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RESEARCH ARTICLE

Heat stress enhances LTM formation in *Lymnaea*: role of HSPs and DNA methylation

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ABSTRACT

Environmentally relevant stressors alter the memory-forming process in *Lymnaea* following operant conditioning of aerial respiration. One such stressor is heat. Previously, we found that following a 1 h heat shock, long-term memory (LTM) formation was enhanced. We also had shown that the heat stressor activates at least two heat shock proteins (HSPs): HSP40 and HSP70. Here, we tested two hypotheses: (1) the production of HSPs is necessary for enhanced LTM formation; and (2) blocking DNA methylation prevents the heat stressor-induced enhancement of LTM formation. We show here that the enhancing effect of the heat stressor on LTM formation occurs even if snails experienced the stressor 3 days previously. We further show that a flavonoid, quercetin, which inhibits HSP activation, blocks the enhancing effect of the heat stressor on LTM formation. Finally, we show that injection of a DNA methylation blocker, 5-AZA, before snails experience the heat stressor prevents enhancement of memory formation.

KEY WORDS: *Lymnaea*, Long-term memory, Heat shock proteins

INTRODUCTION

In our *Lymnaea* (Linnaeus 1758) model system, we have the ability to study the causal neuronal mechanisms that underlie associative learning and memory formation. This is because the sufficiency and necessity of the neuronal circuit has been experimentally shown (Syed et al., 1990, 1992; Scheibenstock et al., 2002). *Lymnaea* satisfy their respiratory requirements using both cutaneous and aerial respiration (Lukowiak et al., 1996). This allows us to train, via operant conditioning, snails not to perform aerial respiration without harming them. In hypoxia, aerial respiration increases and with our conditioning procedure snails that learn and form memory decrease significantly this behaviour. We have previously found that there are both 'smart' and 'average' snails in regards to how quickly they can form long-term memory (LTM) following operant conditioning training (Lukowiak et al., 2010). The strain of snails that we use here has been designated as the *W-strain* and these snails are considered to be average. That is, it takes two 0.5 h training sessions to make LTM in comparison to 'smart' snails where LTM is formed following a single 0.5 h training session. In all cases, memory is operationally defined as there being a significant decrease in the number of attempted openings in the memory test session compared with the initial training session (Lukowiak et al., 1996).

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Memory formation is modified by the level of stress experienced by the animal during learning and the memory consolidation period and this has been known since the writings of Bacon in the 1620s (Bacon, 1620). The so-called Yerkes–Dodson law (i.e. an inverted-U function) describes this phenomenon quite well (see Ito et al., 2015 for a more thorough discussion of this). Stress modifies memory formation, its duration and its recall (Shors, 2004; Kim and Diamond, 2002). Importantly, in *Lymnaea*, memory formulation is modulated by environmentally relevant stressors (Lukowiak et al., 2003a,b, 2008, 2010, 2014a).

Heat is assumed to be a stressor in *Lymnaea* as many important behavioural, physiological and immunological processes in *Lymnaea* are temperature dependent (Teskey et al., 2012). It has been demonstrated that laboratory-reared *Lymnaea* prefer temperatures of around 20°C for optimal growth and mortality (Vaughn, 1953; McDonald, 1969). At higher temperatures, *Lymnaea* have reduced immune function and parasitic infestation increases (Seppala and Jokela, 2011; Hermann et al., 2013; Seppala and Leicht, 2013; Leicht et al., 2013). Thus, it is safe to assume that higher temperatures are an ecologically relevant stressor for *Lymnaea*.

Conversely, a heat stressor (30°C for 1 h) enhances LTM formation in *Lymnaea* (Teskey et al., 2012). That is, snails experiencing the heat stressor formed LTM following a single 0.5 h training session, while LTM was not formed in control experiments (i.e. no exposure to the heat shock). Later, it was found that there was a time-related up-regulation of the heat shock protein gene transcripts HSP40 and HSP70 in *Lymnaea* neurons isolated from the *W-strain* snails (Foster et al., 2015). Both HSP40 and HSP70 were rapidly induced in the CNS (within 30 min of the end of heat stress) and reached maximum levels of expression between 2 and 4 h later. This suggested to us that the enhancing effects of the heat stressor on LTM formation might persist for hours after snails experienced the stressor. In the golden apple snail (*Pomacea canaliculata*), the effects of a similar heat shock (i.e. up-regulation of HSPs) persist for days (Song et al., 2014). Here, we performed experiments designed to determine whether the enhancing effect of the heat shock on memory formation persists over time. In addition, because the heat stressor up-regulated HSPs in neurons, we tested whether induction of the HSPs is a necessary step in the enhancement of LTM. That is, would blocking their up-regulation using the flavonoid quercetin (Hosokawa et al., 1990; Jakubowicz et al., 2008) prevent memory enhancement? Finally, a number of different manipulations in *Lymnaea* that cause enhancement of LTM (e.g. predator detection) or lengthen the persistence of LTM have been shown to be dependent on DNA methylation (Lukowiak et al., 2014b). Changes in DNA methylation state can cause memory duration to change (Chahrour et al., 2008; Landry et al., 2013). For example, use of a DNA methylation blocker, 5-AZA, alters the persistence of LTM in *Lymnaea* (Lukowiak et al., 2014a,b). Our

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working hypothesis was that a stressful stimulus such as a thermal stressor alters the state of DNA methylation in neurons necessary for LTM formation. We tested this hypothesis by injection of 5-AZA into snails (Lukowiak et al., 2014b) before they experienced the heat shock, and determined whether enhanced LTM formation occurred.

MATERIALS AND METHODS

Snails

Lymnaea stagnalis, originally obtained from Vrije Universiteit, Amsterdam, that originated from a strain of snails collected from canals in polders near Utrecht, The Netherlands, in the 1950s, were raised in the Biological Sciences snail facility at the University of Calgary. We have designated these snails as the *W-strain* of *Lymnaea.* We have done this because it is apparent that different strains of *Lymnaea* possess differing abilities to form memory following operant conditioning of aerial respiration, and it is important to easily be able to identify which strain of snail is being used in each study. Adult snails (with a shell length of 2.5–3.0 cm) were maintained in eumoxic (i.e. normal O_2 levels; P_{O_2}>9975 Pa) aquaria in artificial pond water (distilled water containing 26 g l^{-1} Instant Ocean, Spectrum Brands, Madison, WI, USA). Additional calcium sulphate dehydrate was added to create what we refer to as a standard calcium level of 80 mg l^{-1} (Dalesman and Lukowiak, 2010; Knezevic et al., 2011). Snails were maintained at room temperature (~20°C) and fed Romaine lettuce *ad libitum*.

The heat stressor procedure

A tank of water heated to and maintained at 30°C served as a water bath in which a 1 litre beaker filled with 500 ml of 30°C pond water was placed. Up to 12 snails were kept in the beaker for a period of 1 h. This is referred to as the heat stress. The heat stress was given to snails at various times before training, as noted in each figure. In the intervals between receiving the heat stress and training, snails were maintained at room temperature (~20°C).

Operant conditioning

Operant conditioning training, a form of associative learning, to reduce aerial respiration in hypoxic conditions was carried out using two different training and testing procedures. In the first procedure, snails received a single 0.5 h training session (TS1; Sangha et al., 2003; Parvez et al., 2005; Orr and Lukowiak, 2008; Teskey et al., 2012) and then a memory test session (MT) was performed 24 h after the TS1. In the second procedure, snails received two 0.5 h training sessions separated by a 1 h interval (i.e. TS1 and TS2) and then received a memory test session (MT) at various times after TS2. N_2 was bubbled vigorously through 500 ml of artificial pond water in a 1 litre beaker for 20 min to make the water hypoxic. After 20 min, the bubbling was turned down to a low level to maintain hypoxia while not disturbing the snails. Snails were placed in the beaker and allowed to acclimate for 10 min prior to training. During the 0.5 h training session, the snail was gently poked on the pneumostome using a wooden stick each time it attempted to open the pneumostome to perform aerial respiration. This 'poke' resulted in the closing of the pneumostome but not a full body withdrawal response. At the end of the training session, the snail was returned to its home aquarium in eumoxic pond water maintained at room temperature (~20°C). Snails were then tested for LTM formation 24 h following TS1 in the case of a single training session or TS2 when the second training procedure was used (e.g. 24 h or 5 days later). The 0.5 h memory test session (MT) was carried out in exactly the same way as the training session. The criterion for LTM formation was that the number of attempted pneumostome openings in the memory test session was significantly lower than the number of attempted openings in the TS1 for the single training session procedure and significantly lower than the number in TS1 and not significantly greater than that in TS2 when the second training procedure was used (Lukowiak et al., 1996, 1998, 2000).

Drugs

Quercetin and 5-AZA (5-aza-2′-deoxycytidine) were obtained from Sigma Chemical Company (St Louis, MO, USA). 5-AZA was dissolved in sterile saline while quercetin was dissolved in 0.1% DMSO. Based on previous studies (Lukowiak et al., 2014b), we used a dose of 87 µmol l^{-1} 5-AZA. 5-AZA is a DNA methyltransferase inhibitor and has been successively used previously in *Lymnaea*. Because 5-AZA does not diffuse across the skin of the snail, we had to inject it into the haemocoel through the foot. To do this, we cooled the snails down in ice-pond water prior to injection. The injection of saline in a similar manner does not alter the behaviour of the snail. Pilot experiments led us to use quercetin at a concentration of 100 µmol l^{-1} added to the pond water in each experiment.

Statistics

A paired *t*-test was used to assess the number of attempted pneumostome openings during the training session (TS) and the memory test (MT) in the case of the single 0.5 h operant conditioning training procedure. This test was also employed to determine whether quercetin altered normal homeostatic breathing behaviour. Results were considered significant (i.e. LTM was judged to be present) if snails significantly reduced the number of attempted pneumostome openings between MT and TS. When we utilized the two 0.5 h training session procedure and tested memory 24 h or 5 days later, an ANOVA was used followed by a *post hoc* Tukey's multiple comparison test.

RESULTS

We first confirmed that we could replicate the data previously obtained (Teskey et al., 2012) showing that the 1 h heat stress at 30°C caused an enhancement of LTM formation in the *W-strain* of snails (Fig. 1). Thus, two cohorts of naive snails (N=20) received a single 0.5 h training session (TS1). The first cohort (Fig. 1A) did not receive the heat stressor, whilst the second cohort (Fig. 1B) did. As can be seen, the control snails (Fig. 1A) did not exhibit LTM. That is, the number of attempted pneumostome openings in MT was not significantly different from that in TS1. In contrast, the snails that received the heat stressor 1 h before the single 0.5 h training session (TS1) exhibited LTM 24 h later (Fig. 1B). That is, the number of attempted openings in MT was significantly lower than that in TS1. Thus, we confirmed the previous findings of Teskey et al. (2012) regarding enhancement of LTM formation by the heat stressor.

We next determined whether a similar LTM enhancement occurred with the two 0.5 h training session procedure (Fig. 2). In the absence of the heat stressor, such training resulted in LTM persisting 24 h but not 48 h (Fig. 2A). Subjecting a naive cohort of snails to the heat stressor 1 h before this training procedure resulted in LTM that persisted for at least 5 days (Fig. 2B). That is, the number of attempted pneumostome openings in the 5 day MT was significantly lower than that in TS1 and not significantly greater than that in TS2. Thus, we conclude that the heat stressor enhances the LTM resulting from the both the single and the two 0.5 h training session procedures.

In the above two experiments, training commenced 1 h after snails received the heat stress. Next, we questioned whether an

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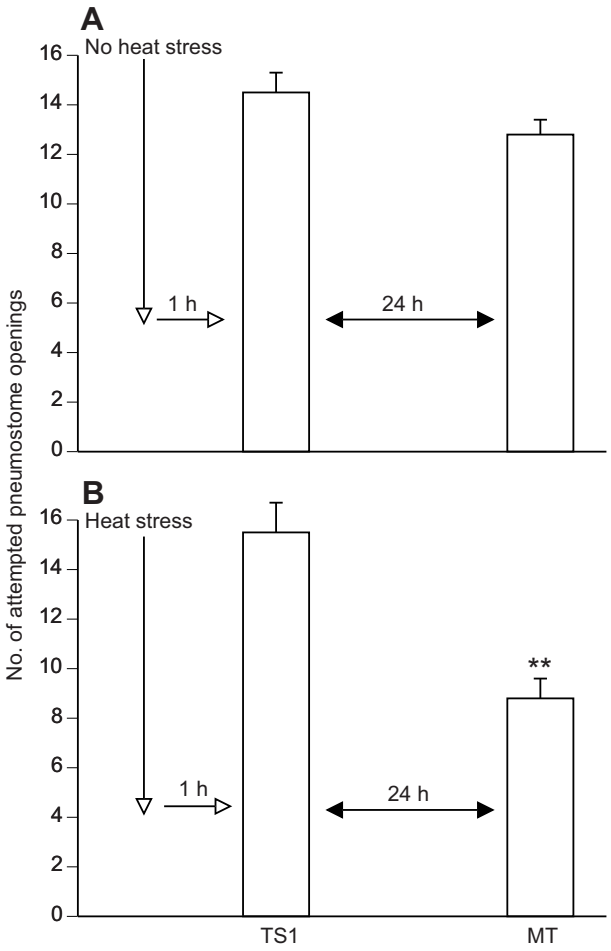

Fig. 1. A heat stressor enhances long-term memory (LTM) formation. (A) Naive *Lymnaea* (*N*=20) received a single 0.5 h training session (TS1) and were tested for LTM 24 h later (memory test, MT). LTM was not present as the number of attempted pneumostome openings in MT was not significantly lower than the number in TS1 (t=0.53, P>0.05). (B) A second cohort of naive snails (*N*=20) experienced the heat stressor (30°C for 1 h) 1 h before receiving the single 0.5 h training session. Memory was tested 24 h later (MT). LTM was present as the number of attempted pneumostome openings was significantly lower than the number in TS1 (t=3.78, P>0.01). Data are means+s.e.m. (**P<0.01).

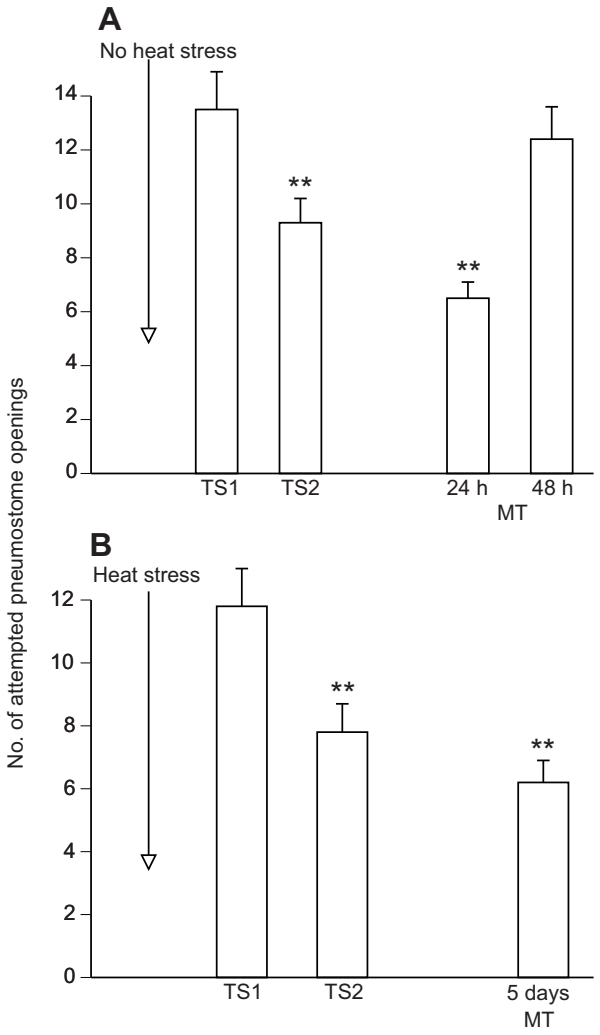

Fig. 2. A heat stressor applied to the typical training procedure results in a 5 day memory. (A) A naive cohort of snails (*N*=20) was trained with the two 0.5 h training session procedure and memory was tested 24 h (*N*=10) and 48 h (*N*=10) later (ANOVA, $F_{2,40}$=13.33, P<0.01). Snails were only tested for memory once. LTM was present in the 24 h MT session as the number of attempted pneumostome openings in TS2 was significantly lower than that in TS1, and the number in the 24 h MT was not significantly greater than that in TS2 (Tukey's multiple comparison test, P<0.01). However, when snails were tested for LTM at 48 h rather than 24 h after TS2, LTM was not present (Tukey's multiple comparison test, P>0.05). That is, while the number of attempted pneumostome openings in TS2 was significantly lower than that in TS1, the number at the 48 h MT was significantly larger than that at TS2 and not significantly different from that at TS1. Thus, in these snails, the typical training procedure did not result in a 48 h LTM. (B) Another cohort of naive snails (*N*=18) was first subjected to the heat stressor and then 1 h later received the two 0.5 h training session procedure. Memory was tested 5 days later. As can be seen, LTM was present (AVOVA, $F_{2,34}$=4.063, P<0.01). A Tukey's *post hoc* test showed that the number of attempted pneumostome openings in MT was significantly lower than that in TS1 (P<0.01) and was not significantly different from that in TS2 (P>0.05). Data are means+s.e.m. (**P<0.01).

interval ranging from 1 to 3 days between experiencing the heat stressor and training would result in enhanced LTM formation (Fig. 3). Three different naive cohorts of snails were used. Each cohort of snails (*N*=20 in each cohort) experienced the heat stress and then training occurred 1–3 days later. In this interval, snails were maintained at room temperature and the subsequent training and memory test sessions were all performed at room temperature. As can be seen, the heat stressor retained its ability to enhance LTM formation following a 1, 2 and 3 day interval. In all three cohorts, the number of attempted openings in MT was significantly lower than the number in TS1. Thus, when we trained snails with the single 0.5 h training procedure up to 3 days after they experienced the heat stressor, we found that these snails exhibited enhanced LTM-forming capabilities. Notice, however, in each of the three cohorts the number of attempted pneumostome openings in each of the single 0.5 h training sessions, while similar to each other, is somewhat less than that in the other figures. This is because a single experimenter performed those three training and test sessions and had a stronger poke than the other experimenters. It is well known that different experimenters often poke differently. It is why a single experimenter trains and tests for memory.

Having shown that the application of the heat stressor causes an enhancement of LTM formation even if training occurred days after exposure to the stressor, we asked whether an epigenetic process such as DNA methylation played a role in LTM enhancement. Building on previous data showing that DNA methylation is

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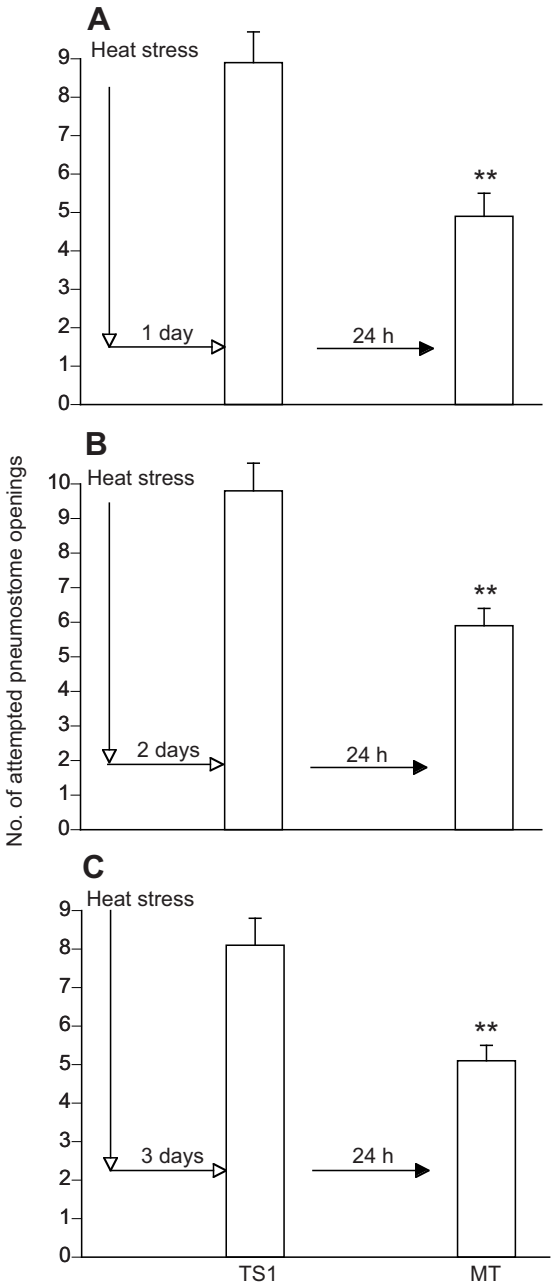

Fig. 3. A heat stressor experienced days previously has the ability to enhance LTM formation. Three naive cohorts (*N*=20 in each cohort) experienced the heat stressor for 1 h and then each was returned to their home aquarium for 1 day (A), 2 days (B) or 3 days (C) at room temperature. Each cohort received the single 0.5 h training procedure (TS1) and was tested for LTM 24 h later (MT). In the 1, 2 and 3 day cohorts, the number of attempted pneumostome openings in MT was significantly lower than that in TS1 (1 day, t=3.158, d.f.=19, P<0.01; 2 days, t=3.015, d.f.=19, P<0.01; and 3 days, t=3.213, d.f.=19, P<0.01), thus demonstrating that LTM formed. Data are means+s.e.m. (**P<0.01).

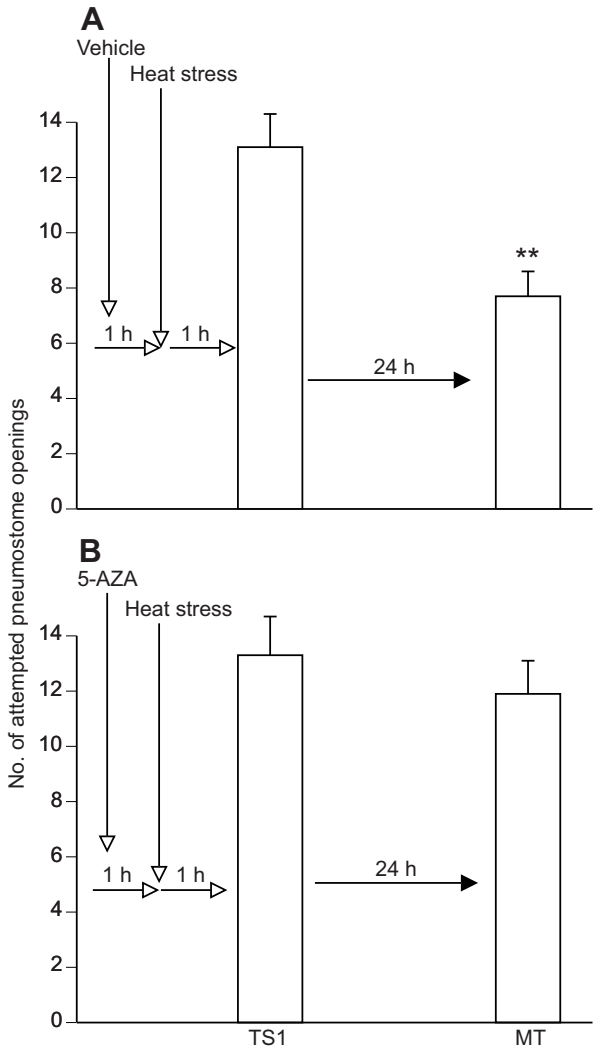

Fig. 4. A DNA methylation blocker impedes the heat stressor-induced enhancement of LTM. (A) In this control experiment, the saline vehicle was injected into snails (*N*=15) 1 h before they received the heat stressor. Then, 1 h after experiencing the heat stress, snails received the single 0.5 h training session (TS1) and LTM was tested 24 h later (MT). The number of attempted pneumostome openings in MT was significantly lower than that in TS1 (t=2.583, d.f.=14, P=0.02). (B) Same procedure as in A, except 5-AZA (87 μmol l^{-1}) was injected into snails (*N*=16), rather than the vehicle. As can be seen, LTM was not formed (t=0.7279, d.f.=15, P>0.05). Data are means+s.e.m. (**P<0.01).

involved in enhancement of LTM formation with other stressors such as predator exposure (Lukowiak et al., 2014b), we utilized a similar strategy here (Fig. 4). As 5-AZA (a DNA methylation blocker used previously in *Lymnaea*) must be injected into snails, we first performed a saline injection control. A cohort of naive snails (*N*=15) was injected with vehicle 1 h before the heat stressor was given. One hour after the heat stressor, snails received a single 0.5 h training session (TS1). Memory was then tested 24 h later. As can be seen (Fig. 4A), these snails exhibited enhanced memory formation showing that the injection of vehicle did not disrupt the ability of the heat stressor to cause memory enhancement. Another cohort of naive snails (*N*=16) was then used, except these snails were injected with 5-AZA 1 h before the heat stressor (Fig. 4B). The snails were then trained 1 h later (TS1); the criterion for LTM formation was not reached at the subsequent MT. Thus, 5-AZA injection blocked the heat stressor-induced memory enhancement.

We then proceeded to perform three further control experiments (Fig. 5) to show that it was only the 5-AZA injection before the heat stressor that prevented LTM enhancement. In the first control group (*N*=18; Fig. 5A), the cohort received the 5-AZA injection immediately (i.e. 5 min) after experiencing the heat stressor. They were then trained 1 h later. We found that LTM was present. In the

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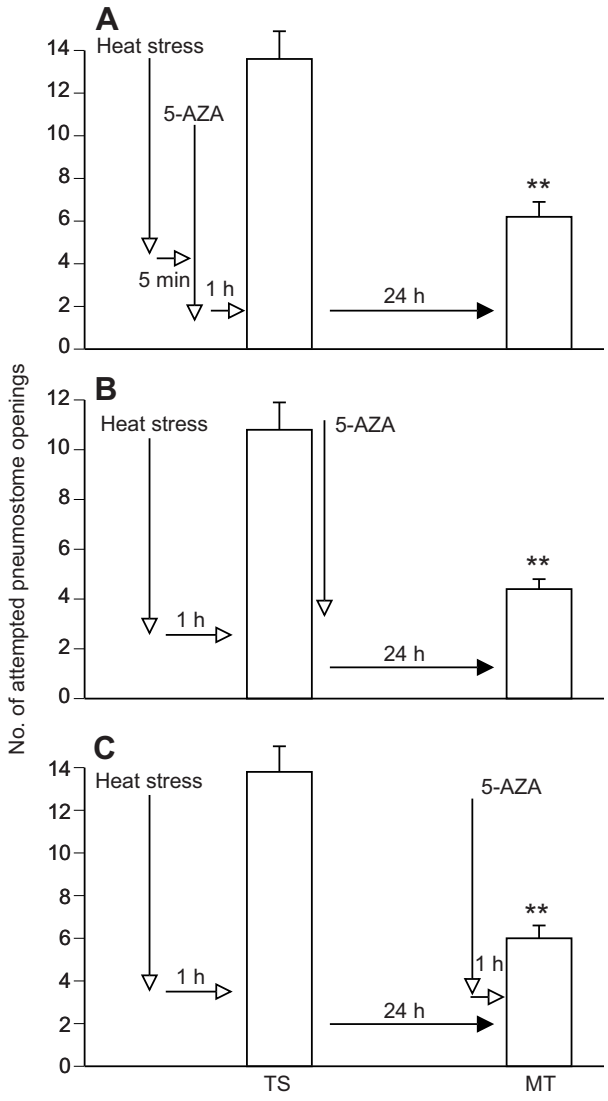

Fig. 5. The DNA methylation blocker 5-AZA only impedes LTM formation when injected before the heat stressor is experienced. (A) In a naive cohort of snails (N=18), the heat stressor was experienced 5 min before snails were injected with 5-AZA. One hour following the injection of 5-AZA, snails received the single 0.5 h testing procedure. In these snails, LTM was formed as the number of attempted pneumostome openings in MT was significantly lower than the number in TS1 (t=5.673, d.f.=17, P<0.01). (B) As in A, except the 5-AZA was injected into snails (N=10) immediately after the single 0.5 h training session (TS1). As can be seem, LTM was formed as the number of attempted openings in MT was significantly lower than that in TS1 (t=5.580, d.f.=9, P<0.01). (C) As in A and B, except 5-AZA was injected into snails (N=17) 1 h before the memory test session (MT). The number of attempted openings in MT was significantly lower than that in TS1 (t=7.469, d.f.=16, P<0.01); thus, LTM formed. Data are means+s.e.m. (**P<0.01).

second control experiment (N=10; Fig. 5B), 5-AZA was injected into the snails immediately after the TS (i.e. after both the heat stressor and the training session). In these snails we found that LTM was present 24 h later. Thus, the injection of 5-AZA after snails experienced the heat stressor and the training session did not block the LTM enhancement. In the final control group (N=17; Fig. 5C), naive snails received the heat stressor and then were trained 1 h later. In these snails, 5-AZA was injected 1 h before the memory test session. As can be seen, LTM was present. Thus, we conclude that it was only with the injection of 5-AZA 1 h before (i.e. as in Fig. 4) the

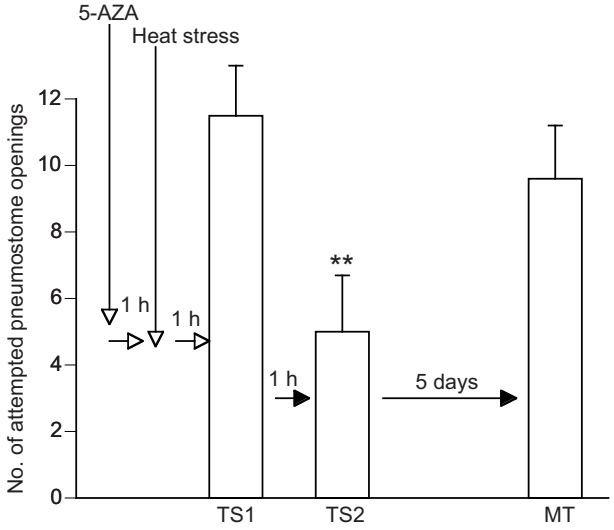

Fig. 6. Injection of 5-AZA before the heat stressor prevents enhancement of LTM formation. Snails (N=12) were injected with 5-AZA 1 h before experiencing the heat stressor. One hour later, they received the two 0.5 h training session procedure and were tested for LTM 5 days later (ANOVA, $F_{11,22}$=5.339, P<0.01). The number of attempted pneumostome openings in the MT was not significantly different from that in TS1 (Tukey's test, P>0.05). Data are means+s.e.m. (**P<0.01).

snails were exposed to the heat stressor that the heat-induced LTM enhancement was blocked.

As shown in Fig. 2B, snails that received the heat stressor and the two 0.5 h training session procedure exhibited LTM 5 days after TS2. We repeated this experiment, but this time snails (N=12) received a 5-AZA injection 1 h before the heat stressor (Fig. 6). When tested for LTM 5 days later, LTM was not observed. Thus, the injection of 5-AZA 1 h before the application of the heat stressor blocked LTM enhancement. All these data (Figs 4–6) are consistent with the hypothesis that the heat stressor causes LTM enhancement involving a DNA methylation process.

It was recently shown (Foster et al., 2015) that the heat stressor caused an increase in the HSP gene transcripts HSP40 and HSP70 in the CNS of the snails. These data are consistent with the hypothesis that these and other 'altered genes' (i.e. activated or suppressed) are necessary for LTM formation. To test the hypothesis that HSPs activated by the heat shock play a role in memory enhancement, we performed experiments utilizing the flavonol quercetin. This flavonol has been shown to block, among other molecular processes, the activation of HSPs (Hansen et al., 1997; Zanini et al., 2007). Here, we first confirmed that quercetin did not significantly alter normal aerial respiratory behaviour (Fig. 7). Quercetin (100 µmol l^{-1}) did not significantly alter either total breathing time (Fig. 7A) or the number of pneumostome openings (Fig. 7B) when snails were placed in control hypoxic pond water and hypoxic pond water containing quercetin. These data are consistent with the notion that experiencing the hypoxic pond water in and of itself does not elicit HSPs necessary for this normal homeostatic behaviour. Thus, any effect we see as a result of quercetin altering the enhancement of LTM formation following the heat stressor would not be due to quercetin's alteration of normal aerial respiratory behaviour. We next asked whether, in the absence of the heat stressor, quercetin would block LTM formation (Fig. 8A). Quercetin pretreatment did not block LTM formation. We then placed another cohort of naive snails (N=16) in quercetin pond water (100 µmol l^{-1}) for 1 h before the heat stress. After 1 h

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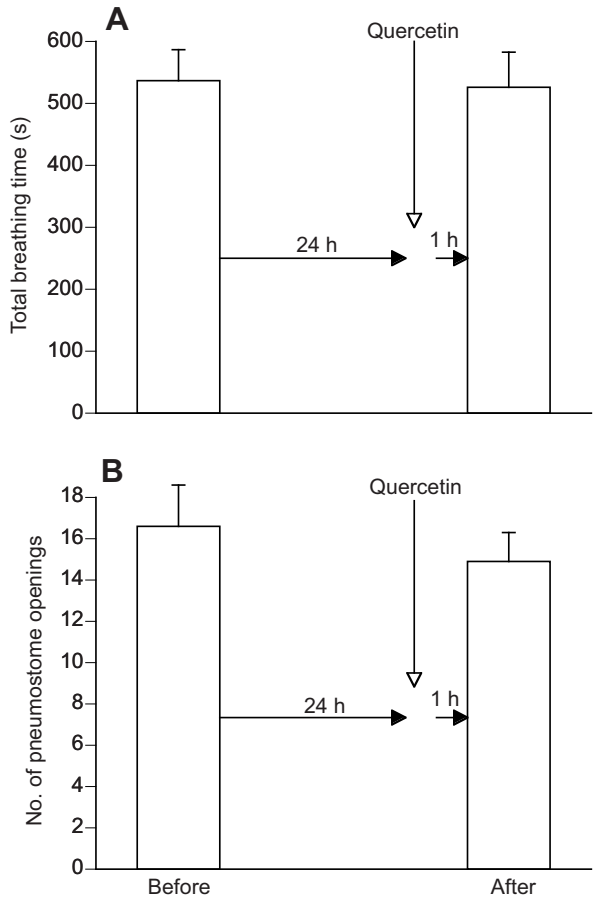

Fig. 7. Quercetin does not alter normal homeostatic aerial respiratory behaviour. The breathing behaviour in snails (N=16) in hypoxic pond water [A, total breathing time; B, number of pneumostome openings (breaths)] before and after a 1 h exposure to quercetin pond water (100 μmol l^{-1}). Total breathing time was not significantly different following quercetin pond water exposure (t=0.19, d.f.=15, P<0.05). Likewise, the number of breaths was not different after 1 h in quercetin pond water (t=1.1, d.f.=15, P<0.05). Data are means+s.e.m. (**P<0.01).

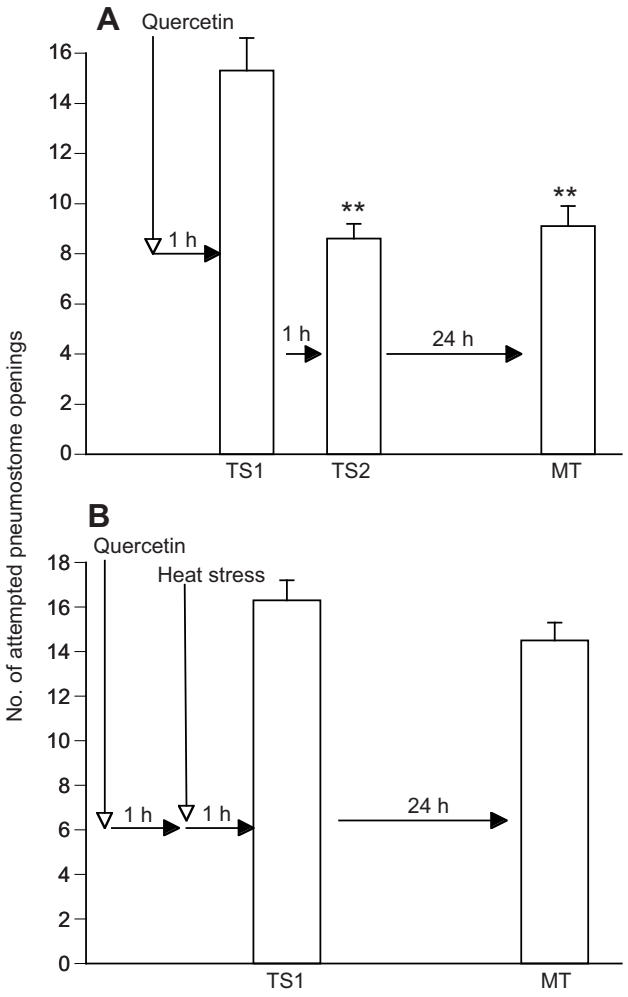

Fig. 8. Quercetin blocks the heat stressor-induced enhancement of LTM. (A) A naive cohort of snails (N=16) pretreated in quercetin pond water (100 μmol l^{-1}) for 1 h before receiving the two 0.5 h training session procedure exhibited LTM when tested 24 h after TS2 (ANOVA, $F_{15,30}$=4.25, P<0.01, Tukey's test, TS1 versus MT, P<0.01). (B) Snails (N=15) received a 1 h exposure to quercetin (100 μmol l^{-1}) before being exposed to the heat stressor. They then received the single 0.5 h training procedure. LTM was tested 24 h later (MT). The number of attempted pneumostome openings in MT was not significantly different from that in TS1 (t=1.618, d.f.=14, P>0.05). Thus, quercetin blocked the heat stressor's ability to enhance LTM. Data are means+s.e.m. (**P<0.01).

we applied the single training session procedure to the snails (TS1; Fig. 8B). When we tested for LTM 24 h later, we did not observe LTM. Thus, quercetin pre-treatment blocked the heat stressor-induced LTM enhancement.

To more strongly support our hypothesis that HSPs play a role in the heat stressor-induced memory enhancement, we performed two further control experiments. In the first (Fig. 9A), we subjected snails to the heat stressor then waited 3 h before quercetin treatment. We then trained snails using the single 0.5 h training procedure and tested for LTM 24 h later. As can be seen, LTM was present. Thus, quercetin applied after the heat stress did not block LTM enhancement. Finally (Fig. 9B), we performed a similar experiment to the one showing that quercetin blocked LTM enhancement, only we used another flavonol, epicatechin. We have previously shown that this flavonol causes LTM enhancement (Fruson et al., 2012; Knezevic and Lukowiak, 2014). Snails (N=16) were placed in epicatechin pond water for 1 h, and then given the heat shock and then 1 h later received the single 0.5 h training procedure. When we tested the snails 24 h later, we found LTM to be present. Thus, epicatechin does not block the enhancing effect of the heat stressor on LTM formation, but quercetin does.

DISCUSSION

In this study, we confirmed previous data (Teskey et al., 2012) that exposing *Lymnaea* to a brief period of heat (1 h at 30°C; the heat stressor) before operant conditioning training enhances LTM formation. In the previous study, only a single 0.5 h training session was used and the maximum amount of time separating the heat stressor from the training session was 4 h. Here, we extend these findings in two important aspects. First, we showed that even with a 3 day interval between experiencing the stressor and training, LTM enhancement occurred. Second, memory enhancement was also demonstrated with the two 0.5 h training session procedure. In this case, LTM persisted for at least 5 days. Typically, the two 0.5 h training session procedure in *W-strain* snails results in a 24 h but not 48 h memory (Lukowiak et al., 2000; Sangha et al., 2003). In addition to showing prolonged memory persistence following the heat stressor, our data strongly implicate DNA methylation and HSP

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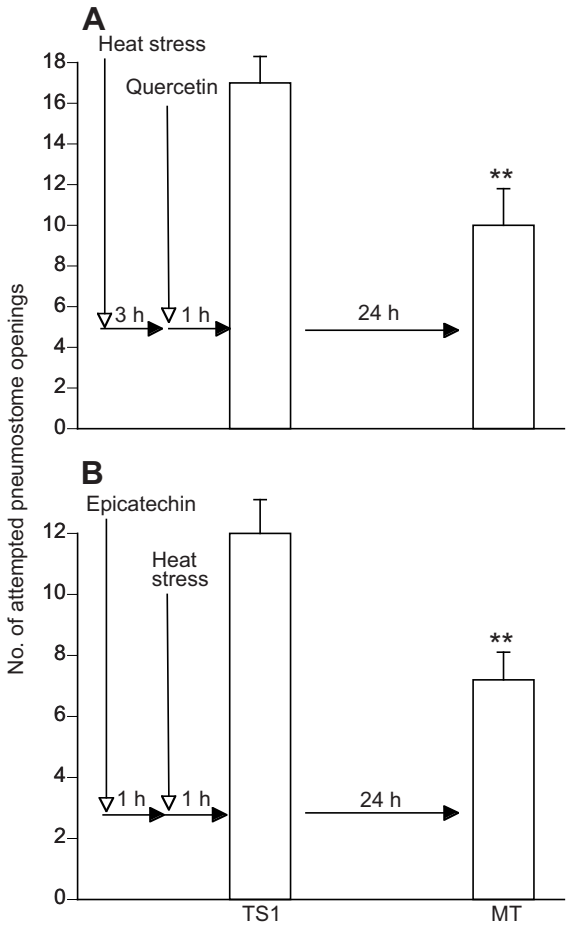

Fig. 9. Exposure to quercetin after the heat stressor and exposure to epicatechin does not alter the heat stressor's ability to enhance LTM formation. (A) A naive cohort of snails (N=15) experienced the heat stressor and then 3 h later was treated with quercetin (100 μmol l^{-1}) for 1 h. The snails then received the single 0.5 h training procedure (TS1) and were tested for LTM 24 h later (MT). In this experiment, the heat stressor enhanced LTM formation as the number of attempted openings in MT was significantly lower than that in TS1 (t=2.182, d.f.=14, P<0.01). (B) Snails (N=16) were pre-treated with epicatechin (50 μmol l^{-1}) for 1 h before experiencing the heat stressor. One hour following the heat stressor, they received the single 0.5 h training procedure (TS1) and were tested for LTM 24 h later (MT). LTM formed as the number of attempted openings in MT was significantly lower than that in TS1 (t=3.0, d.f.=15, P<0.01). Data are means+s.e.m. (**P<0.01).

activation as necessary for this stressor-induced enhancement of memory.

As mentioned in the Introduction, heat alters many important homeostatic functions in *Lymnaea* including immune system functioning, mortality and reproduction (Vaughn, 1953; McDonald, 1969, 1973; Seppala and Jokela, 2011). In addition, the higher temperatures increase parasite virulence in snails (Seppala, and Leicht, 2013; Leicht et al., 2013). *Lymnaea* that have experienced an experimental 'heat wave' (i.e. maintained at 25°C for up to 11 days) show reduced immune function after 7 days and within the first 7 days of the 'heat wave' their growth and reproductive functions increase (i.e. number of snails that laid eggs and the total number of eggs laid; Leicht et al., 2013). Snails also reduce their innate response to predation when reared at higher environmental temperatures (Dalesman and Rundle, 2010). We do not know, however, whether freshly collected *Lymnaea* from ponds experiencing temperature fluctuations from 4 to 29°C, as we have measured near Calgary in the summer, have the same heat intolerance as laboratory-reared snails. This question will be examined in the future.

In *W-strain Lymnaea*, acute heat stress of 30°C for 1 h was sufficient to increase the synthesis of HSPs above constitutive levels (Foster et al., 2015). In other molluscan species (e.g. the golden apple snail, *Pomacea canaliculata*, or Pacific oysters, *Crassostrea gigas*), heat shock similar to that employed here was sufficient to up-regulate HSPs (e.g. HSP70) for days (Clegg et al., 1998; Cheng et al., 2007; Song et al., 2014). The up-regulation of the HSPs was hypothesized to confer thermotolerance in these molluscs for up to 4 days following the heat stressor. We have not ascertained whether the heat shock employed here is effective in inducing heat tolerance in *Lymnaea.* We do know that it causes enhancement of LTM formation for days following the stressor. As mentioned above, heat shock has negative effects, for example, on the immune and reproductive systems in *Lymnaea.* We are uncertain whether the ability to make memory faster and longer lasting, which we consider a positive effect, offsets the change in immunity. In laboratory conditions, it probably is not too detrimental to have a decrease in immune function; however, in field conditions, we are not so certain. As mentioned above, we will attempt to determine whether field-collected *Lymnaea* respond in a similar manner to a heat shock and whether there are negative effects on survivability, reproductive success or ability for enhanced memory formation.

HSP induction involves both transcription and translational processes (Hosokawa et al., 1990). Quercetin appears to alter a transcription factor (heat shock factor 1) so that HSP induction by a stressor is blocked. The HSPs are typically thought of as a class of protein chaperones (i.e. they help to preserve proper protein function). Thus, quercetin will be effective in blocking the negative effects of certain stressors on memory formation if those stressors work via an elaboration of HSPs (Mohammadi et al., 2014). In this regard, Jung et al. (2010) showed that quercetin impaired memory formation in mice by affecting a transcription factor, CREB, that has been shown to play a key role in LTM formation across species (Costa-Mattioli et al., 2009). We know that CREB activation is necessary for LTM formation in *Lymnaea* (Sadamoto et al., 2004). We can conclude that quercetin prevents the enhancing effect of the heat stressor on LTM formation. However, it only blocks LTM enhancement if it is applied before snails experience the heat stressor. Applying quercetin at other times did not block enhancement of LTM formation. Furthermore, not all flavonoids block the heat stressor-induced LTM enhancement. We found that another flavonoid, epicatechin, given before the heat stressor did not block LTM enhancement. As shown in Fig. 9, epicatechin, unlike quercetin, on its own alters LTM formation. Epicatechin enhances LTM (Fruson et al., 2012) and in addition can overcome the suppressive effects on memory formation of low environmental calcium (Knezevic et al., 2011) on LTM formation (Knezevic and Lukowiak, 2014). Thus, we are confident that the effects of quercetin on blocking enhancement of LTM formation are due to the ability of quercetin to block the initiation of HSP production.

Previously, we showed that the DNA methyltransferase blocker 5-AZA blocked the enhancing effects of predator detection on LTM formation in *Lymnaea* (Lukowiak et al., 2014b). In that study, it was concluded that changes in DNA methylation state play a key role in how stressors like predator detection enhance LTM formation and its persistence. It was suggested that stress may modulate memory formation by affecting DNA methylation. The data we obtained here showing that 5-AZA blocked the heat stress-induced LTM enhancement further support the hypothesis that stressors may have their effect on LTM formation via a DNA methylation process.

However, more experiments on many other stressors will need to be performed before we can begin to accept this hypothesis in *Lymnaea*. Our working hypothesis is that the heat stressor enhances LTM formation via DNA methylation changes. Whether these changes in the state of DNA methylation involve the HSPs activated by the stressor remains to be determined.

In conclusion, the data obtained here are consistent with our hypothesis that the heat stressor enhances LTM formation in part by causing the production of HSPs. In addition, because the interval between when snails experience the heat stress and the enhancing effect this stressor has on LTM formation is of the order of days, the hypothesis that it involves epigenetic mechanisms has credence, especially as a DNA methylation blocker given before the exposure to the heat stressor blocks enhancement of LTM. To our knowledge, an interaction between epigenetic processes such as DNA methylation and the elaboration of HSPs as the result of a heat stressor has not been studied in regards to the formation of LTM.

Competing interests

The authors declare no competing or financial interests.

Author contributions

H.S. performed most of the quercetin and 5-AZA experiments, but was helped by H.R., Y.K. and K.L. in blind experiments. E.d.F., C.S., E.S., A.P. and T.S. all performed various heat experiments and controls. Ken Lukowiak designed the experiments and wrote the manuscript with input from all the other authors.

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