Canadian Journal of Zoology

Article

Comparison of behavioural and transcriptional responses to a heat stressor between freshly collected and an inbred strain of *Lymnaea*

Veronica Rivi[a], **Anuradha Batabyal**[b,c], **Cristina Benatti**[a,d], **Fabio Tascedda**[d,e,f], **Joan M. C. Blom**[a,d], and **Ken Lukowiak**[b]

[a]Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, Modena, Italy; [b]Department of Physiology and Pharmacology, Hotchkiss Brain Institute, Cumming School of Medicine, University of Calgary, AB, Canada; [c]Department of Physical and Natural Sciences, FLAME University, Pune, India; [d]Centre of Neuroscience and Neurotechnology, University of Modena and Reggio Emilia, Modena, Italy; [e]Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy; [f]Consorzio Interuniversitario Biotecnologie (CIB), Trieste, Italy

Corresponding author: **Veronica Rivi** (email: veronica.rivi@unimore.it)

Abstract

Different populations of organisms occurring across varying thermal regimes show diversity in responses to heat stress. We use a "common garden experimental" approach designed to deal with phenotypic plasticity to study in *Lymnaea stagnalis* (Linnaeus, 1758) the behavioural and molecular responses to a heat shock in laboratory-inbred snails (W-strain) and freshly collected snails (Stony strain) from ponds. In the W-strain, which has been reared under standardized temperatures for generations, the exposure to 30 °C for 1 h (heat shock, HS) when experienced after a novel "taste" results in a taste-specific aversion known as the "Garcia effect". This learned avoidance requires the upregulation of heat shock proteins (HSPs). In contrast, freshly collected Stony strain, which experiences temperature fluctuations regularly, does not exhibit a Garcia effect. Here, we found that (1) Stony-strain snails have higher basal mRNA levels of HSPs than W-strain ones; (2) in the W-strain, the training procedure to cause the Garcia effect upregulates the mRNA levels of HSPs and key neuroplasticity-related genes such as CREB1 and GRIN1; (3) in Stony-strain snails, the same training procedure fails to alter the mRNA levels of those targets. These data suggest that Stony-strain snails do not perceive the HS as a stressor because of the higher HSP basal mRNA levels, which may confer a higher thermal tolerance.

Key words: invertebrates, HSP70, NMDA, CREB1, *Lymnaea stagnalis*, Garcia effect, pond snail

Introduction

Learning can be defined as the modulation of adaptive behaviours with experience to better deal with ever-changing environments (Burns et al. 2011). To increase their chances of survival and adapt to their environment, organisms form long-term memory (LTM) (Hughes et al. 2017). However, given the high "neuronal costs" of learning and memory formation (in terms of altered gene activity and new protein synthesis), not all events can be encoded into memory (Dalesman et al. 2013). An important factor that may determine whether or not a specific event will be encoded into LTM is the level of stress perceived at the time of the event (Lukowiak et al. 2008). Over the last three decades, invertebrates have proven to be promising models for investigating the diverse ways stress may alter memory formation, consolidation, recall, and forgetting, thanks to their relatively simple nervous systems and tractable behaviours that can be classically and operantly conditioned (Burns et al. 2011; Kandel et al. 2014; Rivi et al. 2020, 2021*b*).

Among them, the pond snail, *Lymnaea stagnalis* (Linnaeus, 1758) represents a valid model organism to unravel the causal mechanisms of learning and memory under diverse stressors, including thermal shock, food deprivation, crowding, low calcium levels, and predator exposure (Dalesman and Lukowiak 2010; Ito et al. 2017; Dodd et al. 2018*a*; Batabyal et al. 2021, 2022; Fernell et al. 2021; Rivi et al. 2021*a*, 2022*a*, 2022*b*, 2022*c*, 2022*d*; Batabyal and Lukowiak 2022). Most of the learning and memory studies involving *L. stagnalis* have been performed in the so-called W-strain snails (an inbred laboratory-reared strain) that originated in Holland and was then distributed to different labs all over the world (e.g., England, Canada, Japan, Hungary, and Italy). However, the ability of organisms to cope with environmental stressors depends on their genetic background (e.g., allele composition) and the level of inbreeding (Leicht et al. 2019). That is, inbreeding, along with an inadvertent selection of traits over the years, may have altered how these snails respond to different environmental stressors that they do not ex-

perience in laboratory-standardized conditions (Armbruster and Reed 2005; Rivi et al. 2022*a*, 2022*b*, 2022*c*; Batabyal and Lukowiak 2022; Batabyal et al. 2022). For example, when exposed to 30 °C for 1 h (i.e., an acute heat shock, HS) following a novel "taste" presentation, W-strain snails form a taste-specific aversion learning known as the "Garcia effect" (Rivi et al. 2021*a*). This learned "taste-specific aversion" requires visceral sickness (e.g., nausea) to be formed (Garcia et al. 1955).

Indeed, while the Garcia effect is an example of conditioned taste aversion, it is "special" in that for the Garcia effect to occur there is a requirement for a novel taste sensation, followed by visceral nausea some hours later (Garcia et al. 1955). From an ecological, real-world perspective, both the Garcia effect and the standard conditioned taste aversion phenomena enable organisms to learn about the toxic nature of certain foods and to prevent further illness and death (Garcia et al. 1974). Given its importance for animal survival, it is not surprising that organisms acquire both the Garcia effect and standard conditioned taste aversion; a single-trial is often sufficient for a robust standard-conditioned taste aversion to occur, representing an ideal learning paradigm for studying the behavioural and molecular basis of learning and memory (Garcia et al. 1985).

Although showing nausea in a snail is extremely difficult (Nakai et al. 2020), we know that prolonged (>3 h) exposure to temperatures of 30 °C or above is lethal (McDonald 1973). Thus, showing that pairing a novel appetitive stimulus with HS results in a Garcia effect supported the hypothesis that HS does result in some sort of nausea in *Lymnaea* (Rivi et al. 2021*a*).

However, when we performed these experiments on a freshly collected wild strain, we found that the same HS used in the W-strain experiments was not sufficient to bring about a Garcia-like effect (Rivi et al. 2022*a*).

It is important to note that the W-strain snails, which exhibit the Garcia effect when the HS was used, have been reared in temperature-controlled regimes (~20 °C) for generations, whereas freshly collected strains are subject to varying seasonal and daily temperature changes (Fernell et al. 2021). For example, in SK and AB (i.e., our major collection sites for wild snails), the temperature of surface water bodies varies from about 4 °C in April/May to nearly 35 °C on some days in July–August (Brown 1979). In addition, in spring and fall, snails can experience large daily temperature fluctuations, with temperatures near zero during the night and up to 20 °C during the same day (Rivi et al. 2022*a*). Thus, in this study, we hypothesized that this inability to produce a Garcia effect in "wild" snails may be due to a differential effect that temperature has to induce the sickness required to bring about a Garcia effect.

Thermal tolerance and sensitivity to an HS show variation across populations of the same species experiencing different thermal regimes (Angilletta 2006). In fact, the upper and lower heat thresholds governing physiological traits depend on the thermal history of the organism being studied, as well as the conditions in which it has been raised and maintained (Ottaviani 2015; Blom and Ottaviani 2017). Thus, results obtained in inbred animals raised and maintained under standardized laboratory temperatures for generations may not be predictive of what happens in the real world. Organisms, in fact, can either acclimate through physiological plasticity (Angilletta 2006; Angilletta Jr. 2009) or evolve adaptive traits over generations through natural selection to match local conditions (Hoffmann et al. 2003; Narum et al. 2013). Such evolutionary adaptation has been demonstrated in various natural populations of both invertebrates and vertebrates as an outcome of thermal stress, which is one of the most important selection pressures (Cousyn et al. 2001; Hairston et al. 2001; Losos 2008). Apart from adaptive mechanisms, organisms can also acclimate or show behavioural plasticity in adjusting to local changes in their environment. For instance, several species of ants show plasticity in thermal tolerance depending on the seasonal fluctuation in temperature as well as under laboratory conditions (Jumbam et al. 2008; Bujan et al. 2020). This is especially important for ectotherm species, like *L. stagnalis*, because their internal temperature is highly dependent on the external temperature (Angilletta 2006).

Thus, the inability to produce a Garcia effect in Stony snails may be due to the fact that in these snails the HS does not cause nausea or stress. If snails do not exhibit nausea following the HS, they will not exhibit the Garcia effect. This hypothesis is supported by our recent data suggesting that both freshly collected and lab-inbred snails are capable of forming a Garcia effect when the bacterial toxin LPS was used in the Garcia effect procedure instead of the HS (Rivi et al. 2023).

Growing evidence demonstrates that differential responses shown by different species and strains of the same species to temperature changes may be due to the differential expression of heat shock proteins (HSPs). HSPs act as chaperone proteins to protect other proteins from changing shape (e.g., denaturation) as a result of temperature fluctuations or other stressors (Ottaviani et al. 2013; Dubrez et al. 2020).

We previously found that the induction of HSP activity is blocked by treating snails with the flavonoid quercetin. Quercetin blocks the upregulation of HSPs and we have shown that quercetin application before the HS blocks the Garcia effect. Thus, we concluded that the heat-induced upregulation of HSPs plays a necessary role in the Garcia effect LTM formation (Rivi et al. 2022*a*). Taking all the findings presented above, in this study we investigated whether there is a difference in the baseline as well as the HS stress-induced upregulation of the mRNA levels of HSPs that confer heat tolerance between the inbred W-strain snails and the Stony-strain snails and that those differences correlate with the ability to bring about a Garcia effect (or lack of one). Thus, we compared the basal mRNA levels of two HSPs, HSP70 and HSP40, between the W-strain snails and Stony-strain snails.

We further asked whether there were differences in the activation of two genes thought to play important roles in learning and memory formation: the subunit 1 of the glutamate NMDA receptors (GRIN1) and the cAMP response element-binding protein 1 (CREB1) (Barco et al. 2003; Hawkins et al. 2006; Rivi et al. 2020; Batabyal et al. 2021). We hypothesized that (1) in the W-strain, which exhibits a Garcia-like effect, the GRIN1 and CREB1 mRNA levels will be upregulated compared to their controls not exposed to the HS. On the other hand, (2) in the Stony-strain snails the training procedure

employed to cause a Garcia-effect memory will not alter the expression of these targets as learning and memory do not occur.

Materials and methods

Model species

The W-strain of *L. stagnalis* has been used in the Lukowiak lab for studying the neuronal basis of aerial respiratory behaviour since 1990. Freshly collected snails were obtained from Stony Lake in SK, Canada (Stony Lake: 51°4701.37″N, 103°2151.85″W), and have been designated as the "Stony-strain". This lake is approximately 250 km east of Saskatoon, SK, Canada. Snails were housed in the same standardized conditions for 3 months (from August to October 2022): 20–22 °C artificial pond water with the addition of $CaCO_3$ to maintain a calcium concentration >50 mg l-1 (see Dalesman and Lukowiak 2010). Snails were housed in aquaria containing artificial pond water at 20 ± 1 °C and fed romaine lettuce ad libitum. The light was controlled on a 16 h:8 h light:dark cycle.

Adult snails having a shell length of 250–300 mm were used in this study.

Carrot slurry

The carrot slurry was prepared by blending two medium peeled organic carrots purchased at a local supermarket with 500 mL of PW. Following blending and straining, a carrot-PW slurry was obtained without any visible pieces of carrot.

Garcia effect training procedure

Snails were trained for the Garcia effect procedure as previously described (Rivi et al. 2022*a*, 2022*b*). Specifically, snails were placed in a 14 cm diameter Petri dish with enough carrot slurry (i.e., the novel appetitive taste) for the snails to be partially submerged. To observe the feeding behaviour, the Petri dishes were placed on a clear Plexiglas stand raised 10 cm above a mirror. Snails were first acclimated for 3 min and then the number of rasps (i.e., feeding behaviour) was counted for 2 min. Animals were returned to their home aquarium for 1 h and were allowed ad libitum access to food (i.e., romaine lettuce). Then, snails were exposed to the HS for 1 h. That is, snails were maintained in a 1 L beaker filled with 500 mL of 30 °C artificial pond water for 1 h. In the control experiment, snails were maintained in 20 °C artificial pond water for 1 h. In both the experimental and control snails, rasping behaviour was again determined for 2 min in carrot slurry 3 h later (Rivi et al. 2021*a*). Immediately after the re-exposure to carrot slurry, animals were euthanized in ice (~ 10 min) and then sacrificed. The central ring ganglia were not pooled (i.e., each ganglion was considered as a single biological replicate) and then maintained in RNA later (Qiagen, Germany) until the RNA extraction procedures. W-strain and Stony-strain snails were handled in the same manner and the same number of times by the same investigator (AB) and had ad libitum access to romaine lettuce before the procedure. This allowed us to ensure that the strains were exposed to the same "level" of stress.

Total RNA extraction, reverse transcription, and qPCR

Animals were anesthetized on ice for 10 min, and the central ring ganglia were dissected and stored at −80 °C before analysis. Total RNA extraction and DNase treatment were performed using GenElute Total RNA Miniprep Kit and DNASE70-On-Column DNase I Digestion Set (Merck KGaA; Darmstadt, Germany) (Rigillo et al. 2018; Rivi et al. 2021*a*, 2022*d*; Cristina et al. 2022). Two-hundred-nanogram sample of total RNA was reverse-transcribed with a High-Capacity cDNA Reverse Transcription Kit (ThermoFisher). Real-time quantitative PCR was carried out on 20 ng mRNA using a Bio-Rad® CFX ConnectTM Real-Time PCR Detection System with SYBR Green Master Mix (Bio-Rad). The cycling parameters were the same as described in Rivi et al. (2021*a*) and consisted of 95 °C for 30 s, 40 cycles of 95 °C for 15 s, and 60 °C for 30 s. The machine read the plate to measure fluorescence at the end of each cycle. Cycle threshold (Ct) values were determined by CFX MaestroTM Software (Bio-Rad). Each sample was run in simplicate (i.e., was analyzed once). Specific forward and reverse primers were used at the final concentration of 300 nmol/L (Table 1). Primer efficiency was between 95% and 101%, and the R2 of primers was greater than 0.99.

The common garden experimental design

First, we measured and compared the molecular and behavioural effects induced by the Garcia effect learning paradigm in two strains of snails exposed to two different thermal environmental conditions: (1) lab-inbred W-strain snails (since the 1950s) that are maintained at standardized thermal conditions (~20 ± 1 °C) over the year and (2) freshly collected Stony-strain snails that live in ponds where temperature varies from 4 °C in spring to greater than 30 °C in midsummer. Then, we compared the basal mRNA levels of two HSPs, HSP70 and HSP40, and two key genes for neuroplasticity, GRIN1 and CREB1, in the central ring ganglia of W-strain snails and Stony-strain snails. The test design entailed two different populations being exposed to either HS or room temperature (RT) and their feeding response (i.e., did they or did they not exhibit a Garcia effect) being measured before and after the HS/RT treatment. Snails from both populations were used after the treatments to measure molecular markers.

Data analysis

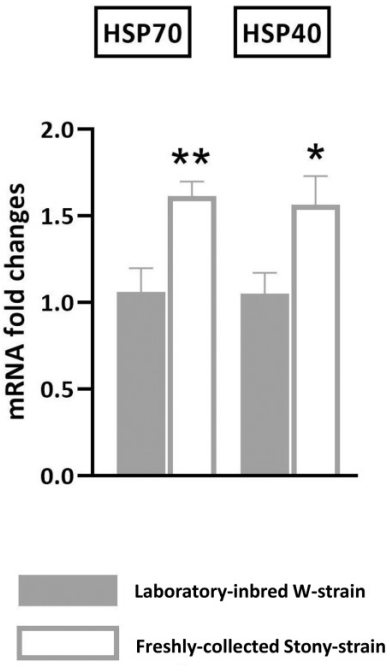
The mRNA levels of each target were normalized to elongation factor 1α and tubulin, which were used as the reference genes. The comparative 2−ΔΔCt method was performed using as a calibrator for the average levels of expression of control HS-unexposed snails. Unpaired Student's t test was used to compare the basal mRNA levels of HSP70 and HSP40 between W-strain and Stony-strain snails (Fig. 1) and expression levels of each target between snails trained for the Garcia effect procedure and their controls (Figs. 2C and 3C). For the behavioural data analysis, we used a linear mixed effect model (R package: lme4: http://lme4.r-forge.r-project.org). As the datasets were normally distributed, we used a standard Gaussian distribution. We had two separate models for the two populations with Trial number (Carrot pre and post) and

Table 1. Primer design nucleotide sequence of the forward and reverse primers used for real-time PCR.

Gene bank accession	Target	Product length (bp)	Type sequence
DQ206432.1	*Lymnaea stagnalis* heat shock protein 70, LymHSP70	199 bp (134–333)	FW: AGGCAGAGATTGGCAGGAT
			RV: CCATTTCATTGTGTCGTTGC
DQ278442.1	*Lymnaea stagnalis* heat shock protein 40, LymHSP40	120 bp (4–124)	FW: AAGGTCTTGAATCCTGATG
			RV: GTGTTTGGTCACCTTCTTT
DQ278441.1	*Lymnaea stagnalis* glutamate ionotropic receptor NMDA type subunit 1, LymGRIN1	150 bp (7–157)	FW: GTGTAAGCAGCCCTCGAACT
			RV: TTCGCTCATCAATACCACCA
AB041522.1	*Lymnaea stagnalis* cAMP responsive element binding protein LymCREB1	180 bp (49–229)	FW: GTCAGCAGGGAATGGTCCTG
			RV: AACCGCAGCAACCCTAACAA
X15542.1	Snail, beta-tubulin LymTUB	100 bp (92–192)	FW: GAAATAGCACCGCCATCC
			RV: CGCCTCTGTGAACTCCATCT
DQ278441.1	*Lymnaea stagnalis* elongation factor 1-alpha, LymEF1α	150 bp (7–157)	FW: GTGTAAGCAGCCCTCGAACT
			RV: TTCGCTCATCAATACCACCA

Note: For each target, the accession number and the size (bp) of the PCR product obtained by amplification of the cDNA (mRNA) are given.

Fig. 1. Freshly collected Stony-strain snails have higher basal levels of HSP70 and HSP40 than the lab-inbred W-strain snails. The basal mRNA levels of HSP70 and HSP40 of the naïve cohort of wild Stony snails ($N = 8$) and naïve lab-inbred W-strain snails were measured and compared. Stony snails showed significantly higher levels of HSP70 and HSP40 compared to the W ones. Comparisons were made by unpaired t test. The solid line is the mean, and the error bars are the SEM. $^{**}p < 0.001$; $^{*}p < 0.05$.



Stimulus (HS and RT) as the fixed effects with interaction and the snail ID as a random effect (Figs. 2A and 3A). All post hoc tests were performed using emmeans function in R (https://github.com/rvlenth/emmeans). Differences with a p value less than 0.05 were considered significant. The mean ± standard error (SEM) has been plotted. Statistical analyses were performed using R statistical software (version 3.6.0) and SPSS v. 26.0 (IBM Corp., Armonk, NY, USA), while graphs were generated using GraphPad Prism v. 9.00e for MAC® (GraphPad Software, Inc., La Jolla, CA, USA).

Results

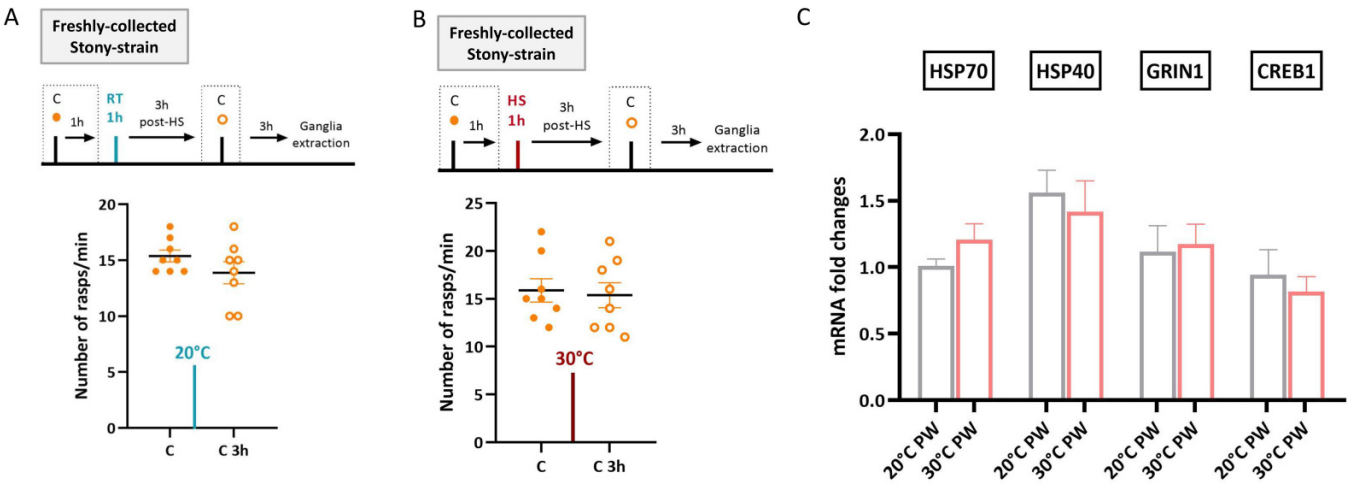
Basal mRNA levels of HSP70 and HSP40

We first investigated whether the two strains maintained at standard laboratory temperatures (~20 °C) exhibit differences in their basal levels of HSP70 and HSP40 (Fig. 1). Thus, eight Stony-strain and eight W-strain snails were sacrificed, the central ring ganglia were extracted, and the mRNA levels of HSP70 and HSP40 were measured and compared. We found that Stony-strain snails had significantly higher HSP70 and HSP40 mRNA levels compared to the W-strain snails (unpaired t test: HSP70: $t = 3.45$, df = 14, $p = 0.004$; HSP70: $t = 2.48$, df = 14, $p = 0.02$).

W-strain snails display a Garcia-like effect and the Garcia-training procedure upregulates HSPs, GRIN1, and CREB1

Next, we investigated the molecular changes induced by the Garcia effect training procedure in the W-strain snails. First, two cohorts of snails were exposed to HS or kept at room temperature (RT, the RT controls) and their response to carrot 1 h before and 3 h after either the RT or HS exposure was recorded. We found a significant interaction effect of the exposure stimulus and the trial (pre or post) (Figs. 2A and 2B; Stimulus*Trial: $F_{1,21} = 24.025$, $P < 0.001$). The feeding response to carrot after the HS was significantly reduced compared to their initial exposure ($t = 8.745$, $p < 0.001$), whereas in the control snails kept at RT the feeding remained the same ($t = 1.813$, $p = 0.084$). Thus, only the snails that were exposed to the HS exhibited the Garcia effect. Subsequently, we measured and compared the expression levels of HSP70, HSP40, GRIN1, and CREB1 in the snails' CNS (Fig. 2C). In snails receiving the HS, there was a significant upregulation of the expression levels of HSP70 and HSP40 (unpaired t test:

Fig. 2. Freshly collected Stony-strain snails do not form a Garcia-like effect following the Garcia-effect training procedure and this training procedure does not affect the mRNA levels of HSPs, GRIN1, and CREB1. The timeline of each experiment is presented above the data. The solid line is the mean, and the error bars are the SEM. (A) A naïve cohort of freshly collected Stony-strain snails ($N = 8$) was exposed to a novel carrot slurry. The number of rasps elicited by this novel appetitive stimulus was counted (C—closed circles) for 2 min, and 1 h later the snails experienced the HS. The number of rasps recorded at 3 h post-HS (C 3 h—open circles) was not significantly different from that elicited by carrot before the HS. Comparisons were made by paired *t* test. (B) Control freshly collected Stony-strain snails ($N = 8$) were exposed to carrot slurry for 2 min and the number of rasps was recorded (C—closed circles). These snails instead of receiving the HS were instead maintained in a beaker maintained for 1 h at room temperature (RT, 20 °C) in artificial pond water. The number of rasps was again recorded 3 h later in carrot slurry (C 3 h—open circles), and it was not significantly different from that recorded initially. Comparisons were made by paired *t* test. (C) The two different behavioural procedures did not alter the levels of HSP70, HSP40, GRIN1, and CREB1 as no differences were found in the mRNA levels of these targets between control snails and the HS-exposed ones. Comparisons were made by unpaired *t* test.



HSP70: $t = 6.72$, df = 14, $p < 0.0001$; HSP40: $t = 6.12$, df = 14, $p < 0.0001$). In addition, we found that the expression levels of GRIN1 and CREB1, which play a key role in memory formation, were upregulated in the W-strain snails trained for the Garcia effect procedure (unpaired *t* test: GRIN1: $t = 3.32$, df = 14, $p = 0.005$; CREB1: $t = 4.37$, df = 14, $p = 0.0006$).

Stony-strain snails do not exhibit a Garcia effect and the Garcia-effect training procedure does not alter the mRNA levels of HSPs, GRIN1, and CREB1

Naive Stony-strain snails received either the HS or RT ($n = 8$ in each procedure) in the Garcia-effect training procedure (Figs. 3A and 3B). We found no significant interaction effect of the stimulus type (HS or RT exposure) and the trial (pre- and post-carrot exposures) on the rasping rate of snails (Stimulus*Trial: $F_{1,21} = 0.247$, $P = 0.624$). Thus, snails did not change their rasping rate irrespective of whether they received the HS or RT stimulus. That is, a Garcia-effect did not occur. Immediately after both behavioural procedures, the Stony-strain snails were sacrificed and the expression levels of HSP70, HSP40, GRIN1, and CREB1 in the central ring ganglia were measured (Fig. 3C).

We found that in the Stony-strain snails, neither the Garcia-effect training procedure nor the control procedure altered the expression levels of HSP70, HSP40, GRIN1, and CREB1 as no differences were found in the mRNA levels of these targets (unpaired *t* test: HSP70: $t = 1.54$, df = 14, $p = 0.15$; HSP40: $t = 0.51$, df = 14, $p = 0.62$; GRIN1: $t = 0.23$, df = 14, $p = 0.82$; CREB1: $t = 0.58$, df = 14, $p = 0.58$).

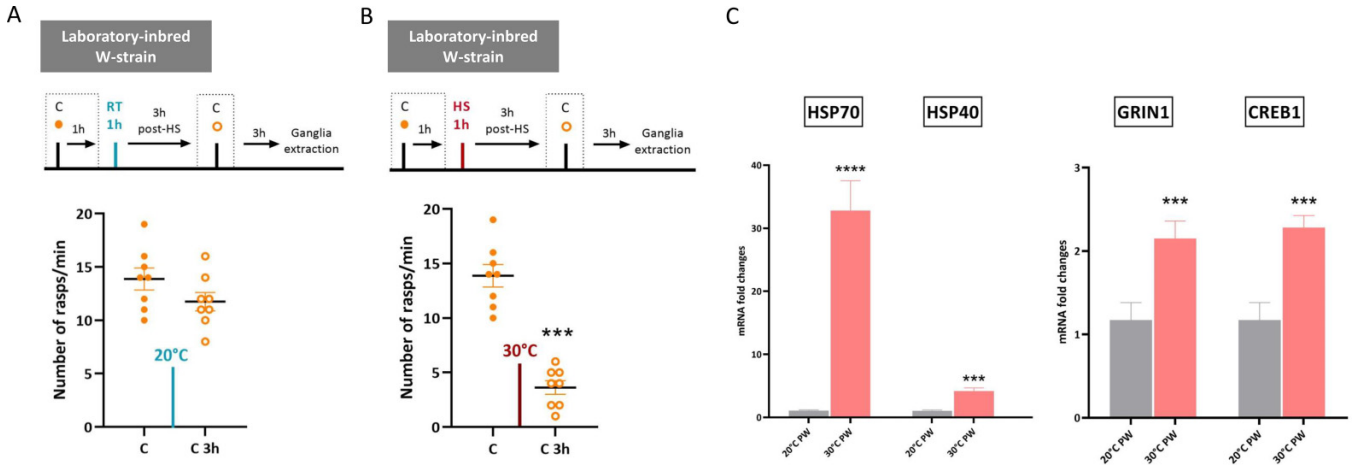
Discussion

The major findings from this study are

- The freshly collected Stony-strain snails have higher basal HSP70 and HSP40 than the inbred W-strain ones.
- Following the Garcia effect training procedure, in W-strain snails the expression levels of HSP70 and HSP40 are upregulated, as are the mRNA levels of two key genes for neuroplasticity, GRIN1, and CREB1. These findings are consistent with the formation of memory.
- The same upregulation of mRNA levels was not observed in the freshly collected Stony-strain snails trained with the same behavioural procedure.

Understanding how and why the two different *Lymnaea* strains used here respond differently to the HS stressor is of great importance in coming to an understanding of the neuronal mechanisms underlying the behavioural ecology of this species. Except for neuronal studies comparing "*smart*" freshly collected snails to W-strain snails, the vast majority of work in the *Lymnaea* model system have employed laboratory-inbred strains, such as the W-strain used here. However, the

Fig. 3. Lab-inbred W-strain snails form a Garcia-like effect, and the behavioural procedure upregulates HSPs, GRIN1, and CREB1. The timeline of each experiment is presented above the data. The solid line is the mean, and the error bars are the SEM. (A) A naïve cohort of lab-inbred W-strain snails ($N = 8$) was exposed to a novel carrot slurry. The number of rasps elicited by this novel appetitive stimulus was counted (C—closed circles) for 2 min, and 1 h later snails received the HS. The rasping behaviour recorded in carrot slurry 3 h post HS (C 3 h—open circles) was significantly reduced compared to the pre-HS exposure. Comparisons were made by paired *t* test. (B) Control W-strain snails ($N = 8$) were exposed to carrot slurry for 2 min, and the number of rasps was recorded (C—closed circles). These snails instead of receiving the HS were maintained in a beaker for 1 h at room temperature (RT, 20 °C) in artificial pond water. The number of rasps was again recorded 3 h later in carrot slurry (C 3 h—open circles), and it was not significantly different from that recorded initially. Comparisons were made by paired *t* test. (C) The behavioural procedure utilizing the HS significantly upregulates the expression levels of HSP70, HSP40, GRIN1, and CREB1 compared to the levels in the room temperature control. Comparisons were made by unpaired *t* test. $^{****}p < 0.0001$; $^{***}p < 0.001$.



W-strain and *L. stagnalis* collected from ponds also have differential responses to environmentally relevant stressors (Orr et al. 2009; Hughes et al. 2017; Dodd et al. 2018*a*, 2018*b*; Batabyal and Lukowiak 2022). Here, we were able to compare the behavioural and transcriptional effects of the HS between W-strain *Lymnaea* and the freshly collected Stony-strain snails. An obvious big difference in the life history of these strains is the temperatures they have experienced during their lives. The W-strain *Lymnaea* only experienced the lab-temperature environment (i.e., relatively constant), while the Stony-strain was subjected to a wide range of temperatures both seasonally and daily. This difference in experience may bestow on this strain a higher thermotolerance that is reflected in the higher basal mRNA levels of at least two HSPs, HSP70 and HSP40, which are the major physiological marker of heat stress (Sorensen and Minchella 1998). In other words, the higher HSPs' basal mRNA levels of the Stony-strain snails may be interpreted as an adaptation to large and rapid temperature variations occurring in the ponds in which they live. Thermal adaptation may be beneficial to balance the cost of upregulating HSPs with temperature fluctuation in the range experienced by *Lymnaea* such as the Stony strain (Sorensen and Minchella 1998; Noer et al. 2020). The differential elaboration of HSPs as seen in the Stony strain may confer survivability if the trend of increasing temperatures experienced on the Canadian prairies continues. On the other hand, the W-strain snails, which have been maintained in relatively constant laboratory environments for approximately 250 generations rarely experience thermal fluctuations higher than 1–2 °C over the year, show lower HSP mRNA levels and higher thermal sensitivity compared to their *wild* sister populations. It is possible that moving the ancestors of our present W-strain snails from polders in the Netherlands in the 1950s to the more tightly controlled environment of the laboratory (i.e., removal of daily and seasonal temperature fluctuations as well as other stressors like predation, parasites, and competition for resources) has resulted in lower stress tolerance specifically in relation to temperature in the current study. However, HSPs are not just upregulated during thermal stress but also are a marker for several other stressors (Tomanek and Sanford 2003). Thus, to further confirm these hypotheses, additional studies are currently underway in our laboratory to study the protein levels as well as understand whether other stressors also show a similar pattern in HSP activity in the W-strain lab versus freshly collected strains (e.g., Stony strain). Similar results to those reported here have been obtained in the doctor fish *Garra rufa* (Heckel, 1843), where higher levels of HSPs have been recorded in strains in elevated water temperature compared with normal river water temperature, suggesting that physiologically increased HSP levels confer thermal tolerance (Oksala et al. 2014).

Previous ectotherm studies, including those of the fruit fly *Drosophila melanogaster* Meigen, 1830 (Hoffmann et al. 2003), the kelp crab *Taliepus dentatus* (Edwards, 1834) (Storch et al. 2009), the intertidal snail *Nucella canaliculata* (Duclos, 1832) (Kuo and Sanford 2009), and the killifish *Fundulus heteroclitus* (Linnaeus, 1766) (Fangue et al. 2006), compared the thermal adaptations to temperature extremes among conspecific

populations living at different latitudes or altitudes.However, most of these studies used critical minimum and critical maximum tolerance limits as a phenotypic response to investigate thermal tolerance. In this study, instead, the Garcia-effect learning procedure provided us with a strong measure for comparing the heat tolerance and induction of HSPs in different population of snails that experience different thermal environments. Importantly, the differences in thermal stress responses reported here between the Stony strain and the W-strain *Lymnaea* may not be generalized to all environmental stressors. For example, we recently observed a difference in predator detection in two freshly collected strains of *Lymnaea* living in adjacent lakes wherein one population had a predator present in their habitat and one did not. Behaviourally, this was reflected in the fact that one strain was *predator-experienced* compared to the other that was *predator-naïve* (Batabyal and Lukowiak 2022; Batabyal et al. 2022). Our data suggest that the difference in predator presence altered the predator detection mechanism between these populations, which correlated well with the presence or absence of crayfish predators (Batabyal and Lukowiak 2022). Along with the difference in baseline HSPs, we also found that the W-strain snails exhibit an upregulation of HSPs following the Garcia-effect training procedure where a novel food was followed by the HS stress. This same procedure did not upregulate HSPs in the freshly collected Stony-strain snails that correlated with the inability of the HS to cause a Garcia-effect-like aversion to the novel food stimulus. The W-strain behavioural data obtained here are consistent with results from our previous work (Rivi et al. 2021*a*, 2022*a*). Additionally, we observe an upregulation of mRNAs that are responsible for long-term memory formation (GRIN1 and CREB1) in the W-strain and not in the Stony strain. These data are suggestive of a mechanistic basis of the Garcia effect memory phenotype wherein CREB1 and GRIN1 upregulation could be a causal factor. However, currently, we only show a correlation between the Garcia effect memory phenotype and the upregulation of some neuroplasticity genes.

Conclusion

Our findings enable us to have a better understanding of the molecular underpinnings of the behavioural differences seen between the two strains of *L. stagnalis* that have vastly different rearing histories*:* (1) the W-strain and the Stony strain. We have gained a better understanding of the evolution of plastic responses to stress in strains of the same species living in different environments that are subject to different selection processes. Further, these data underlie the importance, which may not always be made, of paying attention when making predictions on how different stressors alter adaptive behaviours, including learning and memory, especially if data are extrapolated from laboratory to *wild* populations.

Acknowledgements

We would like to thank Consorzio Interuniversitario Biotecnologie (Trieste, Italy) for the research grant awarded to VR to work at the Hotchkiss Brain Institute (University of Calgary, Canada) as a visiting researcher.

Article information

History dates

Received: 2 May 2023
Accepted: 24 May 2023
Version of record online: 26 June 2023

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Data availability

The raw data that support the findings of this study are available from the corresponding author, VR, upon reasonable request.

Author information

Author ORCIDs

Veronica Rivi https://orcid.org/0000-0002-8413-4510
Anuradha Batabyal https://orcid.org/0000-0002-3373-5519
Cristina Benatti https://orcid.org/0000-0003-0236-9525
Fabio Tascedda https://orcid.org/0000-0002-3422-004X
Joan M. C. Blom https://orcid.org/0000-0002-4974-1964
Ken Lukowiak https://orcid.org/0000-0001-9028-1931

Author notes

Veronica Rivi and Anuradha Batabyal contributed equally to the manuscript.

Author contributions

Conceptualization: VR, AB, KL
Data curation: VR, AB, KL
Formal analysis: VR, AB
Funding acquisition: FT, JMCB, KL
Investigation: VR
Methodology: VR, AB, CB
Project administration: KL
Resources: VR, CB, FT, KL
Supervision: KL
Validation: VR
Visualization: CB, JMCB, KL
Writing – original draft: VR
Writing – review & editing: AB, CB, FT, JMCB, KL

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding information

This research was supported by Regione Emilia-Romagna "L'invertebrato *L. stagnalis* quale Modello per la Medicina Traslazionale" L.R. N. 20/2002 PROGETTI DI RICERCA SUI METODI ALTERNATIVI ALL'UTILIZZO DI ANIMALI and FAR

2016 Department of Life Sciences, University of Modena and Reggio Emilia. Behavioural trials were performed at the University of Calgary and funding was provided by the Natural Sciences and Engineering Research Council of Canada (NSERC-227993-2019) to KL. These sources of funding were not involved in the study design, data collection, analysis, interpretation, writing of the report, or in the decision to submit the paper for publication.

References

Angilletta, M.J. 2006. Estimating and comparing thermal performance curves. J. Therm. Biol. **31**(7): 541–545. doi:10.1016/j.jtherbio.2006.06.002.

Angilletta, M.J., Jr. 2009. Thermal adaptation: a theoretical and empirical synthesis. Oxford University Press, Oxford. doi:10.1093/acprof:oso/9780198570875.001.1.

Armbruster, P., and Reed, D.H. 2005. Inbreeding depression in benign and stressful environments. Heredity, **95**(3): 235–242. doi:10.1038/sj.hdy.6800721. PMID: 16077737.

Barco, A., Pittenger, C., and Kandel, E.R. 2003. CREB, memory enhancement and the treatment of memory disorders: promises, pitfalls and prospects. Expert Opin. Ther. Targets, **7**(1): 101–114. doi:10.1517/14728222.7.1.101. PMID: 12556206.

Batabyal, A., and Lukowiak, K. 2022. Tracking the path of predator recognition in a predator-naive population of the pond snail. Behav. Ecol. **34**: 125–135. doi:10.1093/beheco/arac107.

Batabyal, A., Rivi, V., Benatti, C., Blom, J.M.C., and Lukowiak, K. 2021. Long-term memory of configural learning is enhanced via CREB up-regulation by the flavonoid quercetin in *Lymnaea stagnalis*. J. Exp. Biol. **224**(13): jeb242761. doi:10.1242/jeb.242761. PMID: 34031691.

Batabyal, A., Chau, D., Rivi, V., and Lukowiak, K. 2022. Risk in one is not risk in all: snails show differential decision making under high- and low-risk environments. Anim. Behav. **190**: 53–60. doi:10.1016/j.anbehav.2022.05.013.

Blom, J.M.C., and Ottaviani, E. 2017. Immune-neuroendocrine interactions: evolution, ecology, and susceptibility to illness. Med. Sci. Monit. Basic Res. **23**: 362–367. doi:10.12659/msmbr.907637. PMID: 29142191.

Brown, K.M. 1979. The adaptive demography of four freshwater pulmonate snails. Evolution, **33**(1): 417–432. doi:10.2307/2407631. PMID: 28568187.

Bujan, J., Roeder, K.A., Yanoviak, S.P., and Kaspari, M. 2020. Seasonal plasticity of thermal tolerance in ants. Ecology, **101**(6): e03051. doi:10.1002/ecy.3051. PMID: 32239508.

Burns, J.G., Foucaud, J., and Mery, F. 2011. Costs of memory: lessons from 'mini' brains. Proc. Biol. Sci. **278**(1707): 923–929. doi:10.1098/rspb.2010.2488. PMID: 21177679.

Cousyn, C., De Meester, L., Colbourne, J.K., Brendonck, L., Verschuren, D., and Volckaert, F. 2001. Rapid, local adaptation of zooplankton behavior to changes in predation pressure in the absence of neutral genetic changes. Proc. Natl. Acad. Sci. U.S.A. **98**(11): 6256–6260. doi:10.1073/pnas.111606798.

Cristina, B., Veronica, R., Silvia, A., Andrea, G., Sara, C., Luca, P., et al. 2022. Identification and characterization of the kynurenine pathway in the pond snail *Lymnaea stagnalis*. Sci. Rep. **12**(1): 15617. doi:10.1038/s41598-022-19652-0. PMID: 36114337.

Dalesman, S., and Lukowiak, K. 2010. Effect of acute exposure to low environmental calcium on respiration and locomotion in *Lymnaea stagnalis* (L.). J. Exp. Biol. **213**(Pt 9): 1471–1476. doi:10.1242/jeb.040493. PMID: 20400631.

Dalesman, S., Sunada, H., Teskey, M.L., and Lukowiak, K. 2013. Combining stressors that individually impede long-term memory blocks all memory processes. PLoS ONE, **8**(11): e79561. doi:10.1371/journal.pone.0079561. PMID: 24223180.

Dodd, S., Rothwell, C.M., and Lukowiak, K. 2018a. Strain-specific effects of crowding on long-term memory formation in Lymnaea. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. **222**: 43–51. doi:10.1016/j.cbpa.2018.04.010.

Dodd, S., Rothwell, C.M., and Lukowiak, K. 2018b. Strain-specific effects of crowding on long-term memory formation in Lymnaea. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. **222**: 43–51. doi:10.1016/j.cbpa.2018.04.010.

Dubrez, L., Causse, S., Borges Bonan, N., Dumétier, B., and Garrido, C. 2020. Heat-shock proteins: chaperoning DNA repair. Oncogene, **39**(3): 516–529. doi:10.1038/s41388-019-1016-y. PMID: 31541194.

Fangue, N.A., Hofmeister, M., and Schulte, P.M. 2006. Intraspecific variation in thermal tolerance and heat shock protein gene expression in common killifish, *Fundulus heteroclitus*. J. Exp. Biol. **209**(Pt 15): 2859–2872. doi:10.1242/jeb.02260. PMID: 16857869.

Fernell, M., Rivi, V., Batabyal, A., and Lukowiak, K. 2021. The temperature-sensitivity of memory formation and persistence is altered by cold acclimation in a pond snail. J. Exp. Biol. **224**: 242513. doi:10.1242/jeb.242513.

Garcia, J., Kimeldorf, D.J., and Koelling, R.A. 1955. Conditioned aversion to saccharin resulting from exposure to gamma radiation. Science, **122**(3160): 157–158. doi:10.1126/science.122.3160.157. PMID: 14396377.

Garcia, J., Lasiter, P.S., Bermudez-Rattoni, F., and Deems, D.A. 1985. A general theory of aversion learning. Ann. N. Y. Acad. Sci. **443**(1 Experimental): 8–21. doi:10.1111/j.1749-6632.1985.tb27060.x. PMID: 3860080.

Hairston, N.G., Holtmeier, C.L., Lampert, W., Weider, L.J., Post, D.M., Fischer, J.M., et al. 2001. Natural selection for grazer resistance to toxic cyanobacteria: evolution of phenotypic plasticity? Evolution, **55**(11): 2203–2214. PMID: 11794781.

Hawkins, R.D., Kandel, E.R., and Bailey, C.H. 2006. Molecular mechanisms of memory storage in *Aplysia*. Biol. Bull. **210**(3): 174–191. doi:10.2307/4134556. PMID: 16801493.

Hoffmann, A.A., Sørensen, J.G., and Loeschcke, V. 2003. Adaptation of *Drosophila* to temperature extremes: bringing together quantitative and molecular approaches. J. Ther. Biol. **28**(3): 175–216. doi:10.1016/S0306-4565(02)00057-8.

Hughes, E., Shymansky, T., Swinton, E., Lukowiak, K.S., Swinton, C., Sunada, H., et al. 2017. Strain-specific differences of the effects of stress on memory in *Lymnaea*. J. Exp. Biol. **220**(5): 891–899. doi:10.1242/jeb.149161. PMID: 28250177.

Ito, E., Totani, Y., and Oike, A. 2017. Necessity knows no law in a snail. Eur. Zool. J. **84**(1): 457–464. doi:10.1080/24750263.2017.1363303.

Jumbam, K.R., Jackson, S., Terblanche, J.S., McGeoch, M.A., and Chown, S.L. 2008. Acclimation effects on critical and lethal thermal limits of workers of the Argentine ant, *Linepithema humile*. J. Insect Physiol. **54**(6): 1008–1014. doi:10.1016/j.jinsphys.2008.03.011. PMID: 18534612.

Kandel, E.R., Dudai, Y., and Mayford, M.R. 2014. The molecular and systems biology of memory. Cell, **157**(1): 163–186. doi:10.1016/j.cell.2014.03.001. PMID: 24679534.

Kuo, E.S.L., and Sanford, E. 2009. Geographic variation in the upper thermal limits of an intertidal snail: implications for climate envelope models. Mar. Ecol. Prog. Ser. **388**: 137–146. doi:10.3354/meps08102.

Leicht, K., Jokela, J., and Seppälä, O. 2019. Inbreeding does not alter the response to an experimental heat wave in a freshwater snail. PLoS ONE, **14**(8): e0220669. doi:10.1371/journal.pone.0220669. PMID: 31393914.

Losos, J.B. 2008. Phylogenetic niche conservatism, phylogenetic signal and the relationship between phylogenetic relatedness and ecological similarity among species. Ecol. Lett. **11**(10): 995–1003. doi:10.1111/j.1461-0248.2008.01229.x. PMID: 18673385.

Lukowiak, K., Martens, K., Rosenegger, D., Browning, K., de Caigny, P., and Orr, M. 2008. The perception of stress alters adaptive behaviours in *Lymnaea stagnalis*. J. Exp. Biol. **211**(11): 1747–1756. doi:10.1242/jeb.014886. PMID: 18490390.

McDonald, S.C. 1973. Activity patterns of *Lymnaea stagnalis* (L.) in relation to temperature conditions: a preliminary study. Malacologia, **14**(1–2): 395–396. PMID: 4804843.

Nakai, J., Totani, Y., Kojima, S., Sakakibara, M., and Ito, E. 2020. Features of behavioral changes underlying conditioned taste aversion in the pond snail *Lymnaea stagnalis*. Invertebr. Neurosci. **20**(2): 8. doi:10.1007/s10158-020-00241-7. PMID: 32385589.

Narum, S.R., Campbell, N.R., Meyer, K.A., Miller, M.R., and Hardy, R.W. 2013. Thermal adaptation and acclimation of ectotherms from differing aquatic climates. Mol. Ecol. **22**(11): 3090–3097. doi:10.1111/mec.12240. PMID: 23452191.

Noer, N.K., Pagter, M., Bahrndorff, S., Malmendal, A., and Kristensen, T.N. 2020. Impacts of thermal fluctuations on heat tolerance and

its metabolomic basis in *Arabidopsis thaliana*, *Drosophila melanogaster*, and *Orchesella cincta*. PLoS ONE, **15**(10): e0237201. doi:10.1371/journal.pone.0237201. PMID: 33119606.

Oksala, N.K.J., Ekmekçi, F.G., Özsoy, E., Kirankaya, Ş., Kokkola, T., Emecen, G., et al. 2014. Natural thermal adaptation increases heat shock protein levels and decreases oxidative stress. Redox Biol. **3**: 25–28. doi:10.1016/j.redox.2014.10.003. PMID: 25462062.

Orr, M., Hittel, K., Lukowiak, K.S., Han, J., and Lukowiak, K. 2009. Differences in LTM-forming capability between geographically different strains of Alberta *Lymnaea stagnalis* are maintained whether they are trained in the lab or in the wild. J. Exp. Biol. **212**(23): 3911–3918. doi:10.1242/jeb.024281. PMID: 19915134.

Ottaviani, E. 2015. Molluscs as models for translational medicine. Med. Sci. Monit. Basic Res. **21**: 96–99. doi:10.12659/MSMBR.894221. PMID: 25925630.

Ottaviani, E., Accorsi, A., Rigillo, G., Malagoli, D., Blom, J.M.C., and Tascedda, F. 2013. Epigenetic modification in neurons of the mollusc *Pomacea canaliculata* after immune challenge. Brain Res. **1537**: 18–26. doi:10.1016/j.brainres.2013.09.009. PMID: 24041774.

Rigillo, G., Vilella, A., Benatti, C., Schaeffer, L., Brunello, N., Blom, J.M.C., et al. 2018. LPS-induced histone H3 phospho(Ser10)-acetylation (Lys14) regulates neuronal and microglial neuroinflammatory response. Brain Behav. Immun. **74**: 277–290. doi:10.1016/j.bbi.2018.09.019. PMID: 30244035.

Rivi, V., Benatti, C., Colliva, C., Radighieri, G., Brunello, N., Tascedda, F., and Blom, J.M.C. 2020. *Lymnaea stagnalis* as model for translational neuroscience research: from pond to bench. Neurosci. Biobehav. Rev. **108**: 602–616. doi:10.1016/j.neubiorev.2019.11.020.

Rivi, V., Batabyal, A., Juego, K., Kakadiya, M., Benatti, C., Blom, J.M.C., and Lukowiak, K. 2021a. To eat or not to eat: a Garcia effect in pond snails (*Lymnaea stagnalis*). J. Comp. Physiol. A, **207**(4): 479–495. doi:10.1007/s00359-021-01491-5.

Rivi, V., Benatti, C., Lukowiak, K., Colliva, C., Alboni, S., Tascedda, F., and Blom, J.M.C. 2021b. What can we teach *Lymnaea* and what can *Lymnaea* teach us? Biol. Rev. Cambridge Philos. Soc. **96**: 1590–1602. doi:10.1111/brv.12716. PMID: 33821539.

Rivi, V., Batabyal, A., Benatti, C., Blom, J.MC., and Lukowiak, K. 2022a. Nature versus nurture in heat stress induced learning between inbred and outbred populations of *Lymnaea stagnalis*. J. Ther. Biol. **103**: 103170. doi:10.1016/j.jtherbio.2021.103170. PMID: 35027189.

Rivi, V., Batabyal, A., Benatti, C., Tascedda, F., Blom, J.M., and Lukowiak, K. 2022b. Too hot to eat: wild and lab-bred *Lymnaea stagnalis* differ in feeding response following repeated heat exposure. Biol. Bull. **243**: 38–43. doi:10.1086/720948.

Rivi, V., Batabyal, A., Wiley, B., Benatti, C., Tascedda, F., Blom, J.M.C., and Lukowiak, K. 2022c. Fluoride affects memory by altering the transcriptional activity in the central nervous system of *Lymnaea stagnalis*. Neurotoxicology, **92**: 61–66. doi:10.1016/j.neuro.2022.07.007. PMID: 35907516.

Rivi, V., Benatti, C., Actis, P., Tascedda, F., and Blom, J.M.C. 2022d. Behavioral and transcriptional effects of short or prolonged fasting on the memory performances of *Lymnaea stagnalis*. Neuroendocrinology, **113**: 406–422. doi:10.1159/000527489. PMID: 36223719.

Rivi, V., Batabyal, A., Benatti, C., Blom, J.M., Tascedda, F., and Lukowiak, K. 2023. Novel taste, sickness, and memory: lipopolysaccharide to induce a Garcia-like effect in inbred and wild strains of *Lymnaea stagnalis*. Physiol. Behav. **263**: 114137. doi:10.1016/j.physbeh.2023.114137.

Sorensen, R.E., and Minchella, D.J. 1998. Parasite influences on host life history: *Echinostoma* revolutum parasitism of *Lymnaea elodes* snails. Oecologia, **115**(1–2): 188–195. doi:10.1007/s004420050507. PMID: 28308452.

Storch, D., Santelices, P., Barria, J., Cabeza, K., Pörtner, H.-O., and Fernández, M. 2009. Thermal tolerance of crustacean larvae (zoea I) in two different populations of the kelp crab *Taliepus dentatus* (Milne-Edwards). J. Exp. Biol. **212**(Pt 9): 1371–1376. doi:10.1242/jeb.030205. PMID: 19376958.

Tomanek, L., and Sanford, E. 2003. Heat-shock protein 70 (Hsp70) as a biochemical stress indicator: an experimental field test in two congeneric intertidal gastropods (genus: *Tegula*). Biol. Bull. **205**(3): 276–284. doi:10.2307/1543291. PMID: 14672982.