1   **Oiling the gears of memory: quercetin exposure during memory formation,**
2   **consolidation, and recall enhances memory in *Lymnaea stagnalis***

3

4   Running Title: Memory enhancement and quercetin

5

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25   **Summary Statement:** Quercetin enhances long-term memory formation acting on
26   the different phases of memory formation, consolidation, and recall.

27

28   **Keywords:** CREB, long-term memory, invertebrates, flavonoids

29

30 Abstract

31 Memory formation (short-term, intermediate-term, and long-term) is an integral
32 process of cognition which allows individuals to retain important information and is
33 influenced by various intrinsic and extrinsic factors. A major extrinsic factor
34 influencing cognition across taxa is diet, which may contain rich sources of molecular
35 agents with antioxidant, anti-inflammatory, and memory enhancing properties that
36 potentially enhance cognitive ability. A common and abundant flavonoid present in
37 numerous food substances is quercetin (Q) which is also known to upregulate cyclic
38 AMP response element binding protein (CREB) in several animals including our
39 model system *Lymnaea stagnalis*. Since CREB is known to be involved in long term
40 memory (LTM) formation, we investigated the role of Q-exposure on memory
41 formation, consolidation, and recall during operant conditioning of aerial respiratory
42 behaviour in *Lymnaea*. Snails were exposed to Q 3h before or after training to
43 ascertain its effects on LTM. Additionally, we investigated the effect of the combined
44 presentation of a single reinforcing stimulus (at 24h post-training or 24h before
45 training) and Q-exposure on both LTM formation and reconsolidation. Our data
46 indicate that Q-exposure acts on the different phases of memory formation,
47 consolidation, and recall leading to enhanced LTM formation.

48

49 Introduction

50 Learning is the ability to acquire new knowledge, whereas memory is the process of
51 storing, recalling, and reconstructing that knowledge over time (Kandel et al., 2014;
52 Zlotnik and Vansintjan, 2019). Memories are not only essential for survival but are
53 also major determinants of “*who we are*” (Milner et al., 1998). In fact, by regulating
54 decision-making processes and influencing emotional reactions, they shape our very
55 identity (Brem et al., 2013; Wilson and Ross, 2003).

56 Memory, based on the duration of its persistence, can be classified into short-term
57 memory (STM, lasting minutes), intermediate-term memory (ITM, lasting 2–3 h),
58 and long-term memory (LTM, persisting for at least 24h and sometimes for the entire
59 lifetime; Braun and Lukowiak, 2011; Lukowiak et al., 2000; Sangha et al., 2003a,b).
60 LTM is functionally dynamic and includes several temporal phases, such as encoding,
61 consolidation, retrieval, and reconsolidation (Bisaz et al., 2014; Marra et al., 2013;

Lukowiak et al., 2000; Rosenzweig et al., 1993). In recent decades, extraordinary progress has been made in demonstrating the molecular and cellular mechanisms underlying LTM, providing insight into how memories might also be enhanced. In this regard, studies in invertebrate models such as *Aplysia californica* (Conte et al., 2017; Hawkins et al., 2006; Philips et al., 2006) and *Drosophila melanogaster* (Davis, 2011; Isabel et al., 2004; Matsuno et al., 2015; Widmer et al., 2018) have paved the way to the elucidation of the conserved molecular and cellular basis of memory across taxa. A key feature of LTM is that a newly encoded memory initially exists in a fragile state and, over time, becomes increasingly resistant to disruption. With retrieval or reactivation, a consolidated memory can re-enter a labile state until it is restabilized through a process of reconsolidation (Nader et al., 2000; Alberini, 2009, 2011; Bisaz et al., 2014; Squire et al., 2015). During both consolidation and reconsolidation processes, memory is susceptible to modification by environmental factors, such as stress and lifestyle choices (McKenzie and Eichenbaum, 2011).

One key lifestyle choice that impacts the strengthening or weakening of the memory trace is diet (Swinton et al., 2018). Growing evidence suggests that a group of dietary-derived phytochemicals, known as flavonoids, may improve LTM formation, consolidation, storage, and retrieval (Ayaz et al., 2019; Fruson et al., 2012; Haleagrahara et al., 2011; Havsteen, 2002; Knezevic and Lukowiak, 2014; Letenneur et al., 2007; Socci et al., 2017). A multitude of pharmacological studies reported that regular dietary consumption of flavonoids and flavonoid-rich foods can improve various cognitive dysfunctions and dementia-like alterations in different animal models (Bakoyiannis et al., 2019; Macready et al., 2009; Mastroiacovo et al., 2015; Socci et al., 2017; Spencer, 2007a; Yevchak et al., 2008). The positive cognitive effects of flavonoids in mammals have been attributed to the protection of neural functioning, stimulation of neuronal regeneration, and increased blood flow to the brain (Macready et al., 2009; Mastroiacovo et al., 2015; Schroeter et al., 2007; Spencer, 2007; Vauzour et al., 2007; Williams and Spencer, 2012; Yevchak et al., 2008).

93 Quercetin (Q, 3,3',4',5,7-pentahydroxyflavone) is one such flavonoid which is
 94 widely distributed in fruits and vegetables, like apples, berries, onions, asparagus,
 95 capers, and red leaf lettuce (Manach et al., 2004, 2005). Q has antioxidant properties
 96 and is able to scavenge free radicals and protect neuronal cells from neurotoxicity
 97 caused by oxidative stress (Ahmad et al., 2015; Cushnie and Lamb, 2005; Eggler et
 98 al., 2008; Kong et al., 2000; Morel et al., 1993a; Robak and Gryglewski, 1988).
 99 Unfortunately, to date, the vast majority of the studies investigating the effects of Q
 100 on memory has been performed on aged individuals or animal models of
 101 neurodegenerative diseases (Ansari et al., 2009; Bhutada et al., 2010; Broman-Fulks
 102 et al., 2012; Haleagrahara et al., 2011; Jayasena et al., 2013; Karimipour et al., 2019;
 103 Khan et al., 2018; Mandel and Youdim, 2004; Morel et al., 1993; Nakagawa et al.,
 104 2016; Reznichenko et al., 2006; Spencer, 2007, 2008; Williams and Spencer, 2012;
 105 Yoshida et al., 1990). Thus, there is a paucity of studies about the memory-enhancing
 106 effect of Q on healthy individuals. However, studies involving Q treatment on
 107 cognitively impaired animal models showed several molecular mechanisms that can
 108 enhance memory. Among them, the extracellular signal-regulated kinase (ERK1/2)
 109 and the protein kinase B (PKB/Akt) signalling pathways are important as they activate
 110 the response element-binding protein (CREB) family of transcription factors which, in
 111 turn, play a key role in regulating the genes necessary for synaptic plasticity
 112 (Finkbeiner et al., 1997; Spencer, 2007; Spencer et al., 2003; Vauzour et al., 2007). In
 113 particular, the transcriptional activator CREB1 is a core component of the molecular
 114 switch that converts STM to LTM (Finkbeiner et al., 1997; Kida, 2012; Silva et al.,
 115 1998; Suzuki et al., 2011). That is, by increasing the expression levels of various
 116 neurotrophins, CREB regulates memory formation, consolidation, and reconsolidation
 117 in a wide range of animal models, from invertebrates to mammals (Alberini, 2009,
 118 2011; Bozon et al., 2003; Stevens, 1994; Yin and Tully, 1996). Therefore, it is not
 119 surprising that CREB and the signalling pathways leading to its activation are
 120 attractive targets for treatment aimed at improving memory function, in both diseased
 121 and healthy individuals (Barco et al., 2003; Khan et al., 2018; Tully et al., 2003).
 122
 123 Importantly for us here in the pond snail *Lymnaea stagnalis* (Linnaeus 1758) it was
 124 found that treatment with Q resulted in the upregulation of the orthologous gene of

125 CREB1 (*Lym*CREB1) 3h after exposure to Q (Batabyal et al., 2021). In recent years,
 126 several studies from our lab have focused on how environmentally relevant stressors
 127 and bio-active compounds alter LTM formation in snails. Using *Lymnaea* as a model
 128 organism, it was shown for the first time in invertebrates that the flavonoid (-)-
 129 Epicatechin present in cocoa and green tea enhances LTM following operant
 130 conditioning of aerial respiratory behaviour (Fernell et al., 2016; Fruson et al., 2012;
 131 Knezevic and Lukowiak, 2014). Using operant conditioning of aerial respiratory
 132 behaviour it has been shown that memory formation can be altered (i.e., enhanced or
 133 inhibited) by a number of different factors (Lukowiak et al., 1996; Lukowiak et al.,
 134 2000, 2010). Consistent with the studies in vertebrate models (Debiec et al., 2002;
 135 Kida et al., 2002; Milekic and Alberini, 2002), new RNA and protein synthesis are
 136 required for LTM formation and reconsolidation in *Lymnaea* (Sangha, Scheibenstock,
 137 and Lukowiak, 2003).

138
 139 Here we investigated the role of a plant flavonoid, Q, on memory formation,
 140 consolidation, and reconsolidation. Following configural learning, *Lymnaea* exposed
 141 to Q show an upregulation of *Lym*CREB1 which significantly enhanced LTM
 142 formation (Batabyal et al., 2021). Those results paved the way for our detailed study
 143 on the effects of Q on different LTM phases following operant conditioning of aerial
 144 respiratory behaviour. Here we assessed for the first time in an invertebrate model the
 145 effects of the exposure to Q on LTM formation, consolidation, and recall. We found
 146 that following the exposure of snails to Q 3h before or after training and 3h before a
 147 24h memory test that LTM formation was enhanced.

148

149 **Material and methods**

150 **Animals**

151 *Lymnaea stagnalis* were bred and raised in the snail facility at the University of
 152 Calgary from a strain of *Lymnaea* originally obtained from Vrije Universiteit in
 153 Amsterdam. We have designated these snails as the W-strain of *Lymnaea* to identify
 154 them from the other strains used in our laboratory. Other strains of *Lymnaea* have
 155 different memory abilities following operant conditioning of aerial respiration
 156 (Sunada et al., 2017). Adult snails (with a shell length of 2.5–3.0 cm) were maintained

at a density of one snail per litre in artificial pond water (PW) under eumoxic (i.e., normal O₂ levels; PO₂>9975 Pa) conditions. Artificial PW consisted of distilled water containing 26g l⁻¹ Instant Ocean (Spectrum Brands, Madison, WI, USA), with added calcium sulphate dihydrate to create what we refer to as a 'standard calcium level' of 80 mg l⁻¹ (Dalesman and Rundle, 2010; Knezevic et al., 2011). Animals were maintained at room temperature (20–22 °C) on a schedule of 16 h:8 h light: dark and had *ad libitum* access to romaine lettuce.

164

165 **Quercetin solution**

Quercetin (3,3',4',5,7-pentahydroxyflavone, Q) was obtained from Sigma Chemical Company (St Louis, MO, USA) and was dissolved in 0.1% (final concentration) dimethyl sulfoxide (DMSO). We prepared a Q solution by dissolving 50µl in 500ml of pond water (PW).

170

171 **Aerial respiratory behaviour**

Lymnaea stagnalis is a bimodal breather. In normal oxygenated conditions cutaneous respiration prevails; however, when in hypoxic conditions it switches to aerial respiration via the respiratory orifice, the pneumostome (Lukowiak et al., 1996; Lukowiak et al., 2006). Aerial respiration can be easily monitored via observation of the pneumostome opening and closing. Individually labeled snails were placed in 1 litre beaker filled with 500 ml of PW made hypoxic by vigorously bubbling N₂ for 20 min (≤0.1 ml O₂ l⁻¹). Snails experiencing hypoxia increase their rate of aerial respiration (Lukowiak et al., 1996). Animals were acclimated for 10 min prior to observation. Bubbling was then reduced and continued at a slower rate during the following 30 min of breathing observation. In that way the environment was maintained hypoxic without disturbing snail activity. During the observation period aerial respiratory behaviour was recorded. In particular, we measured the amount of time the pneumostome was open for each snail without any tactile stimulation. This allowed us to compute the total breathing time in seconds for each snail. Following each breathing observation period, snails were returned to their home eumoxic aquaria.

188

189 **A 0.5h training session procedure for operant conditioning**

190 In all of the operant conditioning training sessions and tests for LTM, a gentle tactile
 191 stimulus (using a sharpened wooden applicator) was applied to the pneumostome area
 192 (i.e., the respiratory orifice) every time the snail began to open its pneumostome to
 193 perform aerial respiration (Lukowiak et al., 1996). We refer to this response as an
 194 ‘attempted pneumostome opening’. This tactile stimulus only evoked pneumostome
 195 closure, without causing the animal to withdraw its foot and mantle area (i.e., the
 196 whole-animal withdrawal response) into its shell. Animals were given a 10 min
 197 acclimation period before the start of experiment, during which they could perform
 198 aerial respiration without receiving the negative reinforcement stimulus. The number
 199 of attempted pneumostome openings was recorded for each individual snail over the
 200 following 0.5h training session. To determine whether LTM was formed, an identical
 201 procedure (i.e., a memory test) was performed at different times following the training
 202 session. The time of the training session was defined as ‘T0’, whereas the memory
 203 tests were defined as times after the T0. Between the conditioning (training and
 204 memory) phases snails were returned to their eumoxic home aquaria, where they were
 205 fed *ad libitum* and freely allowed to perform aerial respiration. Breathing behaviour
 206 was not monitored while snails were in the home aquaria.

207

208 **Single poke procedure**

209 Snails were placed in the hypoxic environment for a 10-min acclimation period
 210 without receiving any negative reinforcement stimulus. During the following 0.5h of
 211 exposure to hypoxic PW they received a poke only the first time they began to open
 212 the pneumostome. Hence the term ‘single poke procedure’ (Karnik et al., 2012).

213

214 **Statistical analysis**

215 All our experimental treatments are independent from each other and have specific
 216 stimulus exposure timelines. Thus, we cannot combine the data in a meaningful
 217 manner for any grouped statistical analyses. We thus performed an individual paired-
 218 sample t test for each experiment. In all analysis reported here, a type I error rate of
 219 0.05 was used. All statistical analyses were performed using GraphPad Prism v. 9.0.0e
 220 for MAC® (GraphPad Software, Inc., La Jolla, CA, USA).

221

222 **Results**

223 **Effect of Q-exposure 3h before or after a 0.5h training session on LTM** 224 **formation**

225 We first determined if aerial respiratory behaviour was significantly influenced by Q.
226 Thus, total breathing time (TBT) was measured in standard hypoxia in a cohort of
227 snails (N = 10) 20h before and 3h after the exposure to Q for 1h. We found that TBT
228 before and following exposure to Q was not significantly different ($t = 0.72$, $df = 9$,
229 $p = 0.49$) (**Figure 1A**).

230

231 We next confirmed that in W-strain snails a 0.5h training session does not result in
232 LTM formation (**Figure 1B**). We trained a naïve cohort of snails (N = 10) with a 0.5h
233 training session (T0) and we tested for LTM 24h later (T24). These snails did not
234 exhibit LTM as the number of attempted pneumostome openings at T0 and T24 was
235 not statistically different ($t = 1.94$, $df = 9$; $p = 0.084$).

236

237 Then, we wished to determine if the Q-exposure 3h before training altered the ability
238 of snails to form LTM (**Figure 1C**). Thus, a cohort of naïve snails (N = 11) was
239 exposed to Q for 1h and three hours later received a 0.5h training session (T0). We
240 tested memory 24h later (T24). Statistical analyses revealed that the number of
241 attempted pneumostome openings at T24 was significantly reduced compared to T0 (t
242 $= 7.9$; $df = 10$; $p < 0.0001$). Thus, Q-exposure 3h before the 0.5h training session
243 enhanced LTM formation (i.e., LTM was present 24h after T0).

244

245 Finally, we asked whether Q-exposure 3h after a 0.5h training session (T0) would also
246 enhance LTM formation (**Figure 1D**). A cohort of naïve snails (N = 11) was first
247 trained in hypoxic PW (T0) and three hours later was placed for 1h in eumoxic Q-
248 containing PW. Animals were then returned to their home aquaria and tested for LTM
249 20h later (i.e., at T24). Again, we found that at T24 the snails' respiratory behaviour
250 was significantly reduced with respect to T0 ($t = 8.74$; $df = 10$; $p < 0.0001$). Thus, a
251 LTM lasting 24h was formed as a result of the exposure to Q after training.

252

253 Finally, we did control experiments to test the effect of the vehicle used to dissolve Q
254 (i.e., DMSO) on LTM enhancement. We found that the exposure to DMSO 3h before
255 the 0.5h training session did not result in LTM (T0: Mean $\pm$ sem = 6.90 ± 0.62 ; T24:
256 Mean $\pm$ sem = 6.60 ± 0.60 ; $t = 0.81$, $df = 9$; $p = 0.43$). Similar results were obtained
257 when DMSO exposure occurred 3h after the 0.5h training session (T0: Mean $\pm$ sem =
258 6.40 ± 0.54 ; T24: Mean $\pm$ sem = 6.0 ± 0.49 ; $t = 0.82$, $df = 9$; $p = 0.43$).

259

260 Together these data show that Q-exposure enhanced LTM formation in W-strain
261 snails when snails experience Q either 3h before or after a 0.5h training session.
262 Exposing snails to vehicle only 3h before or 3h after training did not result in LTM.
263 Finally, Q-exposure did not alter normal homeostatic aerial respiration. Thus, we
264 concluded that Q exposure is capable of enhancing LTM formation.

265

266 An obvious experiment to perform was to train a naïve cohort of snails in hypoxic PW
267 + Q and determine if this also resulted in enhanced LTM formation. Quite
268 unexpectedly, we found that the combination of hypoxic PW and Q severely
269 suppressed snails' aerial respiratory behaviour, resulting in a sleep-like quiescent state
270 that persisted for at least 2h after ending the exposure (data not shown). This
271 phenomenon is currently under investigation in our lab.

272

273 **Effect of the single-poke procedure and Q-exposure on LTM**

274 The demonstration that Q-exposure both 3h before and after a 0.5h training session
275 procedure enhanced LTM formation and consolidation led us to investigate the effects
276 of Q exposure on memory reconsolidation. Previous studies demonstrated that 24h
277 after a training procedure that only results in an intermediate-term memory (ITM) a
278 residual memory trace is present despite the LTM phenotype being absent (Parvez et
279 al., 2005, 2006). Here (**Figure 2A**, $N = 11$), we found that a 0.5h training session
280 followed by the 'single poke' procedure 24h after (i.e., T24) was not sufficient to
281 enhance LTM at T48 (i.e., 48h after T0). In fact, there was no statistical difference
282 between the number of attempted openings at T48 compared to T0 ($t = 1.79$, $df = 10$;
283 $p = 0.1$).

284

285 We next asked (**Figure 2B**) whether the combined exposure to Q and the single poke
 286 procedure performed 24h after a 0.5h training session procedure (i.e., at T24) would
 287 result in LTM when memory was tested 24h later (i.e., T48). Thus, a naïve cohort of
 288 snails (N = 11) was first trained in a 0.5h training session (T0) and 20h later was
 289 exposed to Q for 1h. Three hours later (i.e., 24h after T0 - T24), animals were placed
 290 in the hypoxic environment and received a poke only the first time they began to
 291 open the pneumostome (i.e., ‘single poke’ procedure).
 292 LTM was then tested 24h later (i.e., T48). In these snails a 48h LTM was shown ($t =$
 293 3.02, $df = 10$; $p = 0.012$), since the number of attempted pneumostome openings at
 294 T48 was significantly less than that in T0. Thus, combining Q-exposure 3h before the
 295 ‘single-poke procedure’ resulted in an LTM when tested 48h after T0.

296

297 This result led us to hypothesize that Q-exposure 3h after the ‘single-poke’ procedure
 298 would also result in enhanced LTM formation. Thus, a naïve cohort of animals (N =
 299 12) was tested using the same above behavioural procedure, but in these animals the
 300 exposure to Q occurred 3h after the ‘single poke’ procedure performed at T24 (**Figure**
 301 **2C**). We found that at T48 the snails’ respiratory behaviour was significantly reduced
 302 with respect to T0 ($t = 8.25$, $df = 11$; $p < 0.01$), indicating that LTM persisted for at
 303 least 48h.

304 Finally, we showed in a cohort of snails (N = 10) trained as above that when Q-
 305 exposure was delayed by 20h after the ‘single-poke’ procedure, LTM was not present
 306 (**Figure 2D**). That is, the number of attempted pneumostome openings between the
 307 T0 and T48 was not significantly different ($t = 0.3$, $df = 9$; $p = 0.77$). These data show
 308 that the ‘single poke’ procedure acts on a residual trace present 24h after T0 and when
 309 combined with Q-exposure 3h before or after, enhanced LTM formation.

310

311 **Effect of the single-poke procedure and Q-exposure before a 0.5h training**
 312 **session and LTM formation**

Continuing with the effects of the ‘single poke’ procedure, we asked what effect, if any, the ‘single poke’ would have if the snails received it before a 0.5h training session. In a naïve cohort of snails (**Figure 3A**; N = 10) the single poke procedure 24h before T0 did not result in LTM when tested 24h later (i.e., T24). A paired t-test revealed that the number of attempted pneumostome openings at T24 was not significantly different from those at T0 ($t = 0.58$, $df = 9$, $p = 0.59$). Thus, LTM was not present. We then asked whether Q exposure 24h before a 0.5h training session (i.e., T0) resulted in enhanced LTM formation. Thus, a naïve cohort of snails (N = 10) was exposed to Q 24h before T0 and was then tested for LTM 24h later (**Figure 3B**). A paired t-test revealed that the number of attempted pneumostome openings at T24 was not significantly different from those at T0 ($t = 1.33$, $df = 9$, $p = 0.21$). That is, Q-exposure 24h before a 0.5h training session did not result in LTM formation.

We next asked if a combination of the ‘single poke’ procedure and Q-exposure 24h before a 0.5h training session (i.e., T0) would result in enhanced LTM formation. These data are presented in **Figure 4**. A naïve cohort of snails (N = 10) was first exposed to Q for 1h and 3h later was subjected to the ‘single poke’ procedure (**Figure 4A**). Animals were then returned to their home aquaria for 20h before being trained with a 0.5h training session (T0). These snails were then tested for LTM 24h later (i.e., T24). We found that the number of attempted openings at T24 was significantly less than at T0 ($t = 7.23$; $df = 11$; $p < 0.0001$). Thus, LTM was present. These data show that Q-exposure, combined with the single poke procedure 24h before training, resulted in LTM formation. We next presented to a naïve cohort of snails (N = 10) the single poke procedure first and then 3h later we exposed the snails to Q for 1h (**Figure 4B**). These snails then were trained with a 0.5h training session (T0) and were tested for LTM 24h later (i.e., T24). The sequential presentation of the single poke and then Q exposure resulted in LTM. That is, the number of attempted pneumostome openings in T24 was significantly less than in T0 ($t = 2.84$, $df = 9$; $p = 0.02$). Thus, the ‘single-poke’ procedure and Q-exposure 3h later was sufficient to cause LTM formation following a 0.5h training session 20h later. Not shown here, are control experiments where the vehicle is used rather than Q along with the single-poke procedure; in all cases enhanced LTM formation was not observed.

345

346 **Discussion**

347 This study tested the hypothesis that Q-exposure during critical periods of memory
348 formation enhanced LTM formation following operant conditioning of aerial
349 respiratory behaviour. Thus, Q-exposure 3h before or after the different phases of
350 learning and memory (e.g., consolidation and recall) lead to enhance LTM formation.
351 In addition, Q-exposure improved LTM formation following the ‘single poke’
352 procedure. These data are all consistent with Q-exposure upregulating *LymCREB1*
353 which then synergistically interacts with the other molecular events necessary to form
354 LTM. Q-exposure has previously been shown to act in the snails’ nervous system to
355 both block the up-regulation of heat shock proteins (HSPs) following a heat shock and
356 to up-regulate *LymCREB1* (Foster et al., 2015; Sunada et al., 2016; Rivi et al., 2021a;
357 Batabyal et al., 2021). Since *LymCREB1* plays a key role in conditioned taste aversion
358 memory formation in *Lymnaea* (Sadamoto et al., 2004), our data extend those
359 findings to include memory formation following operant conditioning of aerial
360 respiratory behaviour. Here we showed that Q-exposure alters four different phases of
361 memory: 1) acquisition (i.e., a learning event), 2) consolidation processes after
362 acquisition, 3) memory recall, and 4) memory reconsolidation. In all these memory
363 phases Q-exposure enhanced LTM persistence.

364 Memory is stored in specific neural networks termed ‘memory engrams’ (Semon,
365 1921) that can be modified by factors such as stress, increased training and, in the
366 data shown here, the exposure to certain bio-active products such as Q. While not
367 examined here, we hypothesize that Q-exposure alters the activity of an identified
368 neuron, RPeD1 (Right Pedal Dorsal 1), known to be a necessary site for LTM
369 formation, extinction, and reconsolidation (Lukowiak et al., 2006; Sangha et al., 2005;
370 Sangha et al., 2003a,b; Sangha, Scheibenstock, and Lukowiak, 2003; Scheibenstock et
371 al., 2002). Previously, it has been shown that predator detection (using crayfish
372 effluent - CE) alters the behaviour of this neuron leading to enhanced LTM formation
373 (Orr and Lukowiak, 2008). However, for that to occur snails had to be trained in CE.
374 The exposure to CE 1h before or 1h after a single 0.5h did not cause LTM
375 enhancement. In a similar vein, another bio-active substance, epicatechin, also had to

376 be experienced either during or immediately after training to cause LTM enhancement
377 (Fernell et al., 2016)

378

379 Our working hypothesis was that Q-exposure, which causes an up-regulation of
380 *LymCREBI* 3 hours after exposure (Batabyal et al., 2021), results in enhanced LTM
381 formation when Q-exposure occurs within 3 hours of the different phases of learning
382 and memory formation. That is, boosting *LymCREBI* activity during the 3-hour
383 window prior to or after the single 0.5h operant conditioning training session may
384 amplify the on-going and necessary molecular processes that underlie LTM. The
385 experiments reported here were all designed to test this hypothesis. Our data also add
386 to our knowledge that bio-active, naturally occurring substances, such as Q, can alter
387 cognitive ability (e.g., enhanced LTM formation). Finally, the data also illustrate the
388 advantages of using our model system in gaining an understanding of how substances,
389 such as Q, alter cognition. Q, as well as many other compounds (e.g., epicatechin),
390 easily penetrate through the integument of the snail and, because snails have an open
391 circulatory system, can easily and quickly reach the CNS (Fruson et al., 2012; Rivi et
392 al., 2021b, Rivi et al., 2020).

393

394 The highly conserved CREB gene and CREB-like proteins have been characterized in
395 *Lymnaea* nervous system (Frank and Greenberg, 1994) Ribeiro et al., 2003; Sadamoto
396 et al., 2004) and it has been demonstrated that the conditioned taste aversion (CTA)
397 training increased *LymCREBI* expression levels (Sadamoto et al., 2010). Moreover,
398 the levels of phosphorylated CREB1 are also increased following appetitive classical
399 conditioning of feeding behaviour (Ribeiro et al., 2003). In particular, in appetitive
400 conditioning studies it was demonstrated that between 10 min and 1 h after training,
401 many translational-dependent processes are activated to form ITM. While ITM only
402 requires the synthesis of new proteins from pre-existing mRNA transcripts, LTM is
403 dependent on altered gene activity, new protein synthesis and transport of the
404 protein(s) to the site of plasticity (Braun and Lukowiak, 2011; Lukowiak et al., 2000;
405 Rosenzweig et al., 1993). ITM and LTM are independent but interrelated events
406 which occur in parallel and has specific temporal phases and transition periods (Marra

et al., 2013). As CREB1 is a regulator of mRNA transcription, it most likely does not play a necessary role in ITM formation but does so for LTM formation (Sadamoto et al., 2004). Studies in *Drosophila* and rodent models also demonstrate that neurons with higher CREB levels are preferentially recruited for LTM engrams as *de novo* transcription and translation is required during formation and consolidation of LTM (Han et al., 2007; Miyashita et al., 2018).

413

In the W-strain of *Lymnaea* used here a 0.5h training session is only capable of causing a memory that persists for approximately 3h and this has been termed intermediate-term memory (ITM, Sangha et al., 2003a,b). However, Parvez et al., (2005, 2006) found that there was a residual memory trace 24h after training, even though the LTM phenotype was absent. Thus, it was apparent to us that we could ascertain whether the exposure to Q 3h before or after this period caused LTM enhancement when combined with the presentation of a stimulus associated with learning (i.e., the single poke procedure). As we showed here, exposing snails to Q 20h after a 0.5h training session was sufficient for the single-poke procedure performed 3h later to cause LTM formation when tested after T0. Similarly, we showed that using the single-poke procedure 24h after the initial 0.5h training session and then 3h later exposing snails to Q resulted in a LTM when tested 48h after the initial training session (i.e., T0). Both the consolidation and reconsolidation processes of LTM in our model system, as well as in vertebrates, have been shown to be dependent on altered gene activity (i.e., transcription) and *de novo* protein synthesis (i.e., translation) (Anokhin et al., 2002; Debiec et al., 2002; Judge and Quartermain, 1982; Kida et al., 2002; Milekic and Alberini, 2002; Nader and Einarsson, 2010). Moreover, there are several lines of evidence implying that memory consolidation and reconsolidation use similar molecular pathways that converge on the activation of CREB1 (Alberini, 2011; McKenzie and Eichenbaum, 2011; Nader and Einarsson, 2010; Tronson and Taylor, 2007).

Together these data are consistent with our hypothesis that Q, which induces an upregulation of *LymCREB1*, interacts synergistically with the molecular processes that underlie LTM formation, consolidation, and reconsolidation. Thus, Q-exposure,

438 in essence, boosts those processes if the boost occurs within a specific ‘window of
439 opportunity’. Outside of that window, an enhancement of LTM formation is not
440 observed.

441

442 We further showed, using the single-poke procedure, that the exposure to Q 3h before
443 or after the single poke procedure enabled a 0.5h training session, given some 20h
444 after the Q-exposure combined with the single-poke procedure, to result in LTM
445 formation. These data suggest that the Q-induced upregulation of *LymCREBI* persists
446 for sufficient time, such that the one 0.5h training session became capable of inducing
447 the molecular changes necessary for LTM formation. We plan to investigate this in
448 detail in our future experiments.

449

450 Our findings reported here on the enhancement of LTM formation with Q-exposure
451 are also consistent with our recent findings on configural learning in *Lymnaea*
452 (Batabyal et al., 2021). In that study, Q-exposure 3h prior to, immediately before or
453 3h following configural learning training, enhanced LTM formation. Usually, the
454 configural learning procedure only results in memory persisting for 3h (i.e., ITM) but
455 following Q-exposure an LTM was formed that persisted in some cases for 48h
456 (Batabyal et al., 2021).

457

458 Previous studies demonstrated that a 0.5h is sufficient to produce an ITM trace lasting
459 3h, which is dependent on new protein synthesis (Braun and Lukowiak, 2011;
460 Lukowiak et al., 2000; Sangha et al., 2003a,b). It is at this 3h post training time, in
461 appetitive food conditioning, that *Lymnaea* show a transition from ‘late-ITM’ to LTM
462 that requires RNA synthesis (Marra et al., 2013). During the 3h interval between
463 training and Q-exposure the molecular machinery required for ITM to be converted to
464 LTM is induced by Q via upregulation of *LymCREBI*. These data are consistent with
465 previous studies from both invertebrates and vertebrates showing the key role of
466 CREB1 in LTM formation and consolidation after various learning paradigms
467 (Alberini, 2011; Sangha, Scheibenstock, and Lukowiak, 2003). We conclude that Q-

468 exposure 3h before or after training, while acting on different mnemonic phases (i.e.,
469 memory formation and consolidation, respectively), enhances the persistence of LTM
470 in *Lymnaea*.

471 Our data are also consistent with the hypothesis that dietary sources of Q could
472 improve cognitive ability (Finkbeiner et al., 1997; Kida, 2012; Silva et al., 1998;
473 Suzuki et al., 2011). Further, our data illustrate that *Lymnaea* is a suitable model to
474 elucidate neuronal and molecular mechanisms induced by Q-exposure during the
475 different phases in the process of transforming learning into memory and its recall.
476 We hypothesize that *LymCREB1* activation is differentially involved and regulated
477 depending on the time of Q-exposure. Other studies suggested that flavonoids may
478 modulate kinase activity [e.g., mitogen-activated protein kinase (MAPK) cascade] and
479 signalling cascades lying downstream of these kinases (Spencer, 2007). The protein
480 kinase/MAPK signalling cascades have been shown to be necessary for both ITM and
481 LTM formation in *Lymnaea* (Ribeiro et al., 2003). Therefore, alteration of protein
482 kinase activity following Q-exposure may potentially be the mechanism by which
483 memory formation is enhanced in *Lymnaea*.

484 Findings presented here provide the groundwork for future molecular analysis of how
485 Q-exposure acts at the neuronal level and the mechanisms involved in the alterations
486 to memory formation, storage and, recall, paving the way for interesting translational
487 neuroscience studies. Our current data provides the first support for Q-modulated
488 enhancement of cognitive function in an invertebrate model after an operant
489 conditioning procedure. Thus, strengthening the case for *Lymnaea* as a model for
490 translational neuroscience (Rivi et al., 2021b, Fodor et al., 2020).

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499

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501   performed behavioural data collection, analysis and writing first draft. AB, CB,
502   JM CB, and FT critically revised manuscript. KL coordinated study, provided funding,
503   and critically revised manuscript. All authors take responsibility of the data provided
504   and consent to the publication of the manuscript.

505

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891

892 Figure legends

894 **Fig. 1.** Quercetin exposure and aerial respiration.

895 (A) Aerial respiratory behaviour in *Lymnaea* is not significantly altered by Q. We
896 plotted and compared the total breathing time (TBT) between the first observation
897 period (Obs1 – closed circles) and the second one performed 24h later, 3h after
898 the exposure to Q (Obs2 – open circles).

899 (B) Snails given a 0.5h TS in hypoxic PW do not form LTM. A naïve cohort of snails
900 (N = 10) received a 0.5h training session (T0 – closed circles) and LTM was
901 tested 24h later (T24 – open circles).

902 (C) The exposure to Q 3h before a 0.5h training session enhances LTM formation. A
903 naïve cohort of snails (N = 10) was exposed to Q for 1h and 3h later received a

0.5h training session (T0 – closed circles). 24h later animals were tested for LTM (T24 – open circles).

(D) The exposure to Q 3h after a 0.5h training session enhances LTM formation. A naïve cohort of snails (N = 11) received a 0.5h training session (T0 – closed circles) and 3h after was exposed to Q for 1h. Snails were tested for LTM 24h later (T24 – open circles).

The timeline for each experiment is presented above the data. The solid line is the mean, and the error bars are the s.e.m. Comparisons were made by paired t-test. ns = not significant; ****p<0.0001.

913

914

915 **Fig. 2.** The single poke procedure, quercetin exposure, and enhanced LTM formation.

916 (A) Snails receiving the single poke procedure 24h before the 0.5h training session do
917 not form LTM. A naïve cohort of snails (N = 11) received a 0.5h training session

918 (T0 – closed circles). 24h later animals received the ‘single poke’ procedure and
 919 24h later were tested for LTM (T48 – closed squares).
 920 **(B)** The exposure to Q 3h before the ‘single poke’ procedure occurring 24h after the
 921 0.5h training session enhanced LTM formation. A naïve cohort of snails (N = 11)
 922 received a 0.5h training (T0 – closed circles) and 20h later was exposed to Q for
 923 1h. 3h later snails received the single poke procedure and at T48 were tested for
 924 LTM (closed squares).
 925 **(C)** The exposure to Q 3h after the ‘single poke’ procedure occurring 24h after a 0.5h
 926 training session enhanced LTM formation. A naïve cohort of snails (N = 11)
 927 received a 0.5h training (T0 – closed circles) and 24h later was subjected to the
 928 single poke procedure. 3h later snails were exposed to Q for 1h and 20h later (at
 929 T48 – closed squares) were tested for LTM.
 930 **(D)** The exposure to Q 20h after the single poke procedure does not result in enhanced
 931 LTM formation. 24h after the 0.5h training session (T0 – closed circles), naïve
 932 snails (N = 10) were subjected to the single poke procedure. Q exposure occurred
 933 20h later, 3h before LTM was tested at T48 (closed squares).
 934 The timeline for each experiment is presented above the data. The solid line is the
 935 mean, and the error bars are the s.e.m. Comparisons were made by paired t-test. ns =
 936 not significant; *p < 0.05; **** p < 0.0001.

937

938 **Fig. 3.** The single poke procedure or Q-exposure 24h before a 0.5h training session
939 does not result in LTM formation.

940 **(A)** When applied 24h before a 0.5h training session, the single poke procedure does
941 not result in LTM formation. Snails were trained (T0 – closed circles) 24h after
942 receiving the single-poke procedure and LTM was tested at T24 (open circles).

943 **(B)** When applied 24h before the 0.5h training session, Q-exposure does not result in
944 LTM formation. Snails were exposed to Q for 1h and 24h later received a 0.5h
945 training session (T0 – closed circles). LTM was tested at T24 (open circles).

946 The timeline of each experiment is presented above the data. Comparisons were
 947 made by paired t-test. The solid line is the mean, and the error bars are the s.e.m. ns =
 948 not significant.
 949

950
 951 **Fig. 4.** The single poke procedure combined with Q-exposure 3h before a 0.5h
 952 training session results in enhanced LTM formation.

953 (A) The exposure to Q 3h before the single poke procedure performed before the
 954 single training results in LTM. A naïve cohort of snails (N = 10) was exposed to Q for
 955 1h and 3h later received the single poke procedure. 20h later, animals were trained
 956 (T0 – closed circles) and LTM was tested at 24h (T24 – open circles).

957 **(B)** The exposure to Q 3h after the single poke procedure performed before the single
 958 training results in LTM. A naïve cohort of snails ($N = 9$) received the single poke
 959 procedure and 3h later was exposed to Q for 1h. 20h later, animals were trained (T0 –
 960 closed circles) and LTM was tested at T24 (open circles).

961 The timeline of each experiment is presented above the data. Comparisons were
 962 made by paired t-test. **** $p < 0.0001$; * $p < 0.05$.

A **B** **C** **D**

A **B** **C** **D**

A

B

A **B**