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ORIGINAL PAPER

Training *Lymnaea* in the presence of a predator scent results in a long-lasting ability to form enhanced long-term memory

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Abstract *Lymnaea* exposed to crayfish effluent (CE) gain an enhanced ability to form long-term memory (LTM). We test the hypothesis that a single CE exposure and operant conditioning training leads to long lasting changes in the capability of snails to form LTM when tested in pond water four weeks later. We trained both juvenile and adult snails with a single 0.5 h training session in CE and show that LTM was present 24 h later. Snails trained in a similar manner in just pond water show no LTM. We then asked if such training in CE conferred enhanced memory forming capabilities on these snails four weeks later. That is, would LTM be formed in these snails four weeks later following a single 0.5 h training session in pond water? We found that both adult and juvenile snails previously trained in CE one month previously had enhanced LTM formation abilities. The injection of a DNA methylation blocker, 5-AZA, prior to training in adult snails blocked enhanced LTM formation four weeks later. Finally, this enhanced LTM forming ability was not passed on to the next generation of snails.

Keywords *Lymnaea* · Long-term memory · Epigenetic change · Predator scent

Abbreviations

CE	Crayfish effluent
CNS	Central nervous system
CREB	cAMP response element-binding protein
F1	First generation
LTM	Long-term memory
MT	Memory test session
PW	Pond water
SEM	Standard error of the mean
TS	Training session
5-AZA	5-Aza-2′-deoxycytidine

Introduction

A strain of pond snails, *Lymnaea stagnalis*, originally collected in The Netherlands has been maintained in many laboratories since the early 1950s (>250 generations). These snails, which we refer to here as *W-strain* snails, during this time have not been exposed to any sympatric predator (predator living in the same geographical area; Orr et al. 2009a). However, they still have the ability to detect kairomones from crayfish (a sympatric predator). Their detection of crayfish effluent (CE) by receptors in the osphradium (Il-Han et al. 2010) initiates various 'anti-predator' behaviours (Orr et al. 2007, 2010). Such behaviours include moving to the surface, decreased righting-response time, changes in cutaneous oxygen consumption, increased shadow-sensitivity, increased exploration and increased aerial respiration. Importantly, predator detection also enhances LTM formation following operant conditioning of aerial respiratory behaviour (Orr and Lukowiak 2008; Lukowiak et al. 2010; Dalesman and Lukowiak 2012). The detection of these kairomones primes the molecular mechanisms responsible for LTM formation (Orr and Lukowiak 2008; Braun et al. 2012). The enhancement of memory formation due to sensing CE can be blocked by two means: (1) severing the osphradial nerve so that the

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sensory information from the osphradium does not reach the CNS (Il-Han et al. 2010); and (2) by the DNA methylation blocker 5-AZA (Lukowiak et al. 2014). Pre-treatment of snails with 5-AZA before exposure to CE prevents the formation of LTM. Thus, DNA methylation triggered by the exposure to CE is necessary for memory enhancement. It is, however, unclear how long the CE-induced changes persist that cause enhanced memory formation.

Juvenile *W-strain Lymnaea* do not have the capability of forming LTM following operant conditioning of aerial respiratory behaviour (McComb et al. 2005). However, if juvenile *W-strain* snails are trained in CE they gain the capability to form LTM (Orr et al. 2010; Sunada et al. 2010). Thus, CE has the ability to alter the nervous system of juvenile *Lymnaea* such that associative learning and LTM formation of aerial respiratory behaviour can now occur. Again, it has not yet been tested how long the CE-induced changes persist in the juvenile snails.

In the (Orr and Lukowiak 2008) study they found that exposing snails to CE immediately after training snails in pond water did not result in enhanced memory formation. They also found that exposing snails to CE 1 h before operant conditioning training in normal pond water was also not sufficient to cause enhanced memory formation. Additionally, a yoked control procedure in CE did not result in enhanced memory formation. They concluded that enhanced CE-induced memory formation was an activity-dependent process (i.e., operant conditioning training) that only occurs in the presence of CE.

We have termed the adult *W-strain* snails used here as 'average' in their ability to form LTM following operant conditioning. That is, in this *W-strain* of snails it takes two 0.5 h training sessions separated by a 1 h interval to result in LTM (Lukowiak et al. 2000, 2003a, b, 2008; Orr et al. 2009b). As mentioned above, juvenile Dutch *W-strain* snails are incapable of showing LTM following similar training procedures. However, we have now shown that there are also strains of *L. stagnalis,* which we have termed 'smart' (Orr and Lukowiak 2008; Orr et al. 2009b; Dalesman et al. 2011; Dalesman and Lukowiak 2011). In these smart snails only a single 0.5 h training session in pond water is required to cause LTM formation. In addition, juvenile *Lymnaea* of these 'smart' strains are competent to form LTM (unpublished observations). Finally, at the neuronal level significant differences have been described in the properties of RPeD1 between 'smart' and 'average' snails (Braun et al. 2012). RPeD1 is a neuron where necessary changes occur for LTM formation (Scheibenstock et al. 2002). Basically in naive smart snails RPeD1 is in a 'primed' state for forming LTM compared to RPeD1 activity seen in naive average snails (Braun et al. 2012).

Juvenile snails also exhibit changes in shell morphology (e.g., thickening or elongation) as a result of predator detection along with the detection of conspecific deaths (e.g., crushed snails; Crowl and Covich 1990; Alexander and Covich 1991; Rundle and Bronmark 2001; Rundle et al. 2004). These morphological changes, as well as a number of behavioural changes seen with predator detection allow snails to potentially avoid predation. Whether changes in memory forming ability in *W-strain Lymnaea* (Orr et al. 2010) also contribute to predatory avoidance is unclear. However, the fact that predator detection causes long-lasting changes in shell morphology suggests to us that there may also be long-lasting changes caused by training in CE in the neural circuits that mediate learning and memory formation. We will directly test this hypothesis here in both juvenile and adult snails subjected to training in CE. We will further attempt to determine if these changes require DNA methylation. Finally, we ask whether the hypothesized changes in juveniles will persist into the next generation of snails.

Dalesman et al. (2011) showed that snails from habitats as close as 0.5 km apart possessed differing cognitive abilities (i.e., average vs. smart) that were maintained over the course of years and rearing in laboratory conditions. Whether or not these differences in cognitive ability are the result of epigenetic differences in the various strains remains to be determined. However, it is worth noting that one of the first suggestions that DNA methylation (or demethylation) might have an important biological role was made by Griffith and Mahler (1969), who proposed that it could provide a basis for long-lasting memories (>50 years) in the brain.

Materials and methods

Snails

Lymnaea stagnalis used in this study (i.e., the *W-strain*) were breed and reared in a facility in the Department of Biological Sciences at the University of Calgary. They 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 Utrecht in the early 1950s. The snails used here have not been exposed to any naturally occurring predator since that time. Snails were maintained in artificial pond water (PW = deionized water + 80 mg/L $CaSO_4$ + 0.26 g/L instant ocean), fed romaine lettuce ad libitum and at room temperature (≈20 °C) until training (see Dalesman and Lukowiak 2010 for further details).

Two different ages of *W-strain Lymnaea* were used here (juvenile and adult). Age was determined by shell length (McComb et al. 2005), measured from the apex of the spiral to the distal prominence of the aperture opening.

The shell length criterion for juveniles was 15 ± 2 mm (mean ± SEM: 15.27 ± 0.09; $n = 153$) and for adult's 23 ± 5 mm (mean ± SEM: 20.44 ± 0.27; $n = 65$).

Crayfish effluent (CE)

*C*rayfish of the genus *Cambrus* was housed in a 110 L aquarium and fed a mixed diet of lettuce and snails ad libidum. To create CE water (crayfish effluent) a 2 L container was filled with PW and the crayfish was transferred inside for a 1 h period (Orr et al. 2010). Snails were only exposed to CE and not the crayfish directly.

Respiration

Lymnaea stagnalis are bimodal breathers. They have the ability to exchange oxygen through their skin (cutaneous respiration) as well coming to the surface and opening their pneumostome (breathing tube) to exchange the contents of their lung with atmospheric air (aerial respiration). In hypoxic conditions e.g., with bubbling N_2 through a beaker containing PW, aerial respiration predominates over cutaneous respiration (Lukowiak et al. 1996).

Operant conditioning

Lymnaea were transferred from their eumoxic aquaria to a housing aquarium at least three days before being individually marked, which in turn was at least 48 h before the experiment. Animals were placed into a 1 L beaker filled with 500 mL either of CE for the first cohort or control PW (i.e., no CE). Prior to the experiment, N_2 was strongly bubbled into the water to make it hypoxic. N_2 was then continuously bubbled at a slower rate throughout the experiment (no disruption of the water surface), to maintain hypoxia without disturbing the snails. Before each session, animals were given a 10 min acclimatization period in which they could freely open their pneumostome. After this period, the snails were gently pushed under water to convey the start of the training. Every time an animal began to open its pneumostome, a gentle tactile stimulus was applied on it with a sharpened wooden applicator stick. The stimulus was strong enough to induce the rapid closure of the pneumostome but at the same time gentle enough to do that without inducing a full body withdrawal response (Inoue et al. 1996, 2001). A single training session of 0.5 h was used. LTM was tested 24 h later.

Following the training and memory sessions snails were returned to their housing aquaria for four weeks. During this time, the juvenile snails matured into the adult state (McComb et al. 2005) while the already adult snails just got four weeks older. The same operant conditioning paradigm was used again on these *Lymnaea,* except that no training was done in CE water but instead all training and testing was performed in control PW.

Operational definition of memory

Memory is considered to be present when the mean number of attempted pneumostome openings in the memory test session (MT) was significantly less than the mean number of attempted pneumostome openings during the training session (TS; Lukowiak et al. 1998, 2000; Orr et al. 2010).

Yoked control

Snails that received a yoked control training session were treated in an identical manner to the operant conditioned animals. The only difference was that for these snails, the tactile stimuli were not contingent on their pneumostome openings. That is, the times of attempted pneumostome openings of the operant conditioned snails were recorded and reproduced in the same exact temporal manner with the yoked snails (Lukowiak et al. 1996; Lukowiak et al. 2003a, b; Haney and Lukowiak 2001). These snails were given a 0.5 h yoked TS followed 24 h later by a MT (this time with contingency between pneumostome openings and tactile stimuli) (Fig. 1).

Exposed control

Juvenile snails, called exposed control snails, were only exposed to CE (but not trained) for 40 min, which is the equivalent time of exposure of the acclimatization plus training session for both operant conditioned and yoked control snails. They were then tested for memory 24 h later in the same way described above (Fig. 1).

First generation (F1)

After the experimental protocol with juvenile snails, the now adult snails from each group (except snails trained in PW) were kept separated in their own resting tank until they copulated and laid eggs. The offspring were then tested, once they reached adulthood, with a 0.5 h operant conditioning (TS) protocol followed 24 h later by a MT in pond water.

Treatment for DNA methylation

5-AZA (the DNA methyltransferase inhibitor, 5-Aza-2′-deoxycytidine) was obtained from Sigma Chemical Company (St Louis, MO, USA). The 5-AZA was dissolved in sterile 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). A dose of 87 umol l^{-1} 5-AZA was chosen (see Lukowiak et al.

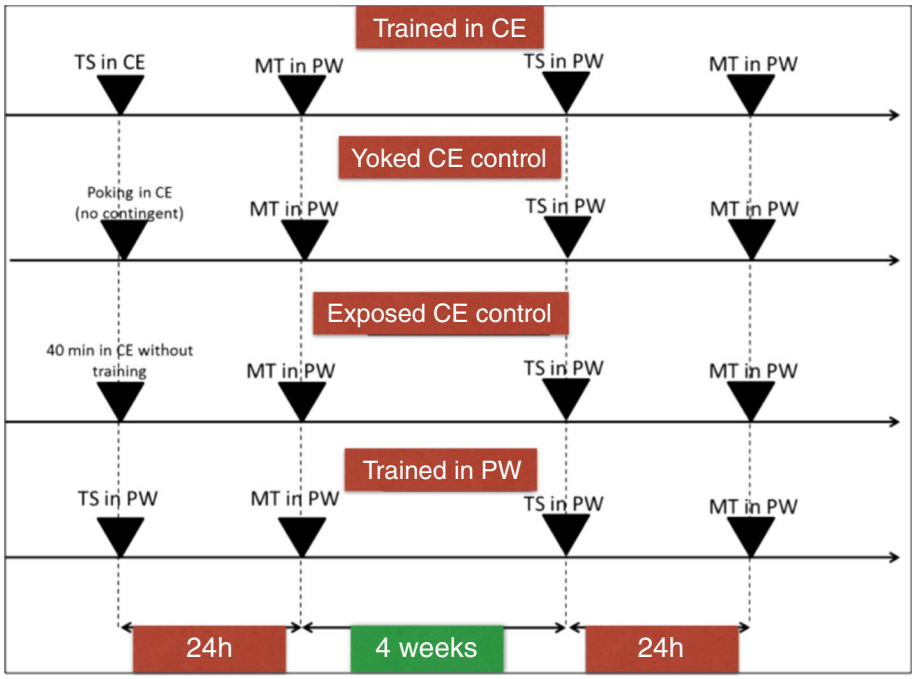

Fig. 1 Course of the experimental protocol, group by group. Training session (TS) and memory test (MT) paradigm consist of 10 min acclimation and 30 min "poking" the pneumostome as it begins to open. In the Trained in crayfish effluent (CE) group snails were trained in CE in only the first training sessions. All subsequent training and memory test sessions were performed in pond water. Yoked control group presents no contingency "poking" as their first training: the temporal pattern of the TS of the trained in CE group is reproduced. The exposed control group is only put in CE for 40 min in their first 'training' session. In the trained in PW group snails received all training and testing in pond water, these snails never had any contact with CE. Between each session (either 24 h or 4 weeks), the snails were returned to the housing tank

2014) for all studies described here. 5-AZA was injected into the haemocoel through the foot of the snail. The concentrations and injection times were determined and optimized based on our previously published data (Lukowiak et al. 2014).

Statistics

All data were analyzed using the R software on windows. For each cohort of animal (juvenile to adult or adult to adult) we computed two way repeated measure ANOVAs, taking into account the treatment (CE, Yoked, PW, Exposed or 5-AZA) and the session (TS or MT) either on the data before the four weeks period or after. If a significant difference was found, post hoc paired *t* tests was run to compare training session (TS) vs memory test (MT) of each group with Bonferroni corrections to assess which group learned. For each cohort we also computed the change in performances between testing and training, before and after the four week period, and used a two way repeated measure ANOVA taking into account treatment and time (before vs after the four weeks period) to compare this data, thus taking into account experiment wide error.

Results

The purpose of these experiments was to test whether operant conditioning of aerial respiration in CE as either an adult or juvenile *W-strain Lymnaea* not only initially caused enhancement of memory formation but also resulted in a long-lasting (four weeks) change in the ability of snails when trained in pond water to form LTM. A secondary purpose was to determine if snails trained as juveniles in CE that showed enhanced memory forming ability as an adult in pond water could pass on the enhanced memory forming ability to the next generation.

We first examined what operant conditioning of aerial respiratory behaviour in CE of juvenile snails would produce. Cohorts of naive juvenile snails were either: (1) Trained in CE; (2) Given yoked control training in CE; (3) Only exposed to CE for 40 min; or (4) Trained in pond water (Fig. 1). All snails were tested for LTM 24 h later in pond water (PW, i.e., no CE). These data are shown in Fig. 2. Juvenile snails that were trained in CE showed LTM when tested 24 h later. That is, the mean number of attempted pneumostome openings in the memory test session (MT) was significantly less than in the TS. Neither the

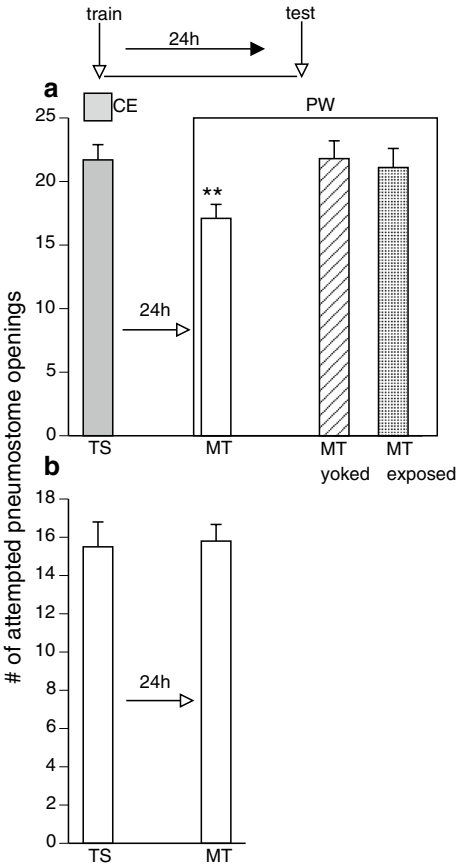

Fig. 2 Operant conditioning of juvenile *Lymnaea* only results in LTM when they are trained in CE. One cohort of juvenile snails were trained in CE (TS, *gray*); **a** second cohort received yoked control training in CE; while the third cohort just exposed to CE (i.e., no tactile stimuli were delivered). All snails were tested for memory 24 h later (MT) in pond water. Snails trained in CE ($n = 48$) exhibited LTM none of the other cohorts did (treatment effect: $F_{(2,187)} = 0.38$, $p = 0.38$; session effect $F_{(1,187)} = 1.48$, $p = 0.23$; interaction treatment χ session $F_{(2,187)} = 3.33$, $p = 0.038$, *post-hoc* *t* test: $p = 0.0032$). Neither yoked nor the CE-exposure only control groups ($N = 35$–40 respectively) showed LTM ($p = 0.44$ for yoked group). **b** Juvenile snails trained in PW ($n = 34$) did not exhibit LTM ($p = 0.76$). The *grey* bar indicates that the snails were trained in CE (crayfish effluent). *Errors* bars represent SEM. $^{**}p < 0.01$

CE exposed control group nor the CE yoked control group showed any significant decrease in the mean number of attempted pneumostome openings in their MT compared to TS of the operant conditioned group.

Another cohort of naive juvenile *W-strain* snails was trained in PW with a single 0.5 h training session (Fig. 2b). They did not exhibit LTM. That is, there were no differences between the mean number of attempted pneumostome openings during the TS and the MT (Fig. 2b). All of our data are similar to previous work (McComb et al. 2005; Orr et al. 2010). These data confirm that only the juvenile snails trained in CE exhibited LTM (Fig. 2).

All these snails were then maintained in their home aquaria for a further four weeks during the time they became adults, based on the size of their shell and ability to copulate and lay viable eggs (McComb et al. 2005).

Now adult snails received a single 0.5 h operant conditioning training session in PW (Fig. 3). It needs to be emphasized that CE was not used in this training sequence. We then tested for LTM 24 h later. In snails that were trained in CE as juveniles we found that a single 0.5 h training session resulted in LTM when tested 24 h later. That is, these snails showed a significantly decreased number of attempted pneumostome openings in the MT compared with TS. These snails now possessed the phenotype of a smart snail (Orr et al. 2009b; Dalesman et al. 2011) in that a single 0.5 h training session is sufficient to result in LTM formation.

However, in the other three cohorts of now adult snails, LTM was not observed following the single 0.5 h training session. That is, these snails did not possess the 'smart' snail phenotype for producing LTM. Thus, neither the juvenile snails that received yoked control training in CE, nor those just exposed to CE, gained the capability in adulthood to form LTM following a single 0.5 h training session. Neither of these two cohorts showed a significant decreased in the number of attempted pneumostome openings between their respective TS and MT. Finally, the cohort of juvenile snails that were trained in PW, when tested four weeks later with a single 0.5 h TS also did not form LTM (Fig. 3).

We performed a similar experiment on naive adult *Lymnaea* (Fig. 4). Three different naive cohorts of snails were examined (Fig. 4a–c). One of the cohorts ($n = 14$) received a single 0.5 h training session in CE (Fig. 4a); another cohort ($n = 16$) received yoked-control training in CE, (Fig. 4b) while the final cohort ($n = 17$) received operant conditioning in pond water (Fig. 4c). In the cohort that was trained in CE (Fig. 4a) LTM was present 24 h (MT) after the single 0.5 h TS. That is, the number of attempted pneumostome openings in MT was significantly less than in TS. In the other two, cohorts memory was not present 24 h later. That is in the CE-yoked control cohort (Fig. 4b) MT was not significantly different than TS, and we obtained the same result in the cohort (Fig. 4c) trained in pond water. Thus, as predicted only those snails that received operant conditioning training in CE exhibited LTM 24 h after the single 0.5 h training session. All these adult snails were then returned to their home aquaria for a four week period. Following this four week period all snails received a single 0.5 h training session (TS-2). Notice that in all of the cohorts TS-2 was not statistically different than the number of attempted openings in TS1. We then asked whether any of the three cohorts possessed the 'smart' snail phenotype. We found that the adult snails trained in CE (TS; Fig. 4a) four weeks previously,

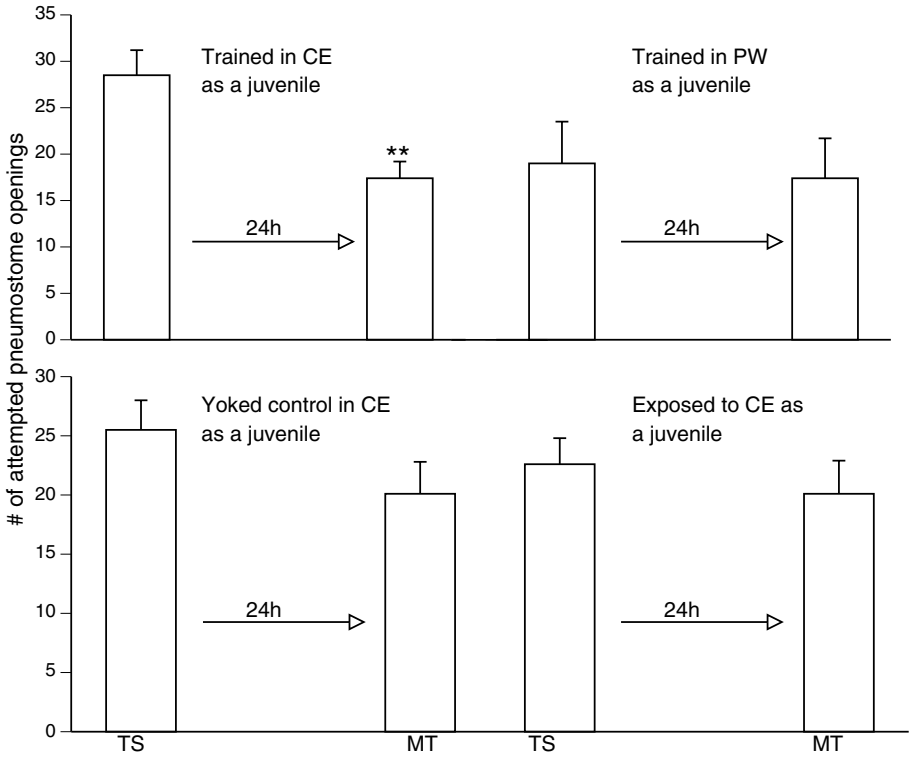

Fig. 3 Snails trained in CE as a juvenile have enhanced memory forming capabilities as an adult. All snails were 'trained' as juveniles in the various conditions (CE, PW, yoked control in CE and just exposed as a juvenile to CE as shown in Fig. 2). 4 Weeks later all snails received a single 0.5 h TS in pond water and a MT 24 h later in pond water. Only snails trained as juveniles in CE (*top left*) exhibited LTM 24 h after the single training session (treatment effect: $F_{(3,93)} = 3.19$, $p = 0.027$; session effect $F_{(1,93)} = 8.25$, $p = 0.0051$; interaction treatment χ session $F_{(3,93)} = 2.19$, $p = 0.095$, *posthoc t* test: $p = 0.0019$). All other cohorts did not exhibit (respectively, $p = 0.11$; $p = 0.59$; $p = 0.48$). Change in performances also confirmed the results in Figs. 2 and 3 (time effect: $F_{(1,59)} = 7.96$, $p = 0.0065$; treatment effect: $F_{(3,152)} = 5$, $p = 0.0024$; interaction time χ treatment $F_{(3,59)} = 1.75$, $p = 0.17$). *Errors* bars represent SEM. $^{**}p < 0.01$

formed LTM (MT-2) after the single 0.5 h training session in pond water (TS-2). Noticed that in the other two cohorts (Fig. 4b,c) LTM was not observed in MT-2. Thus, the two control cohorts did not possess the 'smart' snail phenotype. We thus conclude that training of adult snails in a single 0.5 h CE session has long-lasting effects on the snail such that when these snails receive a single 0.5 h training session in pond water they now have the ability to form LTM. Finally, we employed another naive cohort of adult snails ($n = 14$; Fig. 4d). These snails were first injected with a DNA methylation blocker, 5-AZA, 1 h before training in CE. When we tested these snails for LTM 24 h later, LTM was not observed. These data confirm earlier findings regarding the ability of 5-AZA to block CE's enhancing effect on LTM formation (Lukowiak et al. 2014). When we tested these snails four weeks later, the single 0.5 h training session (TS-2) did not result in LTM formation 24 h later. We attempted a similar series of experiments with juvenile snails but none survived the treatment with 5-AZA. Together these data show that the enhancing effects of CE on LTM formation are long-lasting, are activity-dependent (i.e., need contingent pairing of the 'poke' to the attempted pneumostome opening) and can be prevented from occurring if a DNA methylation blocker is used.

Finally (Fig. 5) we asked whether the off-spring of the snails that we initially trained as juveniles (Fig. 2) and then tested as adults in pond water four weeks later (Fig. 3) would show an altered phenotype to form LTM. These snails after being tested as adults were returned to their respective home aquaria were they were observed to copulate and lay eggs. We then tested the offspring of each group (i.e., the F-1 s) for their ability to form LTM following a single 0.5 h training session in pond water as an adult. We found that the offspring from each group did not show any differences in their memory capabilities (Fig. 5). That is, none of the offspring showed enhanced memory forming capabilities. The number of attempted pneumostome openings between the TS and the MT was not significantly different in the offspring from snails trained in CE, yoked control or exposed control groups. All the offspring behaved in a similar manner irrespective of their specific training and CE exposure procedure their parents experienced.

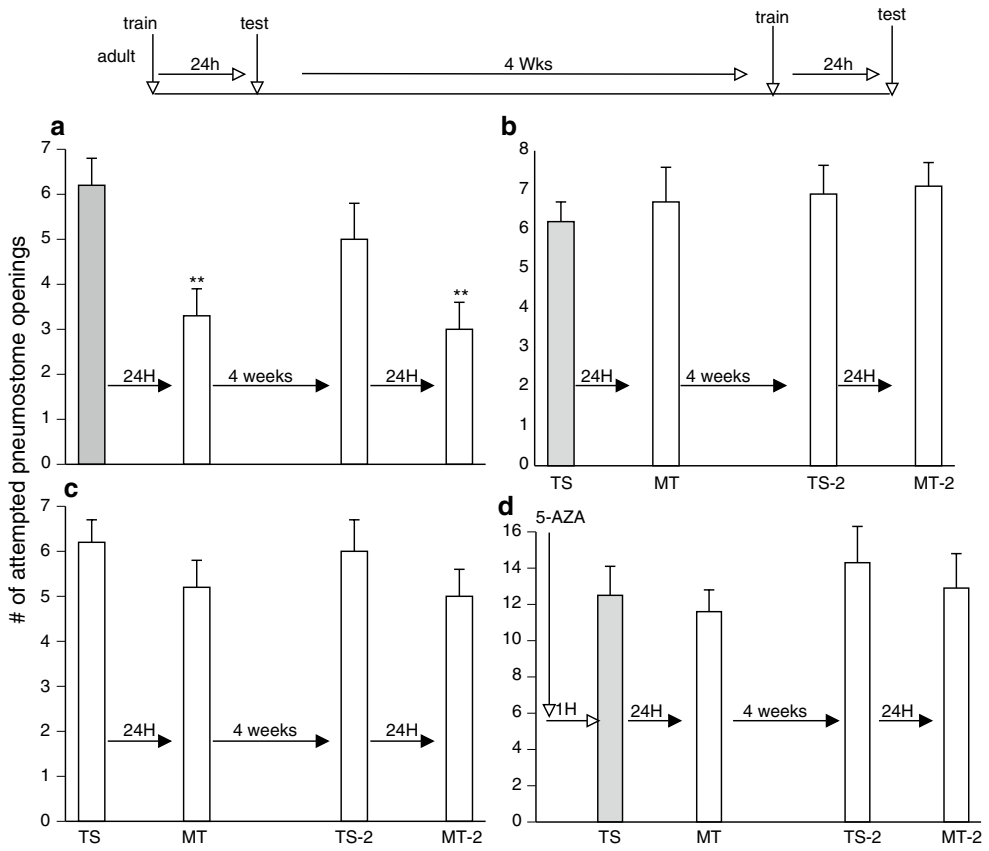

Fig. 4 CE-training in adults causes long lasting enhancement of memory formation. **a** Adult snails ($n = 14$) trained in CE. These snails exhibited LTM (treatment effect: $F_{(3,102)} = 45.35$, $p < 0.0001$; session effect $F_{(1,102)} = 4.42$, $p = 0.038$; interaction treatment x session $F_{(3,102)} = 2.69$, $p = 0.0501$, *posthoc t* test: $p = 0.00073$). 4 weeks later they were trained in pond water (TS-2) and exhibited LTM 24 h later (treatment effect: $F_{(3,102)} = 86.58$, $p < 0.0001$; session effect $F_{(1,102)} = 5.12$, $p = 0.026$; interaction treatment x session $F_{(3,102)} = 1.08$, $p = 0.36$, *posthoc t* test: $p = 0.006$). **b** As in a, except snails ($n = 16$) received yoked control training in CE. LTM was not present (TS and TS-2; respectively, $p = 0.58$ and $p = 0.89$). **c** As in a, all training and testing sessions ($n = 17$) were in pond water. LTM was not observed ($p = 0.11$ and $p = 17$). **d** As in a, only 1 h before training in CE snails were injected with the DNA methylation blocker 5-AZA ($n = 14$). LTM was not present 24 h ($p = 0.45$) or 4 weeks later ($p = 0.066$). Change in performances also confirmed these results (time effect: $F_{(1,102)} = 0.0069$, $p = 0.93$; treatment effect: $F_{(3,152)} = 4.21$, $p = 0.0075$; interaction time χ treatment $F_{(3,59)} = 0.3$, $p = 0.83$)

Discussion

We set out to determine how long the effects of operant conditioning training in CE persist. We trained both juvenile and adult snails in CE along with the necessary controls. Our data confirm that with training in CE there is an immediate enhancement of their ability to form LTM. Our data further show that in both experimental cohorts of snails the effect of training in CE persisted for at least four weeks. When trained with a single 0.5 h session in pond water four weeks after training in CE these snails gained the smart snail phenotype in that they possessed enhanced memory ability. The prolonged enhancement of the ability to form LTM brought about by training in CE even persisted in juvenile snails whose nervous system undergoes a maturing process in that interval. We further found that although the effects of CE persisted through the maturation process their effects were not passed on to the next generation. Finally, in adult snails injected with a DNA methylation blocker before training in CE neither the initial enhancement of memory (i.e., 24 h) nor the smart phenotype observed four weeks later was observed.

Juvenile snails form long term memory with detection of crayfish odor

Juvenile *W-strain Lymnaea* are incapable of forming LTM following operant conditioning, even if the training procedure consists of two 45 min training sessions separated by a 1 h interval (McComb et al. 2005). It was also shown that the activity of the central pattern generator (CPG) that drives aerial respiration in *Lymnaea* (Syed et al. 1990, 1992) is different in juveniles than in adults (McComb et al. 2003). It was found that RPeD1 (the neuron that initiates

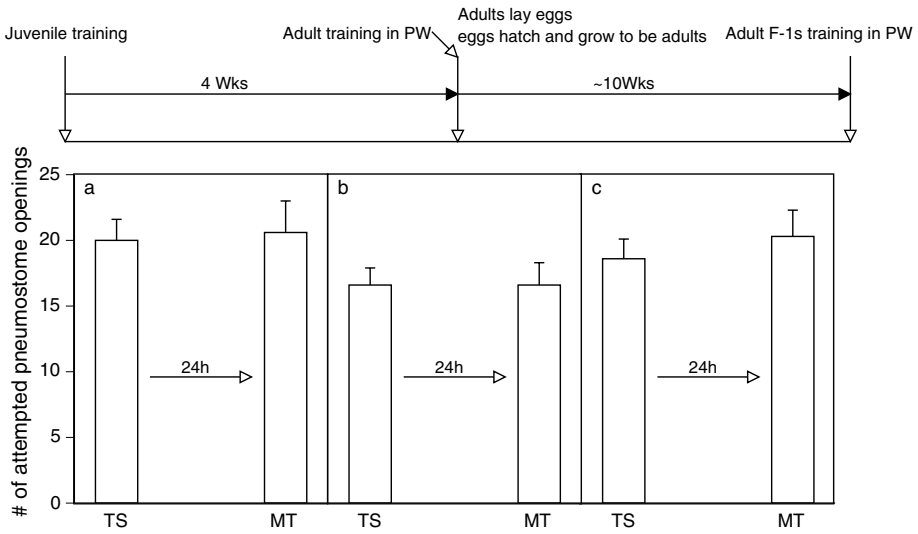

Fig. 5 Observed epigenetic changes were not inherited. F1 snails from parents shown in Figs. 2 and 3 do not exhibit the 'smart' snail phenotype. Each cohort of snails was kept separate and allowed to mate. They laid eggs and the hatchlings grew into adults. These F1 adults received a single 0.5 h TS memory was not observed 24 h later (treatment effect: $F_{(2,50)} = 1.74$, $p = 0.19$; session effect $F_{(1,50)} = 0.43$, $p = 0.52$; interaction treatment x session $F_{(2.50)} = 0.17$, $p = 0.84$). The cohort depicted in a was obtained from the juveniles originally trained in CE. The *b* cohort was the yoked control cohort; while the cohort in *c* was derived from snails exposed to CE

rhythmic neuronal activity in the CPG) in juveniles is more excitable than in adults; yet juveniles perform aerial respiration less often than in adults. Finally, there are age-dependent changes in synaptic connectivity both within the CPG and in the input from the periphery (i.e., osphradium; Il-Han et al. 2010) that favour respiratory rhythmogenesis to be expressed in adults. In adult *Lymnaea,* the peripheral pneumostome area, including the osphradial ganglion, exerts a suppressive regulatory control over the respiratory CPG (McComb et al. 2003) and this suppressive input is not present in juvenile *Lymnaea*.

The electrophysiological changes in the CPG circuit brought about by CE exposure are, however, similar in sign in both adult and juvenile snails (Sunada et al. 2010). That is, in both adults and juveniles exposure of the snail to CE causes significant reduction in RPeD1 activity. This is a key neuron necessary for learning and memory formation in *Lymnaea* (Scheibenstock et al. 2002; Sangha et al. 2003a, b) and decreased excitability in this neuron is a hallmark of LTM formation (Spencer et al. 1999, 2002; Braun and Lukowiak 2011; Braun et al. 2012). Even in 'smart' snails following operant conditioning training RPeD1 excitability is significantly decreased even further (Braun et al. 2012).

Adult snails trained as juveniles exhibit the 'smart snail' phenotype

What we have shown here is that following training in CE these 'average' snails four weeks later possesses the phenotype of 'smart' snails. They are now capable of forming LTM following a single 0.5 h training session in pond water. However, it was only with operant conditioning training in CE that conferred the 'smart' snail phenotype to these 'average' snails. Moreover, the smart snail phenotype could be prevented if a DNA methylation blocker was injected into the adult snails before training in CE. Snails receiving CE-yoked training, CE exposure only or training in pond water 4 weeks previously did not gain the competency to form LTM following a single 0.5 h training session in pond waster (i.e., the smart snail phenotype). We suggest: (1) that activity dependent training in CE brings about long-lasting changes in these snails conferring on them the smart phenotype; and (2) the ability to acquire the smart phenotype is dependent on a DNA methylation process.

The DNA methylation changes underlying enhanced memory formation do not persist into the next generation

We then went on to test a second hypothesis: The offspring of snails trained as juveniles in CE and exhibiting a smart-snail phenotype, as an adult would produce smart snail offspring. Our data were not consistent with this hypothesis. We found that the offspring of these "newly smart" snails were not 'smart'. That is, while the parents because of their training in CE, as juveniles were 'smart', this was not passed along to their offspring. Thus, in F1 snails from "newly smart" snail parents a single 0.5 h training session did not result in LTM. These F1 adult snails behaved just like the typical *W-strain* snail. It needs to be pointed out

that when the offspring of Alberta or UK naturally 'smart' snails are grown under laboratory conditions that they still retain their enhanced memory forming capabilities (Orr et al. 2009b; Dalesman and Lukowiak 2010). It was not laboratory rearing that 'dumbed' the F1 '*newly smart* snails' down; rather the enhanced memory forming capability acquired by the parents was not passed down to their offspring. The lack of heritability of the newly acquired enhanced LTM forming capability means that the epigenetic changes hypothesized to occur in neurons necessary for enhanced memory formation did not occur or did not persist in the gametes. It has been reported, however, that epigenetic changes that occur as a result of exposure to traumatic stress in life can be passed on to the next generation in mice via changes in sperm small non-coding RNAs (Gapp et al. 2014). In addition, we know that maternal dietary changes affect DNA methylation in the developing fetus in both rodents and humans leading to life-long behavioural and health outcomes (Waterland and Jirtle 2003; Dolinoy et al. 2007; Dominguez-Salas et al. 2014). While we did not see such changes in our study it is possible that with repeated exposure to CE and training such changes in the offspring might occur.

Crayfish effluent and operant conditioning training elicits epigenetic changes

A previous paper (Il-Han et al. 2010) showed that a serotoninergic (5HT) pathway, via the osphradial nerve, is activated when the snails detect a crayfish predator (i.e., CE). Using a serotonin antagonist (mianserin) the authors blocked the enhancing effect of CE on LTM formation. On the other hand, when they injected the snail with 5HT, the *W-strain* snails showed an enhanced ability to form LTM similar to what was observed with CE. The authors postulated that CE exposure and 5HT injection primed the consolidation process necessary for LTM formation via an epigenetic effect. That is, CE detection or 5HT injection ultimately lead to an epigenetic effect (e.g., DNA methylation) on the genes in neurons, such as RPeD1, which are necessary for LTM formation. More recently, Lukowiak et al. (2014) directly tested this hypothesis and showed that CE's or the injection of 5HT enhancing effect on LTM formation can be blocked using 5-AZA-2 deoxycytidine, a DNMT (DNA Methyl Transferase) inhibitor. Together these data suggest to us that in both juveniles and adults trained in CE, epigenetic changes (e.g., DNA methylation) occur that alter neuronal activity in the neurons necessary for LTM formation and that these changes persisted for at least four weeks. Further support of this hypothesis comes from our data showing that injection of the DNA methylation blocker 5-AZA before training in CE prevented the 'smart' phenotype from occurring. The idea that epigenetic changes (e.g., DNA methylation) play an important role in the formation and long-lasting maintenance of LTM was first enunciated by Griffith and Mahler in 1969. In the case of the juveniles, these changes persist through the maturation process that occurs in the circuits mediating rhythmic activity that drives aerial respiratory behaviour. We have not yet examined whether RPeD1 in these snails also possess the phenotype seen in smart snails. Experiments in the future will directly address this question.

Knowing that the enhanced LTM formation following predator detection (i.e., training in CE) was dependent on DNA methylation we asked whether the 'smart' snail phenotype seen in both juveniles and adults four weeks after training in CE would occur in 5-AZA treated snails. We found that adult snails injected with 5-AZA before training did not exhibit the 'smart' snail phenotype four weeks later. We attempted the same experiments in juvenile snails. However, in our hands the juvenile snails did not survive this treatment. We conclude based on the data from the adult snails that a DNA methylation is necessary for adult snails trained in CE to gain the 'smart' snail phenotype four weeks later.

It also needs to be remembered that LTM formation in *Lymnaea* (Sadamoto et al. 2003; Rosenegger et al. 2008, 2010; Rosenegger and Lukowiak 2010, 2013; Takigami et al. 2014) as well as in other model systems (Yu et al. 2011; Giese and Mizuno 2013; Landry et al. 2013) depends on activity of a CREB promoter that leads to altered gene expression which following new protein synthesis leads to changes in synaptic strength and intrinsic membrane properties that form the neuronal basis of LTM. In *Aplysia* it has been demonstrated that an epigenetic modification (e.g., histones) could be mediated by neurotransmitters, such as 5HT, that were involved in LTM formation (Guan et al. 2002). That is, while it has been appreciated for many years that specific neurotransmitters, such as 5HT played a necessary role in initiating an intricate cascade of kinase activity (e.g., PKA and CaMKII) that lead to changes in gene transcription and new protein synthesis that form the molecular basis of LTM (Takigami et al. 2014); it is only more recently that we've become to appreciate that epigenetic changes (DNA methylation and histone modification) also play important roles in the formation and maintenance of LTM (Holloway and Gonzalez-Maeso 2015). However, it has always been a puzzle as to how memory can persist, for in some cases, the life of a long-lived (> 90 years) organism. One hypothesis relevant to long-lasting memory first proposed in 1969 (Griffith and Mahler 1969) that is again attracting more and more attention is the idea that epigenetic changes (e.g., DNA methylation) play an important role in the formation and maintenance of LTM. The data reported here are all consistent with this notion.

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Complicance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Ethical statement We have followed all applicable University of Calgary protocols necessary for these experiments.

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