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RESEARCH ARTICLE

Long-term memory of configural learning is enhanced via CREB upregulation by the flavonoid quercetin in *Lymnaea stagnalis*

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ABSTRACT

Animals respond to acute stressors by modifying their behaviour and physiology. The pond snail *Lymnaea stagnalis* exhibits configural learning (CL), a form of higher order associative learning. In CL snails develop a landscape of fear when they experience a predatory cue along with a taste of food. This experience results in a suppression of the food response; but the memory only persists for 3 h. *Lymnaea* has also been found to upregulate heat shock proteins (HSPs) as a result of acute heat stress, which leads to the enhancement of memory formation. A plant flavonoid quercetin blocks the upregulation of HSPs when experienced prior to heat stress. Here, we used this blocking mechanism to test the hypothesis that HSP upregulation plays a critical role in CL. Snails experienced quercetin prior to CL training and surprisingly instead of blocking memory formation it enhanced the memory such that it now persisted for at least 24 h. Quercetin exposure either prior to or after CL enhanced long-term memory (LTM) up to 48 h. We quantified mRNA levels of the transcription factor CREB1 in the *Lymnaea* central nervous system and found LymCREB1 to be upregulated following quercetin exposure. The enhanced LTM phenotype in *L. stagnalis* was most pronounced when quercetin was experienced during the consolidation phase. Additionally, quercetin exposure during the memory reconsolidation phase also led to memory enhancement. Thus, we found no support of our original hypothesis but found that quercetin exposure upregulated LymCREB1 leading to LTM formation for CL.

KEY WORDS: Associative learning, Transcription factor, Consolidation, Reconsolidation

INTRODUCTION

Fear induced by predation threat can lead to long-term changes in behaviour, physiology and memory in animals (Barbosa and Castellanos, 2005; Lima, 1998; Lima and Dill, 1990). Prey animals have evolved various anti-predatory strategies to overcome the threat from predation and these rely on multiple cues. Associative learning plays an important role in recognising cues that signal the presence of a predator as it enables organisms to form correlations between stimuli and consequently avoid potential danger (Maren et al., 2013). However, memory for such an acute stressor depends on the cost–benefit associated with the fear cue, especially when a threat is coupled with a positive resource that promotes growth and survival, such as food. Such a phenomenon occurs in the pond snail *Lymnaea stagnalis*, which shows a higher order associative learning called configural learning (CL). In CL, after experiencing a food cue along with a predator cue, snails avoid the food in subsequent encounters (Kagan and Lukowiak, 2019; Swinton et al., 2019). The strong predatory cue results in the formation of a 'landscape of fear' (Laundre et al., 2010) and, under such circumstances, the food cue that was experienced simultaneously with the predator cue now acts as a threat stimulus. That is, the food cue now signals danger and as such enhances long-term memory (LTM) formation in a behaviour not concerned with eating (i.e. operant conditioning of aerial respiratory behaviour; Swinton et al., 2019). Previously, it was shown that predator detection in *L. stagnalis* enhanced LTM formation when snails were operantly conditioned in the presence of a predatory cue (i.e. crayfish effluent, CE) as now a single training instead of two was sufficient to form LTM (Orr and Lukowiak, 2008). Thus, following CL in *Lymnaea*, the food odour signalled a predator threat and caused enhanced LTM formation. Importantly, when the food odour was paired with CE in the CL training procedure, only an intermediate-term memory (ITM, lasting ∼2–3 h) formed, i.e. LTM did not form. This may be because suppressing feeding behaviour after predator detection for a longer period of time does not have a positive benefit for the snails.

When exposed to environmental stressors such as predation or temperature shifts, organisms respond not only by changing their behaviour but also by rapidly synthesizing a suite of proteins called heat shock proteins (HSPs) (Lindquist and Craig, 1988). These HSPs are synthesized to protect/repair other proteins and aid in their correct folding, as they might become denatured as a result of the stress (Feder and Hofmann, 1999; Ikwegbue et al., 2017). Among the HSPs, HSP40 and HSP70 function as co-chaperones and work together to promote the correct folding, assembly and transport of newly synthesized proteins, minimizing their aggregation (Fink, 1999; Liberek et al., 2008). HSPs have also been found to be upregulated in some invertebrate taxa after experiencing predation threat (Jermacz et al., 2020; Pijanowska and Kloc, 2004). The HSPs belong to a family of proteins that have been highly conserved throughout evolution and are expressed by all cells and organisms. Thus, their functions have been extensively studied in a wide range of species (Robert, 2003; Verghese et al., 2012), including *L. stagnalis*. In particular, the heat stress associated with exposure to 30°C pond water (PW) for 1 h was found to enhance memory formation in snails (Teskey et al., 2012; Tan and Lukowiak, 2018). It was also found that this heat stressor in *Lymnaea* led to a rapid (within 30 min) upregulation of the mRNA levels of both HSP40 and HSP70, reaching a peak of expression within 2–4 h of exposure to the thermal stress (Foster et al., 2015). It was further demonstrated that the heat shock stressor-induced enhancement of LTM formation occurred as a result of the upregulation of HSPs by the heat shock

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stressor (Sunada et al., 2016). However, the enhancing effect of the thermal stimulus on memory was obstructed by a flavonol, quercetin (Sunada et al., 2016). Treatment with quercetin before snails experienced the heat stress prevented memory enhancement (Sunada et al., 2016). However, when applied after the thermal exposure, quercetin did not alter the heat stressor's ability to enhance memory formation (Sunada et al., 2016). Recently, in *Lymnaea* it was demonstrated that blocking HSP expression by quercetin obstructed a Garcia-like effect for a novel food substance induced by heat stress (Rivi et al., 2021). However, in addition to blocking HSP upregulation, quercetin has modulatory effects on several signalling molecules, including the cyclic AMP response element binding protein 1 (CREB1) (Babaei et al., 2018; Nijveldt et al., 2001). LymCREB1 plays a key role in memory formation in *Lymnaea* (Sadamoto et al., 2004).

Here, we hypothesized that quercetin obstructs CL by blocking the upregulation of HSPs induced by exposure to the predator scent during the CL training. Thus, we exposed snails to quercetin before and after the CL training procedure and determined whether the CL memory was obstructed. If HSP upregulation is necessary for CL memory formation, exposure of snails to quercetin before CL training should block the CL memory. However, we knew from previous data that once upregulated, the expression levels of HSPs are not altered by quercetin exposure (Sunada et al., 2016). Thus, snails exposed to quercetin after the CL training should still exhibit CL memory.

MATERIALS AND METHODS

Study animal

We used a lab bred strain (the W-strain) of *Lymnaea stagnalis* (Linnaeus 1758) maintained at the University of Calgary since the 1980s. The founding population of our *L. stagnalis* originated from polders in Utrecht in The Netherlands in the 1950s and was then maintained at the Vrije University in Amsterdam. Our W-strains originally came from this population of inbred Dutch *L. stagnalis*. We housed snails in artificial PW (0.25 g l^{-1} of Instant Ocean in deionized water, Spectrum Brands, Madison, WI, USA) supplemented with $CaCO_3$ to ensure calcium concentrations remain above 50 mg (Dalesman and Lukowiak, 2010). Snails were maintained at 20±1°C on a 16 h:8 h light:dark cycle and fed romaine lettuce *ad libitum*. A total of 133 adult snails (shell length 25–32 mm) were used without repetition.

Carrot slurry

We used carrot as the food stimulus for all CL experiments. A carrot slurry was prepared by peeling, blending and straining two medium sized organic carrots (~60–70 g) in 500 ml of PW.

Quercetin solution

Quercetin (3,3′,4′,5,7-pentahydroxyflavone; Sigma Chemical Company, St Louis, MO, USA; purity level >95%) was dissolved in 0.1% (final concentration) dimethyl sulfoxide (DMSO); we prepared quercetin solution by dissolving 50 µl in 500 ml of PW. This concentration of quercetin has been successfully used previously in *Lymnaea* (Sunada et al., 2016).

Predatory odour/CE

We used the odour of crayfish (*Faxonius virilis*) which are natural predators of *Lymnaea*, and the W-strain innately recognise crayfish as predators (i.e. they are a predator-experienced strain) and detect and respond to CE with multitude of anti-predatory behaviours (Orr et al., 2007). The crayfish used for all experiments was 7.5 cm in length and was housed in a 70 l aquarium containing artificial PW. The crayfish was fed a diet of lettuce and snails, and was maintained in the aquarium for 6 months before the current study commenced.

CL procedure

The same CL procedure was used as in previous reports (Swinton et al., 2019). We measured rasping of *L. stagnalis* as the response in the CL experiments. Rasping (i.e. feeding behaviour) is a repeated rhythmic movement of the radulae which is used to scrape off food from the surface of a substrate, and food is ingested in the process. We recorded the number of rasps for 2 min following acclimation. All trial sessions began by ascertaining the spontaneous rate of feeding in PW and after 3 h the feeding rate was determined in the presence of carrot (pre-training). The next day (i.e. 18 h later), snails were trained for CL by exposing them for 45 min to a solution of CE+C (carrot slurry made using 500 ml of CE). The feeding response to carrot was then tested 3, 24 and 48 h after the CL training session (post-training). In all the CL experiments, snails were placed in a 14 cm Petri dish mounted on top of a mirror, with enough PW or carrot slurry to partially submerge them. The mirror allowed for convenient recording of rasping behaviour. Individuals were acclimated for 5 min before each experimental test.

Marking or grading scheme

Snails were also given 'pass' or 'fail' grades on an individual basis to show how well (or how poorly) they learned. The following grading scheme was used to assign a learning outcome: a pass (P) grade was assigned if the feeding behaviour in carrot post-CL training had a greater than 25% reduction with respect to the first carrot exposure (pre-training); and a fail (F) grade was assigned if the decrease was less than 25%. This kind of marking scheme has been successfully used before (Aonuma et al., 2016). We used this scheme for better illustration purposes only.

Total RNA extraction, reverse transcription, and qPCR

Snails were killed by placing them in ice for 10 min and a single central ring ganglion was used for total RNA extraction. Six replicates were analysed for each group (N=6). Total RNA extraction and DNase treatment were performed using GenElute™ Total RNA Miniprep Kit and DNase 70-On-Column DNase I Digestion Set (Sigma-Aldrich) as previously described (Benatti et al., 2017). A 200 ng sample of total RNA was reverse transcribed with a High-Capacity cDNA Reverse Transcription Kit (ThermoFisher). Real-time quantitative PCR (qPCR) was carried out on 20 ng mRNA using a Bio-Rad® CFX Connect™480 Real-Time PCR Detection System with SYBR Green Master Mix (Bio-Rad). Specific forward and reverse primers were used at a final concentration of 300 nmol l^{-1} (Table 1). Single PCR products were subjected to a heat dissociation protocol (StepOne Real-Time PCR System, Applied Biosystems). The cycling parameters were: 95°C 2 min and 94°C 10 s, 60°C 30 s for 40 cycles. Cycle threshold (Ct) values were determined by CFX Maestro™ Software (Bio-Rad).

A control group was included to control for potential bias arising from the exposure of snails to DMSO. Snails of this group were maintained for 1 h in clean PW without experiencing quercetin or DMSO. In this way, we verified that a DMSO concentration of 0.1% did not have any transcriptional effects in the ganglia of snails (Capriotti and Capriotti, 2012). DMSO is an effective solvent that can induce various actions in experimental settings, ranging from metabolic stress to cytotoxic effects depending on the concentration used. Therefore, to ensure the quality of this experiment and the reproducibility of the results, we decided to exclude any potential transcriptional interference caused by DMSO.

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Table 1. Nucleotide sequence of the forward and reverse primers used for qPCR

GenBank accession no.	Target	Product length	Forward primer	Reverse primer
AB041522.1	*LymCREB1*	180 bp (49–229)	GTCAGCAGGGAATGGTCCTG	AACCGCAGCAACCCTAACAA
X15542.1	*LymβTUB*	127 bp (92–219)	CGCCTCTGTGAACTCCATCT	GAAATAGCACCGCCATCC
DQ278441.1	*LymEF1a*	144 bp (13–157)	CTGGGAGCAAAGTCAAGCAT	TTCGCTCATCAATACCACCA

LymCREB1, *Lymnaea stagnalis* cAMP responsive element binding protein; *LymβTUB*, snail β-tubulin; *LymEF1a*, *Lymnaea stagnalis* elongation factor 1-α. The GenBank accession number, fragment size and nucleotide position of the PCR product obtained are given for each target.

Statistical analyses

For quantitative evaluation of changes in mRNA expression, the comparative ΔΔCt method was performed, using it as a calibrator for the average expression levels of control snails. The stability of mRNA expression of two reference genes (elongation factor 1-alpha, *LymEF1α*; and beta-tubulin, *LymTUB*) was assessed using Normfinder®; *LymβTUB* was the most stable gene across groups and was used for gene normalisation. Statistical analyses were performed using separate unpaired *t*-test with the two treatments being vehicle and PW (Fig. 3A) and vehicle and quercetin (Fig. 3B) exposed snails (with $P<0.05$ significance level).

Across all behavioural experiments, the number of rasps was our response variable and the different treatments, i.e. exposure to carrot slurry pre-CL training (C pre), and at 3 h (C 3 h), 24 h (C 24 h) or 48 h (C 48 h) post-CL training, were our predictor variables, with individuals being repeatedly measured for all treatments in a single experiment. For each experiment the model was: response(number of rasps)~treatment(C pre, C 3h, C 24h, etc.)+(1|ID).

We used generalised linear mixed effects modelling for all experimental analyses with a Poisson distribution as our response variable (number of rasps) was count data. The only experiment with a normal data distribution was our control experiment on feeding behaviour before and after exposure to quercetin and was analysed using lme4 (R package: lme4 and lmerTest; Bates et al., 2015; https://cran.r-project.org/web/packages/lmerTest/index.html). All other experiments with non-normal data were analysed using glmmadmb (R package: glmmADMB; http://glmmadmb.R-forge.R-project.org), which also accounted for zero inflated data in some cases. We did not transform any data. In all models, individual snail ID was included as a random effect. Individual ID contributed minimally to the variation in the data (standard deviation across all models <0.33). We performed Tukey's *post hoc* comparisons using glht (R package: multcomp; http://multcomp.R-forge.R-project.org). Our average sample size for each experimental treatment fell within estimated sample size calculated using the package 'webpower' with effect size (>0.7), power (0.80) and significance (0.05) levels specified for repeated measures data (Zhang et al., 2018). All data were analysed using R statistical software version 3.6.0 (http://www.R-project.org/).

RESULTS

Effect of quercetin on CL in *L. stagnalis*

First, we asked whether quercetin had any effect on the snails' feeding behaviour. This is important as feeding is the behaviour examined in the CL experiments. We recorded the rasping behaviour in the presence of carrot slurry before and 3 h after quercetin exposure and found no significant difference between them (*t*-test: $n=12$, $t=0.92$,

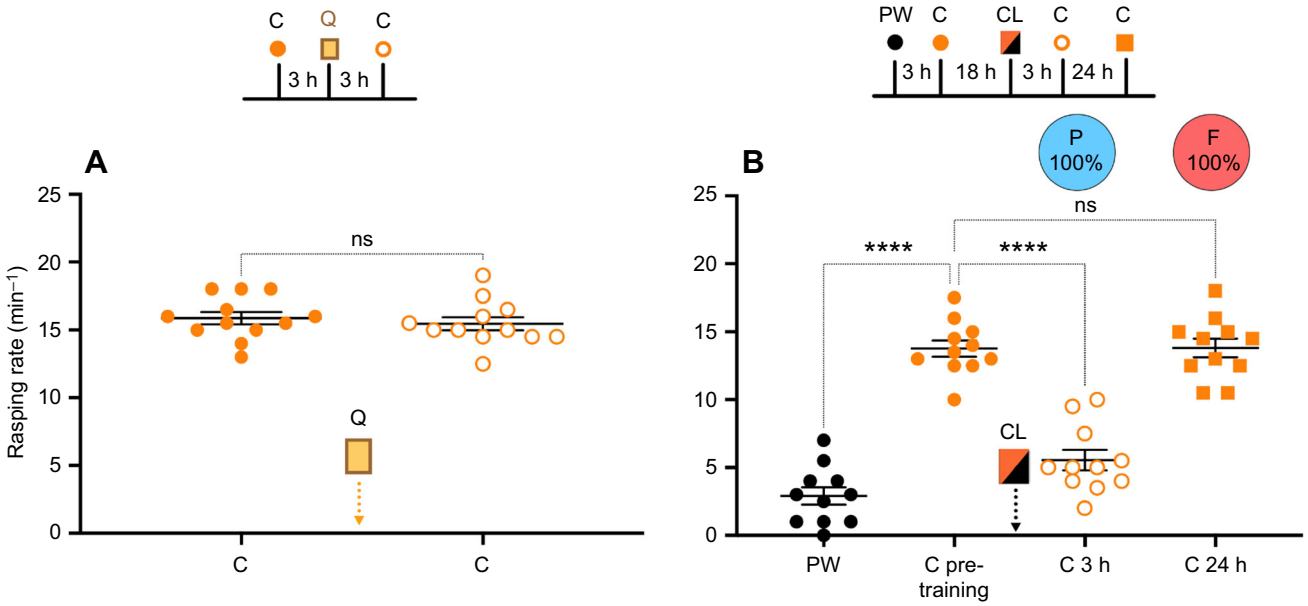

Fig. 1. Feeding and configural learning. The time line for each experiment is shown above the obtained data. (A) Snails ($n=12$) showed no significant difference between rasping rate in the presence of carrot slurry (C) before and after exposure to quercetin (Q) (*t*-test: $P=0.376$). (B) Snails showed a significant increase in rasping rate in the presence of carrot slurry (C pre-training) compared with that in pond water (PW) (glmm: $P<0.001$). Following the configural learning (CL) training procedure, snails showed intermediate-term memory (ITM) but not long-term memory (LTM) as the number of rasps was significantly lower 3 h (C 3 h; $P<0.001$) but not 24 h (C 24 h; $P=0.999$) post-training, when compared with the pre-training rate ($n=11$). Pie charts show the percentage of animals with a 'pass' (P) or 'fail' (F) grade, where pass indicates a ≥25% reduction in rasping rate 24 h after training compared with the pre-training value. Data are presented as means±s.e.m. ****$P<0.001$; ns, not significant ($P>0.05$).

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P=0.376; Fig. 1A). Thus, exposure to quercetin does not alter feeding behaviour in the snails. We next demonstrated that in our hands the CL training procedure (*n*=11) resulted in the suppression of feeding. We first measured their spontaneous rasping behaviour in PW and then 3 h later in carrot slurry (Fig. 1B). We found it to be significantly higher in carrot slurry than in PW (glmm: $z=-8.12$, $P<0.001$, Fig. 1B). Following the 45 min simultaneous exposure to carrot and CE (i.e. the CL procedure), snails showed a significant decrease in rasping behaviour in carrot 3 h later ($z=7.63$, $P<0.001$, Fig. 1B). However, when tested 24 h post-CL training, no memory for CL was observed ($z=-0.041$, $P=0.999$). None of the snails showed a decrease in feeding ≥25% 24 h post-CL training compared with pre-training (fail: 100%). Thus, following the CL training procedure, snails only formed a 3 h ITM for CL.

Next, we tested the effect of quercetin exposure on snails receiving the CL training procedure. Snails were exposed to quercetin either immediately before (Fig. 2A) or after (Fig. 2B) CL training. In snails (*n*=14; Fig. 2A) exposed to quercetin immediately before the CL procedure, both a 24 h and a 48 h memory were now present. That is, their response to carrot slurry at 24 h ($z=9.61$, $P<0.001$) and 48 h ($z=2.95$, $P=0.014$) after CL training was significantly less than their initial pre training (C pre) response. Using our grading scheme, we found that 93% and 57% of snails dropped their feeding response to carrot slurry by 25% or more 24 h and 48 h after CL training, respectively.

We then asked whether quercetin exposure immediately after CL training leads to standard CL memory present at 3 h (*n*=14; Fig. 2B). Interestingly, in these snails, we found that the response to carrot

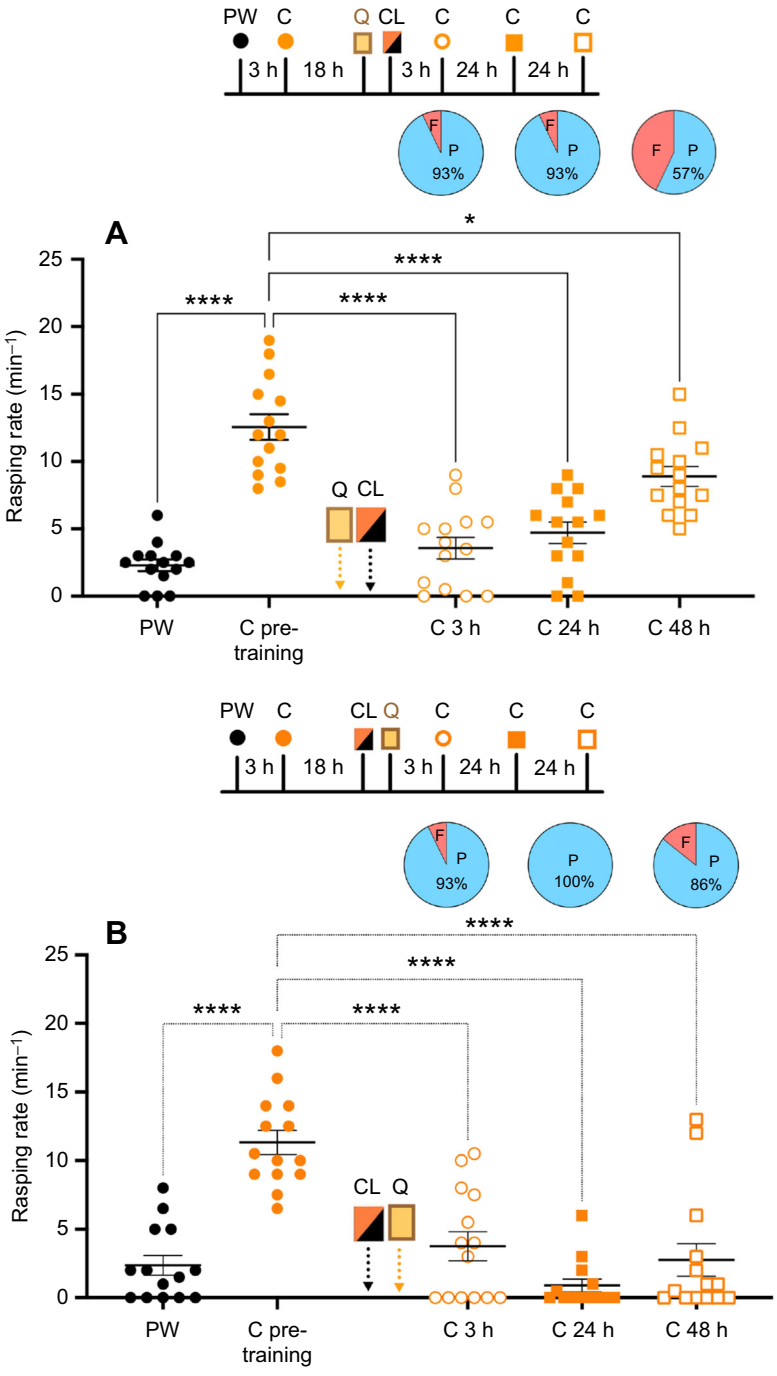

Fig. 2. Quercetin enhances LTM formation for CL. The time line for each of the experiments is presented above the data. (A) Snails (*n*=14) were exposed to quercetin immediately before CL training. These snails showed a significant decrease in feeding behaviour (i.e. memory) 3 h (*P*<0.001), 24 h (*P*<0.001) and 48 h (*P*=0.014) post-training. (B) Snails (*n*=14) were exposed to quercetin immediately after CL training. These snails showed a significant decrease in feeding behaviour 3 h (*P*<0.001), 24 h (*P*<0.001) and 48 h (*P*<0.001) post-training. Pie charts show the percentage of animals with a pass (P) or fail (F) grade, where pass indicates a >25% reduction in rasping rate 24 h and 48 h after training compared with the pre-training value. Quercetin exposure immediate after CL training (B) resulted in a greater proportion of snails with better memory at 48 h post-training compared with snails that experienced quercetin immediately before CL training (A). Data are presented as means±s.e.m. ****P<0.001, *P<0.05; ns, not significant (P>0.05).

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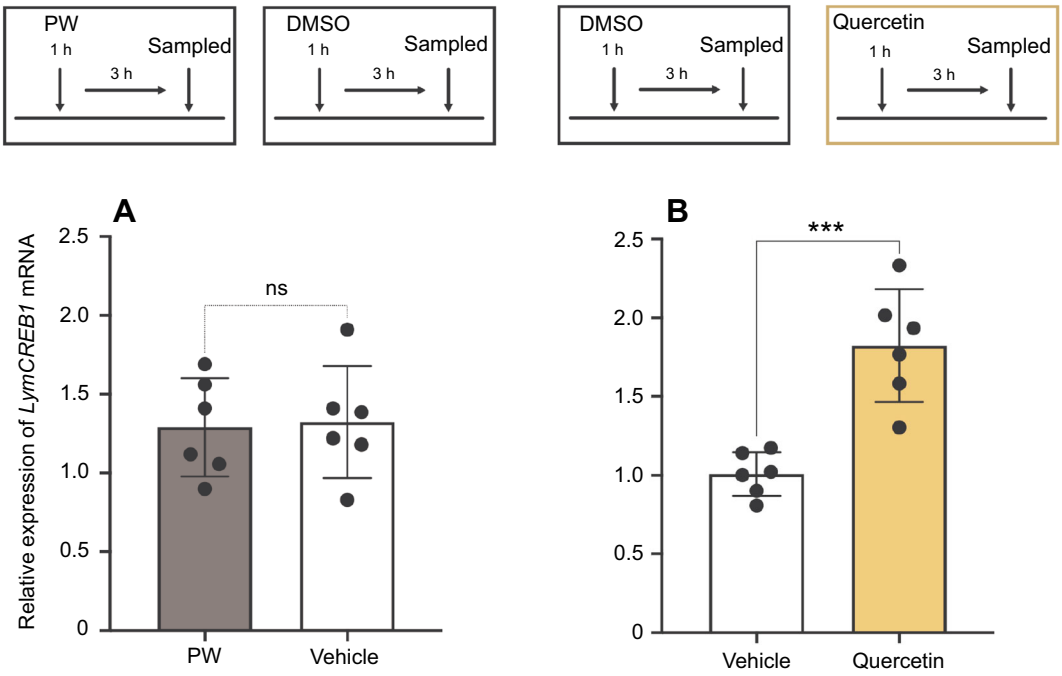

Fig. 3. Upregulation of *LymCREB1* expression following quercetin exposure. The time line for each of the experiments is presented above the data. (A) *LymCREB1* expression showed no significant difference in snails exposed to PW and to vehicle (DMSO) (n=6 in each; t-test: P=0.867). (B) Quercetin significantly upregulated *LymCREB1* expression in the cohort exposed to quercetin compared with that exposed to vehicle (DMSO) (n=6 in each; t-test: P=0.001). Data are presented as means±s.e.m. ***P=0.001; ns, not significant (P>0.05).

slurry was significantly reduced 24 h (z=12.22, P<0.001) and 48 h (z=7.70, P<0.001) post-CL training. Again, the majority of snails suppressed their feeding response 25% or more 24 h (100%) and 48 h (86%) after training. Thus, quercetin exposure enhanced rather than as we hypothesized obstructed memory formation.

We next investigated possible mechanisms underlying this memory enhancement. We measured the expression levels of the *L. stagnalis* orthologue of CREB (LymCREB1) in the CNS of snails exposed to quercetin (Fig. 3). We first determined whether DMSO at a concentration of 0.1% used as a vehicle for quercetin alters the transcriptional activity of LymCREB1. We exposed a group of snails to PW (n=6) or DMSO (n=6) for 1 h and sampled ganglia for gene expression analysis 3 h later. We found no differences between vehicle (DMSO) and naive (PW) control groups in the expression levels of *LymCREB1* (t-test: t=0.17, P=0.867; Fig. 3A). Snails were then randomly assigned to two treatment groups (n=6 per group), one exposed to DMSO for 1 h and the other exposed to quercetin for 1 h (Fig. 3B); 3 h later, total RNA was extracted from the snails and mRNA levels of *LymCREB1* between the two groups were quantified. We selected the 3 h interval as we know from previous studies that quercetin inhibits HSP expression 3 h post-exposure. An unpaired t-test revealed that the expression of *LymCREB1* was significantly induced in snails exposed to quercetin with respect to the DMSO control (t-test: t=−5.19, P=0.001).

That result lead us to test the effect of quercetin exposure 3 h prior to or 3 h after the CL procedure (Fig. 4). To begin with, all snails showed memory 3 h post-CL training. Quercetin exposure 3 h before training (n=12) resulted in LTM at 24 h but not at 48 h (Fig. 4A). That is, we found the response to carrot slurry was significantly lower at 24 h (z=8.86, P<0.001), but not at 48 h (z=0.88, P=0.782). At 24 h post-training, the majority of snails (83%) dropped their feeding response >25% but only 17% of snails dropped their feeding response >25% at 48 h post-training (i.e. 83% failed).

In a second cohort of naive snails (n=12), quercetin exposure 3 h after CL training led to LTM both 24 h and 48 h after CL training (Fig. 4B). That is, the feeding response was significantly lower both 24 h (z=8.86, P<0.001) and 48 h later (z=3.60, P=0.001). Thus, quercetin exposure was more effective when it occurred 3 h after CL training. In this cohort, 100% and 66% of snails dropped their feeding response >25% 24 h and 48 h after training, respectively.

Finally, we tested the effect of quercetin exposure during the memory reconsolidation phase (Fig. 5). In the reconsolidation phase, memory enters a labile state and can be converted to LTM if reinforced. We performed the following two experiments, where snails (n=12; Fig. 5A) were exposed to quercetin immediately after the feeding test 3 h post-CL training (i.e. snails exhibiting ITM) and 24 h post-CL training (i.e. snails not showing any memory for CL; Fig. 5B). When the quercetin exposure occurred immediately after the 3 h feeding test (snails showed memory as they significantly reduced rasping behaviour; z=6.29, P<0.001) they exhibited LTM both 24 h (z=13.12, P<0.001) and 48 h (z=3.38, P=0.005) post-CL training. An overwhelming majority (100% and 91%) of snails decreased their response to carrot slurry >25% at the 24 h and 48 h time points, respectively. However, in the cohort that were exposed to quercetin immediately after the feeding test 24 h post-CL training (n=10; Fig. 5B), the snails did not show LTM 24 h (z=0.46, P=0.986) or 48 h (z=0.16, P=0.999) after CL training. These snails experienced quercetin when there was no LTM present. Thus, we found no memory enhancement in this case (all snails failed at both 24 h and 48 h post-training).

DISCUSSION

This study was designed to test the hypothesis that HSPs are necessary for CL memory; that is, if HSP upregulation was blocked, using quercetin, CL memory formation would not occur. However, to our surprise that was not what took place. Rather, quercetin enhanced CL memory formation. The data obtained enable us to

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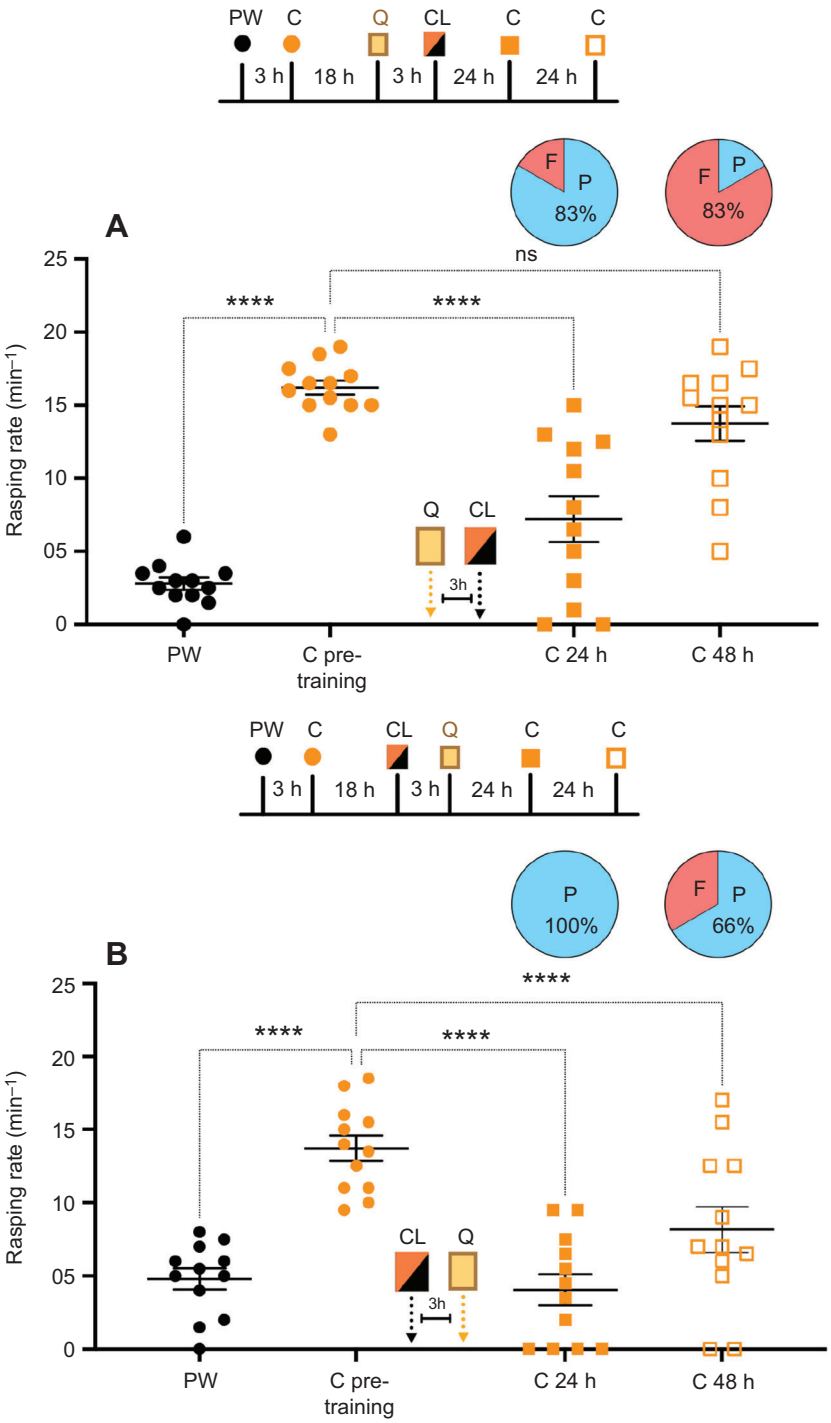

Fig. 4. Quercetin experienced 3 h prior to and 3 h after CL training enhances LTM formation. The time line for each of the experiments is presented above the data. (A) Snails (n=12) exposed to quercetin 3 h prior to CL training showed a 24 h memory (P<0.001) but not a 48 h memory (P=0.782). (B) Snails (n=12) exposed to quercetin 3 h after CL training exhibited both a 24 h (P<0.001) and a 48 h (P=0.001) LTM. Pie charts show the percentage of animals with a pass (P) or fail (F) grade, where pass indicates a >25% reduction in rasping rate 24 h and 48 h after training compared with the pre-training value. A greater proportion of snails in the group exposed to quercetin 3 h after training passed as compared with the group exposed to quercetin 3 h prior to training. Data are presented as means±s.e.m. ****P<0.001; ns, not significant (P>0.05).

conclude that: (1) HSP upregulation is not necessary for CL memory formation and (2) quercetin enhanced CL memory formation.

With quercetin exposure, LTM formed that persisted for up to 48 h. Coincident with these behavioural findings we also showed that quercetin exposure upregulated *LymCREB1* mRNA levels in the CNS. Finally, quercetin was also a potent enhancer of LTM formation if it was experienced during the memory consolidation period.

This study was a follow up from previous work showing that environmental stressors, such as heat shock, leads to the upregulation of HSPs coincident with enhanced LTM formation (Tan and Lukowiak, 2018; Teskey et al., 2012 Sunada et al., 2016). Additionally, if the upregulation of the HSPs was blocked, memory enhancement did not occur (Sunada et al., 2016). Recently, we found that quercetin, when applied before the heat shock, prevented the heat-induced upregulation of mRNAs coding for HSP40 and HSP70 in the *Lymnaea* CNS (Rivi et al., 2021). Those results were consistent with data obtained in other organisms, showing that quercetin blocks HSP induction or upregulation (Hosokawa et al., 1990; Storniolo et al., 2015; Wang et al., 2009). As quercetin blocks HSP upregulation in *L. stagnalis*, we wanted to determine whether HSPs are also involved in predator-induced enhanced learning and memory. CL in *Lymnaea* provided us with the perfect paradigm to

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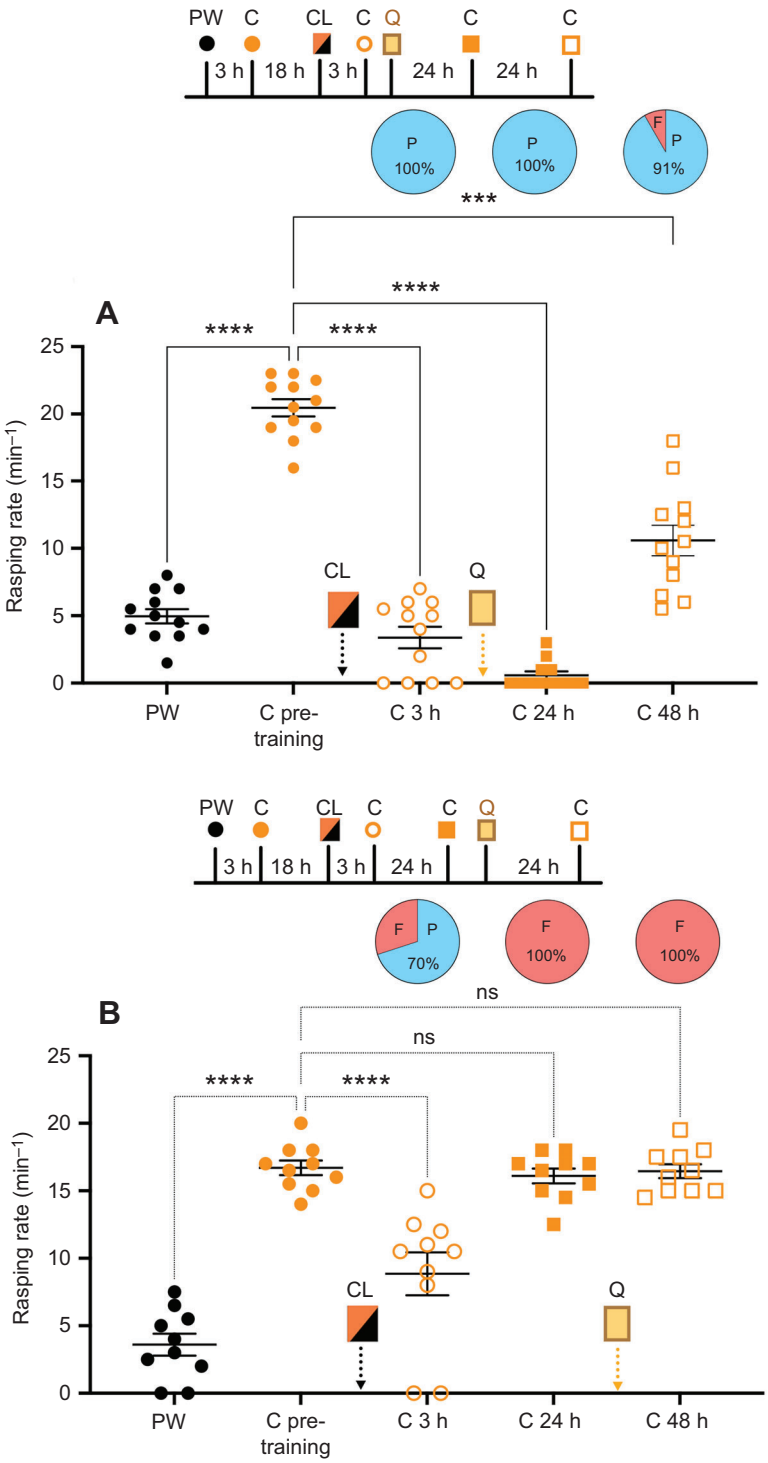

Fig. 5. Quercetin experienced during memory reconsolidation enhances LTM formation. The time line for each of the experiments is presented above the data. (A) Snails (n=12) underwent CL training; 3 h later, when challenged with carrot slurry, they exhibited ITM (P<0.001). The snails were then exposed to quercetin immediately after the memory test. Quercetin exposure resulted in a 24 h (P<0.001) as well as a 48 h (P=0.005) LTM. (B) Following CL training, snails (n=10) exhibited memory 3 h (P<0.001) but not 24 h later (P=0.986). Immediately after the 24 h memory test, snails were exposed to quercetin. When memory was tested 24 h later (i.e. 48 h after CL training), snails showed no memory (P=0.999). Quercetin enhanced LTM formation when memory was present but not when it was absent. Pie charts show the percentage of animals with a pass (P) or fail (F) grade, where pass indicates a >25% reduction in rasping rate 24 h and 48 h after training compared with the pre-training value. Data are presented as means±s.e.m. ****P<0.001; ns: not significant (P>0.05).

test this idea as we could block HSP elaboration by exposing snails to quercetin immediately before or after CL training and then determine whether the expected 3 h memory was present. However, unexpectedly the opposite was found. Quercetin applied immediately before or after CL did not block memory; rather, it enhanced it. That is, LTM persisting for 24 h and 48 h after CL was obtained. The adaptive significance of a CL LTM is debatable as it would mean the snail would avoid a food resource for a longer time after experiencing the food with a predatory cue. This might, however, be advantageous and promote survival in a high threat environment. It is also possible that food deprivation may 'overcome' the CL memory; but this has not yet been determined.

Quercetin in addition to blocking HSP expression also upregulates multiple signalling molecules such as CREB1 (Babaei et al., 2018; Karimipour et al., 2019). CREB1 as a key transcription regulator is responsible for induction of numerous factors involved in neuronal differentiation, synaptic plasticity and learning and memory (Kandel, 2012; Lonze and Ginty, 2002). During LTM formation, the activity of transcription factors is essential and CREB has been found to be a key molecule that is upregulated during classical conditioning of taste aversion

behaviour in *Lymnaea* (Azami et al., 2006; Sadamoto et al., 2004). Our demonstration of the upregulation of *LymCREB1* expression and memory enhancement following quercetin exposure suggests a conserved role of the signalling pathways underling LTM across multiple associative learning paradigms and across taxa. In fact, similar to our results obtained in *L. stagnalis*, evidence from mammals indicates that the upregulation of CREB by quercetin is associated with enhanced hippocampus-dependent learning and memory (Karimipour et al., 2019; Xia et al., 2015). Moreover, studies from rodents indicated that, like consolidation, CREB also is required in the reconsolidation of a conditioned fear memory (Kida et al., 2002). Thus, the enhanced LTM formation by quercetin via upregulation of LymCREB1 suggests an evolutionarily conserved biochemical pathway.

Memory types are distinct and physiologically different as LTM requires altered gene activity as well as new protein synthesis, whereas ITM only requires new protein synthesis (Scheibenstock et al., 2002; Sangha et al., 2003a,b). As shown here, quercetin has the ability to 'upgrade' ITM to LTM. Even though all quercetin exposures independent of time before or after CL training led to a 24 h LTM, snails did not exhibit memory at 48 h post-CL training when quercetin was experienced 3 h before CL training (Fig. 4A). In contrast when quercetin was experienced 3 h after CL training (i.e. when ITM could be observed), a 48 h memory was observed (Fig. 4B). Thus, quercetin experienced prior to CL training is not as effective a memory enhancer compared with when it is experienced post-training during memory consolidation. We do not know for certain the temporal change in *LymCREB1* expression immediately following exposure to quercetin, only that it is upregulated 3 h post-exposure. Thus, we speculate that quercetin-dependent enhancement of LTM formation might be dependent on the entire temporal pattern of LymCREB1 activity following quercetin exposure and upregulation of LymCREB1 might be needed only during the memory consolidation period. When quercetin is presented 3 h prior to CL training, snails are only experiencing a partial increase in LymCREB1 activity during the consolidation phase, which leads to only a 24 h LTM. However, when quercetin is experienced after CL training, snails experience the full temporal range of LymCREB1 activity, leading to a 48 h LTM.

Another interesting aspect of quercetin induced enhancement of LTM formation was observed during reconsolidation. Under normal conditions, 3 h post-CL memory is not reconsolidated to form LTM in *L. stagnalis*, but quercetin exposure led to LTM formation, probably via upregulation of LymCREB1 (Fig. 5A). However, exposure to quercetin at the 24 h time point when the snails have no ITM did not lead to LTM memory enhancement. It has been shown across multiple studies, including *Lymnaea*, that reconsolidation requires new protein synthesis and CREB activity, similar to the memory consolidation phase (Anokhin et al., 2002; Kida et al., 2002; Sangha et al., 2003a,b). Quercetin thus builds on an existing ITM or LTM memory but does not have an enhancing effect when no residual memory is present. Thus, quercetin-induced enhanced LTM formation may only be effective during a memory consolidation or reconsolidation phase.

Although our original hypothesis was negated, we ended up finding that quercetin enhanced LTM formation. Thus, the unique combination of the CL paradigm coupled with a memory-enhancing bioactive compound reinforces the importance of experience-dependent changes in learning and memory. Previous *Lymnaea* studies from our group have also shown enhanced LTM when epicatechin, another flavonoid, is used during or immediately following training for operant conditioning (Fernell et al., 2016; Swinton et al., 2018) and future studies will investigate whether this epicatechin-mediated memory enhancement involves CREB upregulation. In conclusion, we found CL memory formation to be independent of HSP expression. Additionally, we found that quercetin upregulates LymCREB1, leading to enhanced LTM formation when experienced during the consolidation or reconsolidation periods.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
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