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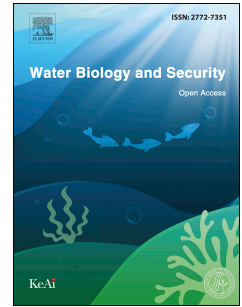
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Hot and cold exposure triggers distinct transcriptional and behavioral responses in laboratory-inbred pond snails

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Abstract

Animals exhibit remarkable behavioral and molecular adaptations to cope with thermal stressors, which are crucial for survival in variable environments that are exacerbated by climate change. Aquatic poikilotherms like our model organism—the pond snail *Lymnaea stagnalis*—face significant challenges due to their dependence on external temperatures. Our study provides valuable insights into the different behavioral and molecular responses of lab-inbred snails to cold and heat shock stressors (i.e., 4 °C and 30 °C), particularly in the context of learning and memory formation. We found that while short-term (1 h) cold exposure transiently upregulated the expression levels of HSP70 and HSP40 in the snail's central ring ganglia, prolonged cold exposure (24 h) resulted in a significant downregulation of LymMIPII and an upregulation of LymMIPR. These data suggest, albeit at the transcriptional level, the existence of a negative feedback loop necessary for sustaining cellular functions when metabolic demands might shift towards conserving energy during prolonged cold exposure. At the behavioral level, we found that, compared to heat shock, cold exposure did not result in a Garcia effect (i.e., a "special form" of conditioned taste aversion). The difference in memory outcomes was associated with changes in the expression levels of selected targets involved in neuronal plasticity and the stress response. While both cold and heat shock upregulated the HSP levels in the snail's central ring ganglia, cold exposure did not affect the expression levels of the neuroplasticity genes LymGRIN1 and LymCREB1, contrasting with heat shock's neurogenic effects. Overall, this study provides insights into *L. stagnalis*'s adaptive responses to thermal stressors, emphasizing different molecular strategies for coping with heat versus cold challenges in aquatic environments. These findings contribute to our understanding of thermal biology and stress physiology in aquatic organisms, underscoring the importance of molecular mechanisms in shaping species' resilience in dynamic environments.

Keywords: temperature sensitivity, *Lymnaea stagnalis*, Garcia effect, HSPs, CREB, energy

42 1. Introduction

43 Climate change is resulting in extreme temperature fluctuations from heat waves and cold waves to incessant drought
44 and floods across the globe, which is threatening species' existence (Duffy et al., 2022; Rocha et al., 2022; Soravia et al.,
45 2021; Wilson & Nicoll, 2001). To cope with such ecological stressors, organisms either acclimate or show behavioral
46 plasticity to local environmental changes (Piyaphongkul et al., 2014; Prahlad & Morimoto, 2009; Quan et al., 2023;
47 Rensing & Ruoff, 2002; Scott et al., 1997), with thermal adaptation being one of the most common strategies observed
48 in species living in diverse temperatures (Fontúrbel et al., 2021; Quan et al., 2022; Tian et al., 2020; van Heerwaarden
49 et al., 2016; Vasseur et al., 2014; Zhou et al., 2018). Poikilotherm organisms, in particular, are vulnerable as their internal
50 temperature depends on external conditions (Guschina & Harwood, 2006; Malik et al., 2023; Wagner et al., 2023).
51 Thermal stress across animal taxa induces the expression of heat shock proteins (HSPs)—molecular chaperones that
52 protect cells from misfolded proteins under stress (Sørensen et al., 2007; Tedeschi et al., 2015, 2016; Tomanek &
53 Sanford, 2003)—whose differential expression is linked to variation in thermal tolerance within and among species (J.
54 Gao et al., 2014; Woodruff et al., 2022).

55
56 We use the great pond snail *Lymnaea stagnalis* as a model species for investigating the effects of different stressors
57 occurring in aquatic environments and as a candidate bioindicator (Lukowiak et al., 2010, 2014; Rivi et al., n.d.; Rivi,
58 Batabyal, Benatti, Tascetta, et al., 2022a; Rivi, Batabyal, Benatti, Blom, et al., 2023b; Rivi, Batabyal, Lukowiak, Benatti,
59 et al., 2023d). Pond snails inhabit stagnant or slow-flowing ponds and lakes in northern North America and Eurasia,
60 experiencing broad temperature fluctuations daily and seasonally (Amorim et al., 2019). In Alberta and Saskatchewan,
61 where we collected wild snails, temperatures in water bodies ranged from about 4 °C in April–May to nearly 35 °C in
62 July–August (Brown, 1979). As *L. stagnalis* is a poikilotherm, cold and hot temperatures affect both its physiology and
63 behavior (Bowler, 2018), as shown by the fact that *Lymnaea* grow between 11 and 28 °C, growth does not occur below
64 10 °C, and the upper lethal limit is around 32–33 °C (Batabyal et al., 2022; Rivi, Batabyal, Benatti, Tascetta, et al.,
65 2022b). *L. stagnalis* overwinter in the wild by moving to the deepest location or the bottom of lakes where the
66 temperature is maintained at 4 °C while the water at the surface freezes (Fernell et al., 2021). Inbred, laboratory-reared
67 snails—like those used in this study—are kept at around 20 °C, rarely experiencing temperature fluctuations greater
68 than 1–2 °C annually. These snails have been maintained at standardized conditions for more than 250 generations
69 (Fodor et al., 2020). Thus, in inbred snails, the exposure to an acute heat shock (1 h at 30 °C), resembling typical mid-
70 summer conditions experienced by wild snails, acutely up-regulates mRNA levels of HSP40 and HSP70 (Rivi et al., 2021;
71 Sunada et al., 2016; Foster et al., 2015; Teskey et al., 2012) as a response to stressful conditions. Although prolonged
72 exposure to 30 °C is lethal for lab-inbred snails, an acute exposure (for 1 h) after the presentation of a novel appetitive
73 taste results in a sickness-like state and, therefore, in a taste-specific and long-lasting form of conditioned taste aversion
74 known as the Garcia effect (Batabyal et al., 2024; Rivi, Batabyal, et al., 2021; Rivi, Batabyal, Benatti, Blom, et al., 2022;
75 Rivi, Batabyal, Benatti, Tascetta, et al., 2022b, 2023b, 2023a). At a behavioral level, we observed long-term memory of
76 the novel food aversion, whereas at the molecular level, the heat shock exposure resulted in a significant upregulation
77 of the expression levels of HSP70 and HSP40 whose inhibition prevents the formation of the Garcia effect, suggesting a
78 key role of these targets in the Garcia effect formation (Batabyal et al., 2021; Rivi et al., 2021, 2024; Sunada et al., 2016).
79 That is, the Garcia effect offers a measure of learning and memory abilities and allows to study how heat tolerance and
80 HSP induction in snails from different thermal conditions, including the cold ones.

81 On the other hand, cold shock can be used to block the snail's memory consolidation following the operant conditioning
82 of aerial respiration, and prolonged exposure to temperatures lower than 10 °C blocks the snail's growth (Orr et al.,
83 2009; Sangha et al., 2003; Martens et al., 2007; Takahashi et al., 2013). Thus, cold and heat shock exposures differentially
84 affect the pond snail's homeostatic behaviors and cognitive functions. Understanding responses to cold and heat shock
85 and tolerance limits is crucial in our changing world, as animal distribution and diversity are closely linked to their stress
86 responses. Animals can navigate temperature fluctuations by altering physiological, psychological, behavioral, and
87 cognitive functions as well as through range shifts to suitable locations (Yin et al., 2024). However, models like *L.*
88 *stagnalis*, which are found in stagnant water bodies and at higher latitudes and altitudes, have fewer dispersal
89 opportunities and are more at risk.

90
91 In this complex scenario, *L. stagnalis* serves as an optimal model to explore the consequences of water temperature
92 fluctuations on their homeostatic behaviors and cognitive functions, as they have been used for ecotoxicology and
93 neuroscience research since the 1970s and exhibit natural cognitive ability differences (Kagan, Batabyal, et al., 2023;
94 Kagan, Rivi, et al., 2023; Rivi, Batabyal, Benatti, Blom, et al., 2023a; Rivi, Batabyal, Benatti, Tascetta, et al., 2023c).
95 This study consisted of two experiments. *Experiment 1* focused on transcriptional effects induced by cold shock
96 exposure (1 h or 24 h) in the inbred snail's central ring ganglia. Previous studies showed that cold, food-scarce winter
97 conditions create a significant bottleneck on animal's poleward persistence, leading to a reversible seasonal phenotype
98 of inactivity, low body temperature, fasting, and low metabolic rate as a survival tactic (Reeve et al., 2022; Speers-
99 Roesch et al., 2018). However, mechanisms underlying winter dormancy remain poorly understood, especially in
100 poikilotherm species like *L. stagnalis*. To elucidate the role of HSPs in the central ring ganglia of *L. stagnalis* under
101 different cold regimens (1 h or 24 h), we assessed transcriptional changes in targets involved in energy homeostasis and
102 neuroplasticity: LymHSP70 and LymHSP40, molluscan insulin peptide II (LymMIP-II) and its receptor (LymMIPR),
103 glutamate ionotropic receptor NMDA type subunit 1 (LymGRIN1), and cAMP response element-binding protein 1
104 (LymCREB1) (Murakami et al., 2013; Rivi, Benatti, Actis, Tascetta, et al., 2022). We hypothesized that short-term
105 exposure to 4 °C would upregulate LymHSP70 and LymHSP40 expression, while long-term exposure would
106 downregulate the insulin pathway to promote an 'energy saving' mode. Given that cold exposure can induce the
107 blockage of memory formation, we tested the hypothesis that exposing snails to 4 °C downregulates neuroplasticity
108 targets like LymGRIN1 and LymCREB1. *Experiment 2* investigated whether a cold shock (4 °C for 1 h) could induce a
109 Garcia effect similar to heat shock (Rivi, Batabyal, Benatti, Blom, et al., 2022) by examining transcriptional effects of
110 these two procedures (cold vs. heat shock) on HSPs and neuroplasticity targets in the snail's central ring ganglia.

111
112
113 This is the first study to investigate both short-term (i.e., 1 h) and long-term responses to cold shock while comparing
114 the biological implications of opposite temperature extremes. Understanding how species like *L. stagnalis* respond to
115 thermal stress is crucial for predicting and mitigating the impacts of climate change on aquatic ecosystems. This is
116 essential for preserving biodiversity and maintaining the stability of ecosystem services (A.K. et al., 2023).

117 The cross-talk between targets involved in stress response, energy balance, and neuroplasticity in mediating thermal
118 tolerance and cognitive functions may help to unravel the critical molecular mechanism through which aquatic
119 organisms cope with temperature variation, offering biomarkers for assessing the health and stress levels of aquatic

120 populations. Furthermore, exploring the neurobiological aspects of thermal stress, particularly its impact on learning
121 and subsequent memory formation, may contribute to enhancing our understanding of how environmental factors
122 influence neural and behavioral plasticity. This knowledge is vital for predicting how aquatic species might adapt to
123 rapid environmental changes. As climate change exacerbates temperature fluctuations and extreme weather events,
124 maintaining the health of aquatic ecosystems becomes increasingly important. This study provides insights into the
125 resilience of aquatic organisms, which can inform conservation efforts and guide the management of water resources
126 (Trégarot et al., 2024; Venegas et al., 2023). Overall, this study underscores the interconnectedness of water biology,
127 neurobiology, and climate change. It calls for integrated approaches to research and conservation that consider the
128 multifaceted impacts of global temperature changes on aquatic organisms and ecosystems.

129

130 **2. Material and Methods**

131 *2.1 Snails and their Maintenance*

132 In this study, we used a laboratory-inbred strain (W-strain) of *L. stagnalis* maintained at the University of Calgary Biology
133 Department. This strain originated from an inbred stock kept at the Vrije University of Amsterdam, originally bred from
134 animals collected in the 1950s in polders near Utrecht, The Netherlands (Koene, 2006). The snails were housed in
135 artificial pond water, which was made from deionized water supplemented with Instant Ocean (0.25 g/L) and calcium
136 carbonate (CaCO₃) to maintain calcium concentrations above 50 mg/L (Dalesman & Lukowiak, 2010). Snails were kept
137 at 20 ± 1 °C with a light-dark cycle of 16 h light and 8 h dark. Six-month-old snails with shell lengths of 20–25 mm were
138 used for the experiments. While experiments on pond snails do not require ethics committee approval, we ensured the
139 well-being of the snails throughout the behavioral procedures.

140

141 *2.2 Experiment 1: Transcriptional Effects of Short- and Long-Term Cold Exposure in L. stagnalis 's Central Ring Ganglia*

142 To investigate the transcriptional effects of short- and long-term cold exposure, 16 naïve snails were placed in a 1-L
143 beaker filled with 500 mL of artificial pond water cooled to 4 °C. The beaker was maintained in a water bath at 4 °C for
144 either 1 h (N = 8) or 24 h (N = 8). Control naïve snails (N = 8) were placed in a 1-L beaker with 500 mL of 20 °C (i.e., room
145 temperature) artificial pond water. After exposure, the snails were returned to their home aquaria for 3 h before being
146 sacrificed. The central ring ganglia were extracted for analysis 3 h post-cold exposure.

147

148 *2.3 Experiment 2: Impact of Cold and Heat Shock on the Behavioral and Molecular Outcomes of a Garcia Effect* 149 *Procedure*

150 To investigate whether a cold shock, similar to a heat shock, could induce a Garcia effect in snails, 18 naïve snails were
151 divided into three groups. We examined their feeding response to a carrot slurry (C), a novel appetitive stimulus eliciting
152 robust rasping behavior, before and 3 h after exposure to different thermal conditions. The carrot slurry was prepared
153 by blending two organic carrots with 500 mL of artificial pond water. Rasping behavior was monitored by placing snails
154 in a 14 cm Petri dish partially submerged in carrot slurry (Rivi, Benatti, et al., 2021). The dishes were positioned on a
155 clear Plexiglas stand elevated above a mirror for better visibility (Rivi, Batabyal, Wiley, Benatti, et al., 2022). After a 3-
156 minute acclimation, the number of rasps was recorded over 2 min. Snails were then returned to their aquaria for 1 h
157 before being exposed to 30 °C (heat shock), 4 °C (cold shock), or 20 °C (room temperature) for 1 h. Three hours later,

158 the rasping behavior was recorded again after a 3-minute acclimation period. During the 3-hour interval, snails were
159 kept in their home tanks without food.

160 Behavioral experiments were performed in the morning, as learning scores are better at this time. After testing for the
161 Garcia effect, snails were euthanized by placing them on ice for 10 min, and the central ring ganglia were dissected and
162 stored at -80°C for analysis.

163

164 2.4 Total RNA Extraction, Reverse Transcription, and Real-Time Polymerase Chain Reaction

165 Before sacrifice, snails were anesthetized on ice for 10 min. The central ring ganglia were dissected and stored at -80
166 $^{\circ}\text{C}$. Total RNA extraction and DNase treatment were performed using the GenElute™ Total RNA Miniprep Kit and
167 DNASE70-On-Column DNase I Digestion Set (Merck Millipore). Each central ring ganglion was used for RNA extraction
168 and a 200-ng RNA sample was reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (ThermoFisher).
169 Real-time quantitative PCR was conducted on 20 ng mRNA using a Bio-Rad® CFX Connect™ Real-Time PCR Detection
170 System with SYBR Green Master Mix (Bio-Rad). The cycling parameters were 95°C for 120 seconds, 95°C for 10 seconds,
171 and 60°C for 30 seconds for 40 cycles. Cycle threshold (Ct) values were determined using CFX Maestro™ Software (Bio-
172 Rad). Primers were 19–23 nucleotides long, with a melting temperature between 58 and 62°C , and a guanine-cytosine
173 (GC) content between 40% and 60%, generating an amplicon of 75–200 bp at a final concentration of 300 nM (**Table 1**).
174 mRNA levels of each target were normalized to the mean of two housekeeping genes, elongation factor 1α , and tubulin,
175 which were stable across groups as confirmed by the analysis using NormFinder (Wang et al., 2012) and were unaffected
176 by any procedure. For quantitative evaluation of changes, the comparative $2^{-\Delta\Delta\text{Ct}}$ method was used, with control animals
177 (exposed to room temperature) as the calibrator.

178

179 2.5 Statistical Analyses

180 Behavioral data were analyzed using a paired Student's *t*-test to compare the number of rasps before and after heat or
181 cold exposure (**Fig. 2**). For gene expression analyses, normality was assessed using the Kolmogorov-Smirnov one-sample
182 test. One-way ANOVA was used to compare the expression levels of each target and significant differences were
183 determined by Tukey's post hoc test. All tests were defined as significant at $p < 0.05$. Data are presented as mean \pm
184 standard error (SEM). Statistical analyses were conducted, and graphs were generated using GraphPad Prism v. 9.0.0e
185 for Mac® (GraphPad Software, Inc.).

186

187 3. Results

188 3.1 Transcriptional Effects of Short- and Long-Term Cold Exposure in *Lymnaea*'s Central Ring Ganglia

189 The aim of *Experiment 1* was to answer the following question: *what are the transcriptional effects induced by exposure*
190 *to a cold shock (4°C) for 1 h or 24 h in the central ring ganglia of lab-bred snails?* We focused our attention on the
191 expression levels of selected targets involved in energy homeostasis, response to stressors, and memory formation in
192 the central ring ganglia of *L. stagnalis* (Murakami et al., 2013; Nakai et al., 2022). A one-way ANOVA demonstrated that
193 there was a significant effect of the cold treatment on the expression levels of LymHSP70 ($F_{2,21} = 46.23$, $p < 0.001$) (**Fig.**
194 **1A**), LymHSP40 ($F_{2,21} = 11.07$, $p = 0.0005$) (**Fig. 1B**), LymMIP-II ($F_{2,21} = 7.88$, $p = 0.003$) (**Fig. 1E**), and LymMIPR ($F_{2,21} =$
195 18.66 , $p < 0.0001$) (**Fig. 1F**). Tukey's post hoc test revealed that short-term exposure to 4°C significantly upregulated
196 LymHSP70 and LymHSP40 mRNA levels compared to control snails ($p < 0.0011$ and $p = 0.0007$, respectively) and snails

197 exposed for 24 h ($p < 0.0011$ and $p = 0.004$, respectively). Notably, no significant differences in LymHSP70 and LymHSP40
198 mRNA levels were observed between control snails and those maintained at 4 °C for 24 h ($p = 0.69$ and $p = 0.72$,
199 respectively). Moreover, we found that the prolonged exposure to 4 °C for 24 h resulted in a significant downregulation
200 of LymMIP-II mRNA levels and a significant upregulation of LymMIP-R compared to control snails (LymMIP-II: $p = 0.04$
201 and LymMIPR: $p = 0.0002$) and those exposed to the cold shock procedure for 1 h (LymMIP-II: $p = 0.013$ and LymMIPR:
202 $p < 0.0001$). No effects induced by the cold exposure (1 h or 24 h) on the expression levels of LymGRIN1 ($F_{2,21} = 1.56$, p
203 $= 0.027$) and LymnCREB1 ($F_{2,21} = 0.13$, $p = 0.88$) were found (**Fig. 1C** and **Fig. 1D**).

204

205 3.2 Impact of Cold and Heat Shock on the Behavioral and Transcriptional Outcomes of a Garcia Effect Procedure

206 After demonstrating that a 1-hour cold exposure significantly upregulated LymHSP70 and LymHSP40 expression,
207 consistent with our previous findings using a heat shock of 30 °C, we explored whether cold shock could induce a Garcia
208 effect and compared the transcriptional effects with those induced by heat shock. Our hypothesis was based on previous
209 research in mammals and *L. stagnalis*, suggesting that prolonged fasting would affect learning and memory differently
210 than a single day of fasting, potentially enhancing memory performance. To investigate this, we assessed the learning
211 abilities and memory performances of snails using the Garcia effect behavioral procedure. Initially, we replicated the
212 Garcia effect by demonstrating that a single pairing of a novel taste (i.e., carrot slurry) followed by a subsequent heat
213 shock (30 °C for 1 h) significantly suppressed the feeding response elicited by carrots for at least 3 h (paired t -test: $t =$
214 4.49 , $df = 5$, $p = 0.006$) (**Fig. 2A**). In contrast, control snails exposed to room temperature pond water (20 °C) for 1 h
215 (without thermal shock) did not exhibit a Garcia effect (paired t -test: $t = 0.90$, $df = 5$, $p = 0.41$) (**Fig. 2C**), consistent with
216 our previous observations. Interestingly, we found that the number of rasps elicited by C at 3 h post cold shock (C-post
217 3 h) did not significantly differ from those recorded before the cold shock (C-pre) (**Fig. 2B**). This suggests that unlike heat
218 shock, cold shock does not result in a sickness state that induces a Garcia effect in lab-inbred snails. Having confirmed
219 our prediction that cold and heat shock induce distinct behavioral responses, we sacrificed the snails following the
220 memory test (C 3h) to analyze the transcriptional effects of the Garcia effect procedure on the mRNA levels of
221 LymHSP70, LymHSP40, LymGRIN1, and LymCREB1 in their central ring ganglia (**Fig. 3**). A one-way ANOVA followed by
222 Tukey's post hoc test revealed a significant effect of the behavioral procedure on the expression levels of LymHSP70
223 ($F_{2,18} = 45.68$, $p < 0.0001$) (**Fig. 3A**), LymHSP40 ($F_{2,18} = 9.09$, $p = 0.002$) (**Fig. 3B**), LymGRIN1 ($F_{2,18} = 9.13$, $p = 0.002$) (**Fig.**
224 **3C**), and LymCREB1 ($F_{2,18} = 29.49$, $p < 0.0001$) (**Fig. 3D**).

225 Specifically, following the Garcia effect procedure, both heat and cold shock stressors induced significant upregulation
226 of LymHSP70 and LymHSP40 compared to control conditions (LymHSP70: heat shock: $p < 0.0001$, cold shock: $p = 0.0002$;
227 LymHSP40: heat shock: $p = 0.003$, cold shock: $p = 0.009$). However, the increase in LymHSP70 expression in the central
228 ring ganglia of snails subjected to heat shock was significantly higher compared to those exposed to cold shock ($p =$
229 0.0009), indicating that heat shock causes a more severe stress response in lab-inbred snails. Moreover, significant
230 upregulation of key neuroplasticity targets such as LymGRIN1 and LymCREB1 was observed only in snails that formed
231 the Garcia effect (i.e., heat shock-exposed) compared to those subjected to cold shock (LymGRIN1: $p = 0.01$, LymCREB1:
232 $p < 0.0001$) and control conditions (LymGRIN1: $p = 0.002$, LymCREB1: $p < 0.0001$), highlighting different neuroplastic
233 responses to thermal stressors in these snails.

234

235 4. Discussion

236 With rising global temperatures that may result in increased temperatures in lakes and ponds, aquatic poikilotherm
237 species, like *L. stagnalis*, are likely to face unprecedented physiological stress in coping with thermal extremes
238 (Benedetti et al., 2021; Calduch-Giner et al., 2022; Fernández et al., 2022; Harvey et al., 2022; Reid et al., 2019). Climate
239 change is causing an increase in the frequency and duration of heatwaves and cold spells across various landscapes
240 where *Lymnaea* species are found (Marx et al., 2021; Meehl & Tebaldi, 2004; Neven, 2000; Rivi, Batabyal, Benatti, Blom,
241 et al., 2022). HSPs are conserved players in the response to thermal stress (both heat and cold) across animal taxa
242 (Mayer, 2010), underscoring their adaptive significance (Anderson & Bell, 2009; Jeyachandran et al., 2023; Oksala et al.,
243 2014; Pluess et al., 2023; Setti et al., 2022).

244

245 In this study, we used the pond snail *L. stagnalis*, a model species for ecological and neuroscience studies, to investigate
246 the behavioral and transcriptional effects induced by a cold stressor and compared it with a heat shock stressor. In
247 laboratory settings, pond snails experience stable temperatures around 20 °C, contrasting sharply with the natural
248 environment's temperature variability (Fernell et al., 2021). While heat shock has been extensively studied in various
249 species, including *L. stagnalis*, cold shock's behavioral and transcriptional effect on learning and memory abilities
250 remained relatively unexplored until *Experiment 1* of this study, where we demonstrated that short-term exposure to 4
251 °C significantly upregulated the mRNA levels of LymHSP70 and LymHSP40 in the central ring ganglia of *L. stagnalis*.

252

253 This suggests that these HSPs are upregulated as part of an immediate response mechanism to cold stress, potentially
254 aiding in the stabilization of cellular proteins and the maintenance of cellular homeostasis under adverse thermal
255 conditions (Harada & Goto, 2017; Jiang et al., 2021; Li et al., 2012; Ma et al., 2021; Matz et al., 1995; Rinehart et al.,
256 2007). This rapid upregulation of HSPs aligns with their role as molecular chaperones that prevent protein misfolding
257 and aggregation, which are common consequences of thermal stress (Lindquist and Craig, 1988; Rinehart et al., 2007).
258 Interestingly, no significant changes in the expression levels of LymHSP70 and LymHSP40 were observed in snails
259 exposed to 4 °C for 24 h, suggesting a transient upregulation of these genes in response to short-term cold exposure.
260 Snails exposed to prolonged cold conditions may have evolved mechanisms to minimize the energetic costs associated
261 with continuous HSP production, thereby optimizing their response to cold stress while mitigating potential fitness costs
262 linked to HSP synthesis (Sørensen et al., 2003).

263 HSPs are the major physiological marker of thermal (both heat and cold) stress and have a high energy demand, which
264 can impair growth and reduce fitness (Sørensen et al., 2003). Furthermore, the findings suggest that *L. stagnalis* may
265 prioritize different molecular responses depending on the nature and duration of the thermal stress encountered.

266

267 Short-term cold exposure triggers an immediate but transient HSP response, likely aimed at acute stress management
268 and cellular protection. In contrast, prolonged cold exposure may necessitate other adaptive mechanisms beyond HSP
269 induction to maintain cellular function and overall metabolic homeostasis over extended periods (Jin et al., 2019, 2020;
270 Lewis et al., 2016). This hypothesis is strengthened by the results of the expression levels of LymMIP-II and LymMIPR.
271 Although short-term cold exposure *per se* did not affect the expression levels of LymMIP-II and its receptor, prolonged
272 cold exposure was associated with a significant downregulation of LymMIP-II and an upregulation of LymMIPR. These
273 effects, albeit only at the transcriptional levels, support the existence of a negative feedback loop necessary for
274 maintaining glucose homeostasis and sustaining cellular functions despite challenging environmental conditions (Rivi,

275 Benatti, Actis, Tascetta, et al., 2022). We hypothesize that the downregulation of LymMIP-II results in a decrease in
276 insulin production or secretion under prolonged cold stress. Similar results have been obtained in severely food-
277 deprived snails, suggesting a common underlying pathway involved in modulating stress responses (Rivi, Benatti, Actis,
278 Tascetta, et al., 2022). Insulin is pivotal in promoting glucose uptake and storage, processes that may be temporarily
279 less critical during extended periods of cold when metabolic demands might shift towards conserving energy rather
280 than actively utilizing glucose (Matsunaga et al., 2016; Nakai et al., 2020; Sim & Denlinger, 2008). This downregulation
281 could also indicate a state of reduced metabolic activity or even a metabolic depression strategy employed by *L.*
282 *stagnalis* to conserve energy resources during prolonged cold exposure. As previous studies in *L. stagnalis* showed that
283 insulin secretion plays a significant role in memory formation (Murakami et al., 2013), the downregulation of LymMIPI-
284 II may also to some extent explain why long-term memory persists in the cold (Fernell et al., 2021) as forgetting in *L.*
285 *stagnalis* requires altered gene activity and new protein synthesis (Sangha et al., 2005).

286
287 Conversely, the upregulation of LymMIPR suggests an increased sensitivity or responsiveness of cells to insulin signals.
288 This adaptive change may enhance the efficiency of glucose utilization in tissues that remain metabolically active despite
289 the cold stress. By upregulating insulin receptors, cells can maintain sensitivity to insulin signaling, optimizing glucose
290 uptake when conditions permit or when metabolic demands increase again (X. Gao et al., 2022; Partonen, 2013; Zhang
291 et al., 2024). The observed changes in LymMIPII and LymMIPR expression levels likely contribute to a negative feedback
292 loop aimed at stabilizing glucose levels within a narrow physiological range. We hypothesize that this feedback
293 mechanism is crucial for preventing hyperglycemia or hypoglycemia, both of which can disrupt cellular functions and
294 overall metabolic balance. Thus, those transcriptional adjustments in insulin-related genes underscore the adaptive
295 flexibility of *L. stagnalis* in response to prolonged cold stress, highlighting the intricate interplay between environmental
296 cues and molecular responses in maintaining metabolic homeostasis.

297
298 Finally, we found that cold exposure (short and long-term) did not induce a change in the expression levels of LymGRIN1
299 and LymCREB1, which are associated with neuroplasticity, memory formation, and forgetting (Abrams, 2012; Bartsch et
300 al., 1998; Batabyal et al., 2021; Fernell et al., 2021). The absence of significant effects on the expression levels of these
301 targets suggests several intriguing insights into the neurobiological responses of *L. stagnalis* to cold stress.

302 Thus, cold shock, even when applied for prolonged periods, may not elicit the same level of molecular activation in
303 these pathways, possibly due to different metabolic demands or cellular responses required to cope with cold stress
304 (Sørensen et al., 2003). Snails may prioritize immediate physiological responses, such as the activation of HSPs to
305 maintain protein stability under cold conditions, over long-term neuroplasticity-related changes. The lack of significant
306 changes in LymGRIN1 and LymCREB1 expression could indicate that snails allocate resources differently in response to
307 cold stress, focusing more on survival mechanisms rather than enhancing cognitive functions or synaptic plasticity.

308 It is also possible that *L. stagnalis* has evolved specific adaptive strategies to cope with cold stress that do not rely heavily
309 on neuroplasticity-related pathways. These adaptations may involve metabolic adjustments, alterations in membrane
310 fluidity, or changes in ion channel activity rather than modifications in synaptic plasticity genes like GRIN1 and CREB1
311 (Acutain et al., 2021; Avery & Krichmar, 2015).

312

313 Understanding the molecular responses of *L. stagnalis* to cold stress, particularly in terms of neuroplasticity and memory
314 formation, provides crucial insights into the adaptive mechanisms of aquatic organisms facing environmental
315 fluctuations amidst ongoing climate changes affecting animal distributions and biodiversity (Zhang et al., 2018).
316 Thus, in *Experiment 2*, we investigated at the behavioral and transcriptional levels whether cold shock (4 °C for 1 h)
317 could elicit a Garcia effect comparable to heat shock. The Garcia effect procedure is a well-founded learning paradigm
318 (Garcia et al., 1955) that offers a behavioral metric to assess thermal tolerance and HSP induction in response to various
319 thermal environments (Rivi et al., 2021). First, we confirmed that a single exposure to a novel taste (carrot slurry)
320 followed by a heat shock induces a Garcia effect, shown by a significant suppression of feeding response to carrot slurry
321 for at least 3 h. This effect was absent in control snails that did not experience thermal shock, which is consistent with
322 previous observations. On the other hand, cold shock (4 °C for 1 h) did not lead to a Garcia effect, as there was no
323 significant difference in feeding response to carrots before and after the cold shock.

324
325 This behavioral distinction highlights that different thermal stressors exert different effects on memory consolidation
326 processes in *L. stagnalis*. The absence of a Garcia effect with cold shock aligns with the transcriptional findings that
327 showed distinct molecular responses between heat and cold shock, particularly in the expression levels of
328 neuroplasticity-related genes like LymGRIN1 and LymCREB1. Heat shock, by inducing a robust upregulation of stress
329 response genes like HSPs, not only triggers learning and memory formation associated with the Garcia effect in lab-bred
330 snails (Rivi et al., 2022a), but also enhances long-term memory formation for the operant conditioning of aerial
331 respiratory behavior when applied before, during, or immediately after training (Alagar Boopathy et al., 2022; Ecroyd
332 et al., 2023; Stetler et al., 2010; Teskey et al., 2012). In contrast, cooling lab-bred snails for 1 h immediately after operant
333 conditioning training is sufficient to block memory formation, whereas the same cooling procedure performed 15 min
334 after training prevents forgetting (Sangha et al., 2003). Moreover, our previous work showed that exposing lab-bred
335 snails to a cold spell for 4 weeks following training extended the persistence of long-term memory for operant
336 conditioning of aerial respiration for at least 4 weeks (Fernell et al., 2021). This finding aligns with recent studies in
337 *Caenorhabditis elegans*, where memories are retained only if the worms are cooled quickly, whereas if they acclimatize
338 to the cold by spending the night in cool conditions before training and then are placed on ice, they forget the
339 information as fast as usual (Landschaft et al., 2024).

340
341 Thus, our current and previous studies suggest that the intensity, timing, exposure duration, and nature of the stressor
342 are critical in shaping behavioral responses and memory consolidation in *L. stagnalis* and other invertebrates. These
343 findings pave the way for future research into the underlying mechanisms behind the different effects of heat and cold
344 shock on memory consolidation.

345
346 This could include exploring the role of specific neurotransmitter systems, synaptic plasticity mechanisms, and other
347 molecular pathways involved in memory formation in response to thermal stressors. Additionally, comparative studies
348 across different populations or species of aquatic organisms could elucidate variation in thermal stress responses and
349 their adaptive significance in natural environments. These findings contribute to our understanding of the adaptive
350 strategies employed by aquatic organisms to cope with environmental challenges and underscore the importance of
351 considering the specific nature of stressors in shaping behavioral and cognitive responses. At the transcriptional level,

352 we found a significant upregulation of stress response genes (LymHSP70 and LymHSP40) both in snails that formed the
353 Garcia effect (i.e., those exposed to the heat shock) and those that did not form the Garcia effect (i.e., those exposed
354 to the cold shock) compared to the control snails maintained at room temperature. However, the upregulation in
355 LymHSP70 was notably higher in snails exposed to heat shock compared to those exposed to cold shock, indicating a
356 more pronounced stress response to heat.

357
358 Furthermore, genes associated with neuroplasticity (LymGRIN1 and LymCREB1) showed significant upregulation only in
359 snails that exhibited the Garcia effect (heat shock-exposed), suggesting that heat shock induces more substantial
360 molecular changes linked to memory formation compared to cold shock or control conditions. This differential gene
361 expression profile highlights the nuanced neurobiological responses to varying thermal stressors in these snails, with
362 implications for understanding adaptive responses to environmental challenges. Understanding how poikilotherm
363 species like *L. stagnalis* respond at the molecular level to temperature fluctuations is crucial for predicting their
364 resilience to environmental changes, particularly in the context of climate change (Alexander et al., 2006; Angilletta Jr.,
365 2009). These insights can inform conservation efforts and management strategies aimed at preserving aquatic
366 biodiversity and ecosystem stability in a changing climate.

367
368 This study provides valuable insights into the transcriptional adaptations of aquatic organisms to temperature
369 fluctuations, which is essential for understanding their resilience to changing environmental conditions.

370 Future studies could explore the broader implications of these molecular and behavioral responses across different
371 populations or species of aquatic organisms. Investigating how genetic variability influences these responses and
372 whether adaptive differences exist in natural populations could provide deeper insights into the evolutionary potential
373 of aquatic species facing climate-driven challenges. Additionally, further investigation is needed into how *Lymnaea*
374 detects water temperature changes. Many animals can detect shifts in temperature through afferent nerve fibers in the
375 skin (Schepers and Ringkamp 2010). However, whether such receptors exist in *L. stagnalis* and how they work has been
376 largely unexplored. Identifying the molecular identity of these receptors could reveal how *L. stagnalis* interprets thermal
377 signals and comparing these mechanisms with those in other mollusks may uncover evolutionary adaptations that help
378 species thrive in diverse environments. Research on *L. stagnalis*'s thermoreceptors could deepen our understanding of
379 the adaptive responses of our model species to both cold and hot stressors and pave the way for comparative studies.
380 Overall, these findings can also contribute to our understanding of thermal biology and stress physiology in aquatic
381 organisms, underscoring the importance of molecular mechanisms in shaping species' resilience in dynamic
382 environments.

383

384 **5. Conclusions**

385 Our study offers important insights into the adaptive responses of *L. stagnalis* to thermal stressors, amidst the backdrop
386 of escalating climate change. We demonstrated that short-term cold exposure triggers an immediate but transient
387 upregulation of key HSPs, underscoring their role in acute stress management. In contrast, prolonged cold exposure led
388 to a strategic shift towards energy conservation and metabolic homeostasis. These findings reveal the nuanced
389 molecular adaptations of *L. stagnalis*, balancing the high energy demands of stress response with long-term survival
390 strategies. Moreover, our behavioral experiments highlighted the different impacts of heat and cold shock on memory

391 formation. While heat shock induced a robust Garcia effect, indicative of enhanced associative learning, cold shock
392 failed to elicit a similar response, aligning with the absence of significant changes in neuroplasticity-related genes. This
393 distinction suggests that the nature and intensity of thermal stressors critically influence the cognitive and behavioral
394 adaptations in aquatic organisms. These findings underscore the complexity of thermal adaptation in *L. stagnalis*,
395 emphasizing the importance of context-specific responses to environmental stressors. Understanding these
396 mechanisms is pivotal for predicting the resilience of aquatic poikilotherms in the face of climate change, providing a
397 foundation for conservation strategies aimed at preserving biodiversity and ecosystem stability. Future research should
398 delve deeper into the molecular pathways and genetic variability underlying these adaptive responses, offering a
399 broader perspective on the evolutionary potential of aquatic species amid global environmental fluctuations.

400

401 **Ethics statement**

402 *Lymnaea stagnalis* are invertebrate animals; thus, the approval of IACUC (Institutional Animal Care and Use Committee)
403 was not required (Italian Legislative Decree D.L. 4 marzo 2014, n. 26 “Attuazione della Direttiva n. 2010/63/UE sulla
404 protezione degli animali utilizzati a fini scientifici”). However, every effort was made to minimize the number of animals
405 used, ensuring adequate food, clean oxygenated water, and low-density conditions.

406

407 **CRedit authorship contribution statement**

408 Veronica Rivi: conceptualization, methodology, investigation, data curation, writing the original draft; Anuradha
409 Batabyal: conceptualization, visualization, writing-reviewing and editing; Cristina Benatti: Visualization and writing-
410 reviewing and editing; Fabio Tascetta: supervision, funding acquisition, writing-reviewing and editing; Johanna Maria
411 Catharina Blom: supervision, funding acquisition, writing-reviewing and editing Ken Lukowiak: conceptualization,
412 supervision, funding acquisition, writing-reviewing and editing.

413

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418

419 **Data availability**

420 Further information and requests for resources and reagents should be directed to and will be fulfilled by Dr. Veronica
421 Rivi.

422

423 **Declaration of competing interest**

424 The authors disclose no commercial or financial conflicting interests upon publication of this article.

425

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430

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755 Table 1

756 The forward (FW) and reverse (RV) primer nucleotide sequences utilized in qRT-PCR, along with the accession number
 757 for each target and the size (bp) of the PCR product obtained through the amplification of cDNA (mRNA).

758

759 Figure legend

760 **Fig. 1. Transcriptional effects induced by the exposure to 4°C for 1 h or 24 h in the central ring ganglia of lab-inbred**
 761 **snails.** The expression levels of LymHSP70 (A), LymHSP40 (B), LymMIP-II (C), LymMIPR (D), LymGRIN1 (E), and LymCREB1
 762 (F) were measured in the central ring ganglia of snails maintained at 20 °C (room temperature) (CTRL group: grey bars)
 763 or at 4 °C for 1 h (light blue bars) or 24 h (hatched blue bars) (N = 8 for each group). Data are presented as means ± SEM
 764 and were analyzed with a one-way ANOVA followed by Tukey post hoc tests. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$,
 765 * $p < 0.05$.

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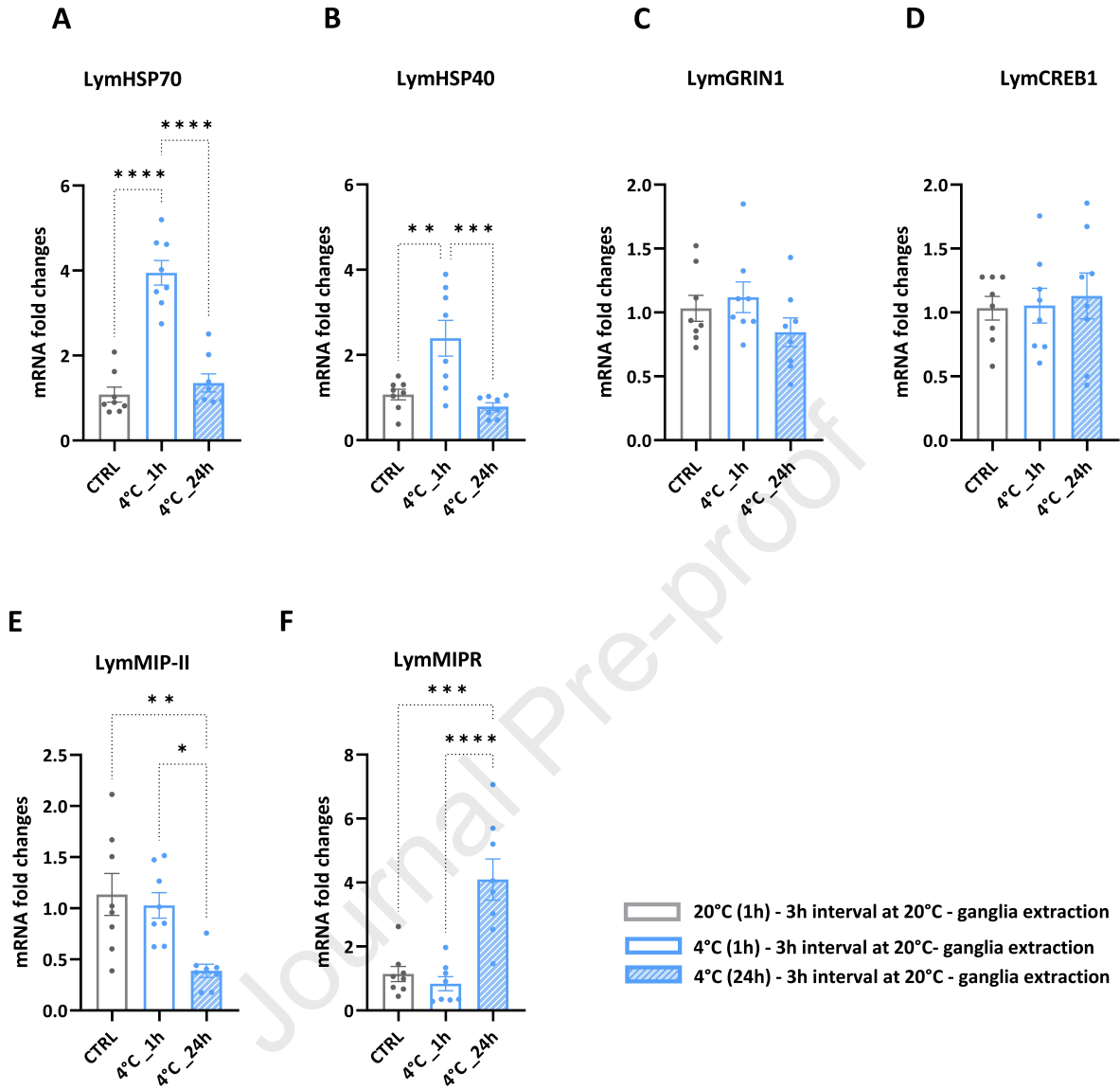
768 **Fig. 2. Cold shock fails to elicit a Garcia effect in laboratory-inbred snails compared to heat shock.** The timeline of each
769 experiment is presented above the data. The number of rasps elicited by the carrot slurry (C) was counted for 2 min (C
770 pre). One hour later, these snails experienced the heat (30 °C; 2A) or the cold (4 °C; 2B) shock stressor for 1 h. Control
771 snails (2C) were maintained at room temperature (20 °C) for 1 h. Three hours later the number of rasps elicited by the
772 carrot slurry (C 3 h) was recorded again for 2 min. Exposure to carrot slurry followed by heat shock induced a Garcia
773 effect, evidenced by a significant reduction in the number of rasps observed in snails tested 3 hours after 30 °C exposure.
774 In contrast, cold shock did not induce a Garcia effect, as no significant differences in rasping behavior in carrot slurry
775 were observed when comparing C pre and C 3h. Data were analyzed using a paired *t*-test. The solid line is the mean,
776 and the error bars are the SEM. ***p* < 0.001, ns = not significant as *p* > 0.05.

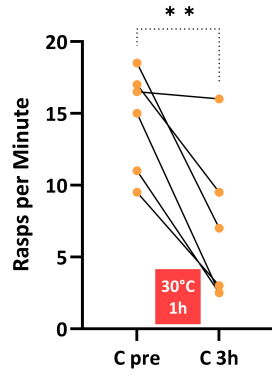
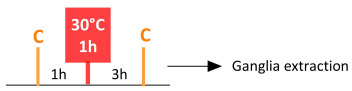
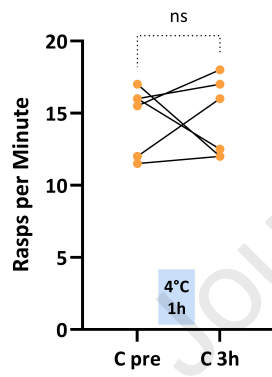
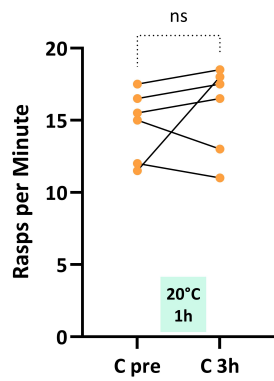
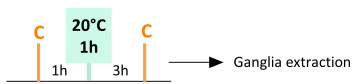
777

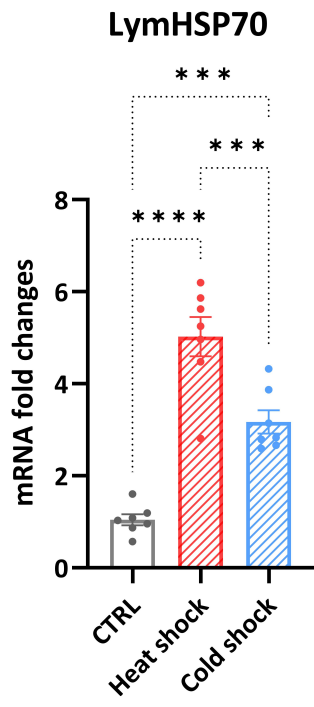
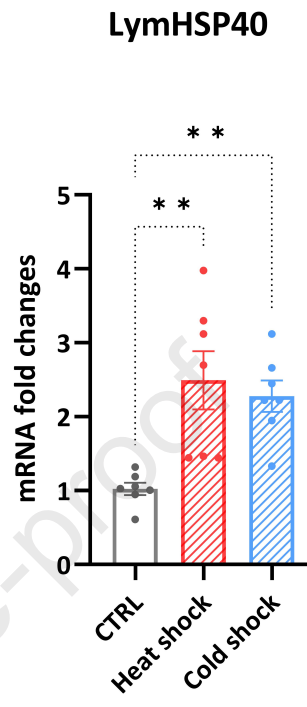
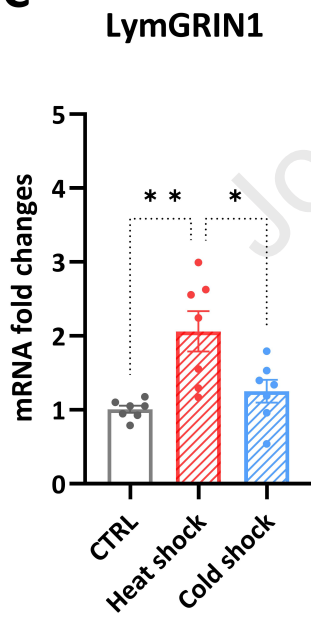
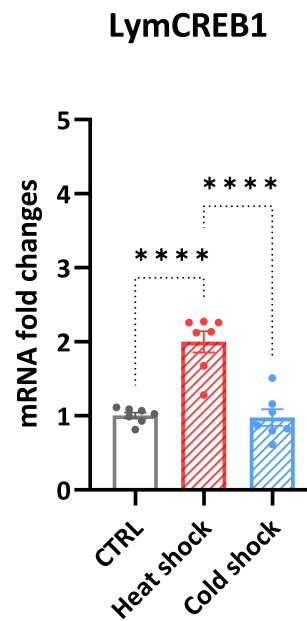
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779 **Fig. 3. Transcriptional effects induced by the Garcia effect procedure in snails exposed to 4 °C or 30 °C.** The expression
780 levels of LymHSP70 (A), LymHSP40 (B), LymGRIN1 (C), and LymCREB1 (D) were measured in the central ring ganglia of
781 *L. stagnalis* trained for the Garcia effect using the heat shock (red hatched bars) or the cold (blue hatched bars) stressors.
782 Control snails (CTRL: grey open bars) were maintained at 20 °C (room temperature) for 1 h. N = 7 for each group. Data
783 are represented as means ± SEM and were analyzed with a one-way ANOVA followed by a Tukey post hoc test. *****p* <
784 0.0001, ****p* < 0.001, ***p* < 0.01 and **p* < 0.05.

Gene bank accession	Target	Product length (bp)	Type sequence
DQ206432.1	<i>Lymnaea stagnalis</i> heat-shock protein 70 LymHSP70	199 bp (134-333)	5' - AGGCAGAGATTGGCAGGAT - 3' 3' - CCATTCATTGTGTCGTTGC - 5'
DQ278442.1	<i>Lymnaea stagnalis</i> heat-shock protein 40 LymHSP40	186 bp (152-338)	5' - AAGGTCTTGAATCCTGATG - 3' 3' - GTGTTTGGTCACCTTCTTT - 5'
X59302.1	<i>Lymnaea stagnalis</i> molluscan insulin-related peptide LymMIP II	186 bp (152-338)	5' - CCAATCATCTTGCAGTTTA - 3' 3' - GTCGTCCAGATCTGTTTCT - 5'
X84994.1	<i>Lymnaea stagnalis</i> putative molluscan insulin-related peptide receptor LymMIPR	78 bp (4137-4215)	5' - ATTGGAGACTTTGGTATGAC - 3' 3' - AACTCCATCTTTGAGAGAC - 5'
AY571900.1	<i>Lymnaea stagnalis</i> NMDA-type glutamate receptor LymGRIN1	140 bp (831-917)	5' - AGAGGATGCATCTACAATTT - 3' 3' - CCATTTACTAGGTGAACTCC - 3'
AB041522.1	<i>Lymnaea stagnalis</i> cAMP responsive element binding protein LymCREB1	180 bp (49-229)	5' - GTCAGCAGGGAATGGTCCTG - 3' 3' - AACCGCAGCAACCCTAACAA - 5'
X15542.1	Snail, beta-tubulin LymTUB	100 bp (92-192)	5' - GAAATAGCACCGCCATCC - 3' 3' - CGCCTCTGTGAACTCCATCT - 5'
DQ278441.1	<i>Lymnaea stagnalis</i> elongation factor 1-alpha LymEF1α	150 bp (7-157)	5' - GTGTAAGCAGCCCTCGAACT - 3' 3' - TTCGCTCATCAATACCACCA - 5'



A**B****C**

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

Highlights

- Aquatic poikilotherms like *L. stagnalis* face water temperature changes
- Short-term cold exposure upregulates HSP mRNA levels: an immediate response to maintain cellular homeostasis
- Long-term cold exposure induces an 'energy-saving' state by regulating insulin signaling
- Heat stress but not cold stress induces a Garcia effect in inbred snails
- *L. stagnalis* shows different molecular strategies for coping with heat versus cold challenges

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