'average' snails that obstructs memory formation in 'smart' snails.

When a combination of stressors is presented to *L. stagnalis*, it is difficult to know what the outcome will be regarding memory formation (Dalesman et al., 2013; Lukowiak et al., 2014a,b). How a combination of stressors impacts memory is an emergent property of how the snails perceive the combination of stressors. This cannot be predicted based on the impact of the stressors on memory formation when the stressors are presented individually. Our data on food deprivation and carrot odour are consistent with these observations. Here we found that freshly collected 'average' snails do not perceive either 3-day food deprivation or carrot odour immediately before training as stressors, when presented individually, that are sufficient to enhance memory formation. However, when 3-day food-deprived TC2 snails were exposed to carrot odour for 30 min immediately before training, the snails showed enhanced memory. Thus, the impact of the stressors, when combined, could not be predicted based on the impact of the stressors individually. We observed a similar phenomenon with the 'smart' snails. Food-deprived WSL snails were able to form LTM with one training session. Non-food-deprived 'smart' snails that were exposed to carrot odour for 30 min before training were also able to form LTM with one training session. However, when the stressors were combined (food deprivation and carrot odour), the 'smart' snails were unable to form memory.

Interestingly, all stressors and combinations of stressors that obstructed memory formation in 'smart' snails enhanced memory formation in 'average' snails (see Table 1). However, there were two individual stressors, CE (or 5-HT as proxy) and KCl, that enhanced memory in 'average' snails, but did not obstruct memory in 'smart' snails. Activation of the 5-HT predator detection pathway, through either a 5-HT injection or training in CE, warns a snail that a predator is nearby. Although a direct comparison between freshly collected 'smart' and 'average' snails has not been made before, response to predators is highly conserved among strains of *L. stagnalis* (Orr et al., 2009a,b; Dalesman and Lukowiak, 2012). From an evolutionary perspective, it is logical that the ability to form memory surrounding a predator encounter is preserved across strains to promote survival, regardless of cognitive ability (Kotrschal et al., 2013; Simpson et al., 2016). Exposure to KCl elicits the WBWR in snails. This response is a snail's last-resort defense mechanism. We have observed, in the laboratory, that when facing imminent predation by a crayfish (i.e. being in the crayfish's grasp), a snail will display the WBWR. Thus, from an evolutionary perspective, it follows that it would be advantageous for preservation of memory surrounding an experience when the WBWR is elicited, such as exposure to KCl. This response is only triggered in situations that a snail perceives as life threatening. Interestingly, when KCl and activation of the predator detection pathway are combined as stressors, 'average' Dutch snails form enhanced memory that is susceptible to a propranolol block of reconsolidation (Hughes et al., 2016). This ability to make a 24 h memory also occurs with either the KCl bath or predator detection alone, but with just the single stressor exposure, propranolol does not obstruct reconsolidation (Hughes et al., 2016). In contrast, the ability to form memory is obstructed in 'smart' snails when both stressors are applied. Again, this result may be understood in the context of the YDH law. Although the effects of predator detection and full-body withdrawal on memory may generally be more conserved between strains than other, non-imminently life-threatening stimuli, perhaps there is still a threshold of 'too much stress'. This threshold could explain why, when both are combined, 'smart' snails, which are either at a higher basal level of stress than 'average' snails or are more sensitive to perceiving stress, are unable to form memory.

We also showed, for the first time, that shell damage alters memory formation in *L. stagnalis*. In 'average' snails, this stressor enhanced memory; in 'smart' snails, memory formation was obstructed. We decided to investigate shell damage as an environmentally relevant stressor after making the observation when collecting snails in the field that snails occasionally had incomplete shells. We postulated that the stressful experience associated with sustaining the shell damage may lead to alterations in memory-forming ability. Alternatively, or in addition, because shell repair is likely to be energetically costly to the snail, the metabolic state that a snail is in after having sustained damage to its shell may lead to an altered ability to form memory. Future work will explore these subtleties and further delve into the causal neuronal mechanisms of the effects of shell damage on learning and memory. The shell clipping that we performed was very similar to the naturally 'clipped' snails we have observed at various collection sites.

The neural network that controls aerial respiratory behaviour in *L. stagnalis* has been fully characterized (Syed et al., 1990, 1992). The neuron RPeD1 has been determined to be the necessary site for memory formation (Scheibenstock et al., 2002; Sangha et al., 2003a,b,c). Memory, as well as exposure to stressors, has been shown to alter the 'state' of RPeD1 (Orr and Lukowiak, 2008; Braun and Lukowiak, 2011; Braun et al., 2012). Furthermore, work from

the Lukowiak laboratory has shown that RPeD1 is in a primed 'state' for memory formation in naive smart snails compared with naive 'average' snails (Braun et al., 2012). In future work, we plan to compare the 'state' of RPeD1 in 'average' and 'smart' snails exposed to stressors that cause enhancement of memory in 'average' snails and obstructed of memory in 'smart' snails. This comparison will enable an understanding of the neuronal correlates to the behavioural findings reported in the present study.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: E.H., T.S. and K.L.; Methodology: T.S., A.P., E.H., C.S., K.L. and E.S.; Investigation: T.S., A.P., E.H., C.S., E.S., K.S.L. and I.P.: Writing - original draft: E.H., T.S. and K.L.; Writing, review and editing: T.S., E.H. and K.L.; Funding acquisition: K.L.; Supervision: K.L.

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