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Stress is not one-size-fits-all: stressor-specific effects on learning and memory in a molluscan model

Veronica Rivi [a, *], Anuradha Batabyal [b], Jasper Hollings [c], Jillian Kitt [c], Fabio Tascedda [a, d, e], Johanna Maria Catharina Blom [d, f], Cristina Benatti [d, f], Ken Lukowiak [c]

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

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Environmental stressors profoundly influence how animals learn and remember, yet different types of stress can trigger distinct behavioural and molecular outcomes. Understanding this is essential for decoding adaptive responses in ecological settings and also for informing translational models of stress-related cognitive dysfunction. The pond snail *Lymnaea stagnalis* offers a powerful platform for such investigations. Despite their simplicity compared to mammals, they exhibit higher-order associative learning, such as the Garcia effect, where a novel taste cue, when associated over a time gap (which can be hours) with sickness (induced by acute heat shock), causes the snails to avoid the novel food. The snails also show configural learning, which occurs when a positive (food cue) and negative stimulus (predator odour) are presented simultaneously, leading to the formation of a 'fear landscape', and the consequent avoidance of the positive food cue. In this study, we extend these existing paradigms by using biologically relevant stressors: a predator-attack-mimicking shell clip (ShC) and acute heat shock (HS). We found striking, stressor-specific differences in behavioural outcomes: ShC supported configural learning but not the Garcia effect, while HS induced the Garcia effect but failed to support configural learning. Along with behaviour, we show distinct transcriptional changes in the central ring ganglia, affecting expression levels of key targets including stress- and plasticity-related genes, serotonergic markers and enzymes of the endocannabinoid system. Together, these results suggest that different stressors establish unique internal stress states ('fear' versus 'sickness') that selectively shape learning pathways. This study highlights a critical principle in neuroscience: stress is not a monolith, and its impact on learning and memory depends on both its nature and the internal state it evokes. This finding holds direct relevance for understanding stress-related psychiatric disorders and maladaptive behaviours.

The dynamic interplay between stress and cognition remains a central focus in translational neuroscience, with wide-ranging implications across psychiatry, neurotoxicology and cognitive health (Albayrak et al., 2024; Dhabhar, 2018). Stress, originally defined by Selye (1973) as a nonspecific response of the body to any demand for change that necessitates physiological, psychological or behavioural adjustment for the wellbeing of the organism (National Research Council (US) Committee on Recognition and Alleviation of Distress in Laboratory Animals, 2008), can profoundly influence how learning and memory are processed (Rivi et al., 2023a; Schwabe, 2025). A major challenge lies in elucidating how different types and intensities of stress affect memory phases such as encoding, consolidation and retrieval (Schwabe et al., 2022). Indeed, depending on its nature and the organism's perception, stress can either enhance or impair

* Corresponding author.
E-mail address: veronica.rivi@unimore.it (V. Rivi).

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memory formation and retrieval (Kim & Kim, 2023). Moreover, stress can contribute to maladaptive cognitive outcomes such as false memories and post-traumatic stress disorder (PTSD; Hauschildt et al., 2012; Jelinek et al., 2009). Numerous mammalian studies have explored these stress–memory interactions, often yielding contradictory results: in some cases, memory is enhanced; in others, it is blocked or unaffected (Atrooz et al., 2021; Goldfarb, 2019; Lipatova et al., 2023; Luethi et al., 2009). These inconsistencies probably stem from the complexity of mammals' nervous systems and the multifaceted ways in which stress affects cognition. To address these challenges, invertebrate models are increasingly employed to uncover evolutionarily conserved mechanisms that govern cognition under stress, while providing complementary advantages (Choudhary & Ibdah, 2013; Rivi, Benatti, Rigillo, & Blom, 2023): reduced neural complexity, individually identifiable neurons and well-characterized behaviour (Brown & Pearson, 2017; Davis, 2011; Gumbert, 2000; Hawkins et al., 2006; Ottaviani & Franceschi, 1996; Perry & Baciadonna, 2017; Rasmussen, 2018; Sengupta & Samuel, 2009). By integrating findings across phylogenetically distant taxa, we can better distinguish universal principles of stress-related memory modulation from species-specific adaptations, thereby building more robust translational frameworks across various fields.

Among invertebrate models, the pond snail *Lymnaea stagnalis* has emerged as a powerful and tractable system for investigating how physiological and psychological stress shape learning and memory (Aonuma et al., 2016; Benjamin & Kemenes, 2009; Fodor et al., 2021; Ito et al., 2013; Kemenes & Benjamin, 1994; Lukowiak et al., 1996, 2008; Rivi, Batabyal, et al., 2021; Rivi, Benatti, et al., 2021; Rivi et al., 2020; Álvarez et al., 2022). Importantly, seminal work from across the globe starting in the early 1980s (e.g. Alexander et al., 1982; Braun & Lukowiak, 2011; Crossley et al., 2019; Dalesman & Lukowiak, 2010; Forest et al., 2016; Kemenes & Benjamin, 1989, 1994; Kemenes et al., 1997; Kobayashi et al., 1998; Lukowiak et al., 2006; Staras et al., 1998; Sugai et al., 2006, 2007; Yamanaka et al., 2000) established *Lymnaea* as a model for studying evolutionarily conserved mechanisms of appetitive, aversive and operant learning-induced, long-term, associative memories. These foundational studies provided the methods and conceptual framework on which later stress studies were built. With its relatively simple and accessible nervous system, *L. stagnalis* allows researchers to link molecular and cellular changes to behavioural outcomes with rare precision, a key advantage for translational relevance (Aonuma et al., 2018; Cristina et al., 2022; Fodor et al., 2021; Koene et al., 2024; McComb et al., 2003; Sadamoto et al., 2012; Wood et al., 2021).

A particularly striking example of this translational potential is *L. stagnalis'* capacity to exhibit a Garcia effect, a 'special' form of conditioned taste aversion (CTA) requiring the organism to become sick after eating something with a novel taste, which results in a long-lasting food avoidance (Rivi, Batabyal, et al., 2021). Unlike 'standard' CTA, in which an appetitive food stimulus is paired closely in time with an aversive stimulus (Chambers, 2018), the Garcia effect occurs even when ingestion of a novel food is followed hours later by an aversive or sickness-inducing experience (Rivi et al., 2023b; Rivi, Batabyal, et al., 2021). This reflects natural scenarios in which toxic substances produce illness only after a delay (Garcia et al., 1955, 1985). Thus, the defining features of the Garcia effect are food novelty and long-delay learning, resulting in a taste-specific aversion that can persist over time.

Although CTA has been widely demonstrated across species, including *L. stagnalis* (Kojima et al., 1996; Sugai et al., 2006, 2007; Wagatsuma et al., 2004), the Garcia effect was long thought to be exclusive to vertebrates (Rivi, Batabyal, et al., 2024), until we provided the first evidence for this phenomenon in an invertebrate model (Rivi, Batabyal, et al., 2021). In particular, we demonstrated that a single exposure to a novel food, followed hours later (up to 48 h) by either a heat-shock stimulus or bacterial lipopolysaccharide injection, caused snails to avoid that food for at least 24 h (Rivi et al., 2022, 2023b; Rivi, Batabyal, et al., 2021; Rivi et al., 2023). Moreover, we systematically tested multiple intervals (1 h, 4 h, 24 h and 48 h) and found robust learning at all delays, consistent with Garcia's original findings in rodents (Garcia et al., 1985; Rivi, Batabyal, et al., 2021). Importantly, experiments with control treatments confirmed that the aversion was due to the pairing of the novel taste with the malaise-inducing stimulus: if the novel taste was presented 1 h after the stimulus, snails actually showed no reduction in feeding. Similarly, when the food was not novel, no Garcia effect was observed (Batabyal et al., 2024; Rivi, Benatti, Actis, Tascedda, & Blom, 2022). Finally, we demonstrated that the nausea-inducing stimuli on their own did not cause an inhibition of feeding, but rather it is the pairing of the novel taste and the stressor that results in a Garcia effect. These findings indicate that *L. stagnalis* can form associations between gustatory cues and internal states of malaise, echoing the 'sickness landscape' described in mammalian models (Dantzer, 2009; Kent et al., 1992).

Lymnaea stagnalis also exhibits a higher form of associative learning, termed configural learning (Batabyal et al., 2021; C. Swinton, Swinton, Shymansky, et al., 2019). This is a higher-order cognitive process where two co-occurring stimuli are encoded as a unified representation, distinct from their components (Kagan & Lukowiak, 2019). We have previously shown that the simultaneous exposure of snails to an appetitive taste cue with a predator-associated chemical signal (e.g. crayfish effluent, CE) leads to the creation of a 'landscape of fear', altering the meaning of the taste cue and converting it into a signal for danger, thereby prompting defensive behaviours, including feeding suppression (Batabyal et al., 2022). Thus, we use the term 'configural learning' to highlight that *L. stagnalis* can encode compound cue configurations as unique representations under specific stress conditions. This broadens the concept of configural processing in invertebrates and demonstrates that higher-order associative processes may be conserved across phyla when the ecological relevance of the stimuli is considered.

This finding reinforces *Lymnaea*'s translational relevance for dissecting stress–memory interactions and underscores the broader principle that stressor identity shapes internal states, which in turn gate access to distinct forms of learning. Notably, we recently demonstrated that another predator-related cue, shell clipping (ShC) mimicking a physical stressor, can be used to induce this higher-order learning in *L. stagnalis* (Rivi, Batabyal, Pele, et al., 2025; E. Swinton, Swinton, & Lukowiak, 2019). Thus, our previous studies demonstrated that different types of stressors, such as biotic predator-related stressors (i.e. CE and ShC) and abiotic heat shock (HS), elicit distinct types of learning and memory phenotypes in *L. stagnalis* (i.e. configural learning and Garcia effect, respectively). However, whether stressors can be interchangeably used to invoke similar effects has not been tested. Additionally, it is important to investigate whether both behavioural and physiological outcomes of the stressors used under the same learning paradigm remain the same or not. Addressing this gap, we asked two key questions. (1) Can HS, a malaise-inducing abiotic stressor, also support configural learning when paired with an appetitive cue? (2) Can ShC, a predator-mimicking biotic stressor, likewise induce a Garcia effect?

ShC was selected over CE because, unlike CE, ShC is not a transient sensitizing cue but a persistent and biologically relevant predator-mimicking stressor. Prior work has shown that a single ShC event delivered 24 h before operant conditioning enhances long-term memory formation, and this facilitation remains evident even after 72 h (Rivi, Batabyal, Pele, et al., 2025; C.

Swinton, Swinton, Shymansky, et al., 2019). In contrast, CE exposure induces more transient behavioural and physiological responses that dissipate within shorter timescales, limiting its suitability for testing long-delay associative phenomena such as the Garcia effect. By leveraging ShC's durability and ecological validity, we hypothesized that it could serve as a robust biotic stressor capable of inducing a Garcia effect (Rivi, Batabyal, Pele, et al., 2025; C. Swinton, Swinton, Shymansky, et al., 2019). Thus, we hypothesized that the ShC could be used in our study to induce a Garcia effect.

Overall, to address these questions, we combined behavioural procedures with gene expression analyses and analysed transcriptional changes in the central nervous system of *L. stagnalis*, focusing on key targets implicated in neuroplasticity, cellular stress responses, serotonergic neurotransmission (given its role in modulating feeding behaviour and antipredator behaviour) and the endocannabinoid system; each of which has been implicated in stress modulation of memory in vertebrate systems (Bacqué-Cazenave et al., 2020; Ibarra-Lecue et al., 2018).

This study was designed to offer new insights into the specificity of stressor effects on higher-order learning processes. By identifying both shared and unique molecular signatures across stress conditions, we aimed to delineate how acute stress experiences reconfigure neural circuits to either facilitate adaptive learning or engender maladaptive behavioural responses, a fundamental question in stress-related neuroscience research. Thus, we hope this work expands the translational relevance of *L. stagnalis* as a model system in cognitive and stress neuroscience. By leveraging its simplicity alongside powerful behavioural paradigms and molecular tools, we can elucidate conserved mechanisms through which stress affects memory, informing broader questions in translational neuroscience, including stress resilience and cognitive flexibility.

METHODS

Study Animal

We used a strain of *L. stagnalis* (i.e. W-strain) maintained under standardized laboratory conditions (20 ± 1 °C on a 16:8 h light:dark cycle and fed romaine lettuce *ad libitum*) at the University of Calgary (Alberta, Canada) since the 1980s. The founding population originated from polders in Utrecht, the Netherlands, in the 1950s, and has been maintained at the Vrije Universiteit in Amsterdam since then. Snails were kept in artificial pond water prepared by dissolving 0.25 g/L Instant Ocean (Spectrum Brands, Madison, WI, U.S.A., https://spectrumbrands.com) in deionized water, supplemented with $CaCO_3$ to maintain calcium concentrations above 50 mg/L, following established protocols (Rivi, Batabyal, Benatti, Tascedda, et al., 2023a). We housed snails at a density of 50–60 individuals per 70 L aquarium to prevent overcrowding. All aquaria were built of glass and equipped with commercial filters with aerators, and the laboratory lighting was of illumination ca. 800 lx. We replaced the pond water every 72 h to maintain water quality and minimize the build-up of metabolic waste. Eggs laid during the experimental timeline were removed from the parent aquaria and kept separately for rearing. Adult animals (ca. 3–4 months old) with shell lengths of 20–25 mm were used in the experiments.

Carrot Slurry and Rasping Behaviour

To ensure food novelty (which is a requirement for the Garcia effect; Garcia et al., 1985), the snails used in these experiments had not been previously exposed to carrots. The carrot slurry used as the novel food stimulus was prepared by blending two fresh medium-sized carrots (ca. 600 g) in 500 mL of artificial pond water as previously described (Kagan et al., 2022). For behavioural testing, individual snails were placed into a 14 cm diameter Petri dish mounted on a clear Plexiglas stand, 10 cm above a mirror to allow visualization of the radula. Following a 3 min acclimation period in the slurry, feeding behaviour was quantified during a 2 min observation period, counting the number of rasps (a rhythmic motor pattern in which the radula scrapes a substrate) leading to food ingestion (Arundell et al., 2006).

Configural Learning and Garcia Effect Procedures

The Garcia effect learning paradigm began with an initial behavioural assessment, in which the number of rasps elicited by the carrot slurry was recorded (C pre) for 2 min following the 3 min acclimation described above. In the Garcia effect procedure, separate groups of snails were exposed to either the HS (30 °C in artificial pond water for 1 h as described in Rivi et al.,. 2023b) or ShC (a clip of the shell margin ca. 10 × 3 mm on the pneumostome side with forceps; E. Swinton, Swinton, & Lukowiak, 2019) 3 h post C pre. This shell clipping region was chosen based on natural crayfish (predator) attacks and to minimize damage to any internal soft tissue of the snail. All animals were retested for carrot response or feeding behaviour 3 h post this procedure.

In the configural learning procedure, all animals were first tested for initial carrot feeding response in the same way as in the Garcia effect procedure (C pre). Post 18 h of C pre, one group of snails was subjected to the ShC and then immediately immersed in the carrot slurry for 45 min. In contrast, a second group was exposed to carrot slurry heated to 30 °C (i.e. the HS) for the same duration. Thus, the stressor and the novel taste were experienced simultaneously. This was the primary difference in the two procedures, where in the Garcia effect the novel food and stressor are separated in time, and in the configural learning the novel food and stressor are simultaneously paired. All animals were retested for carrot responsiveness 3 h after the stress exposure (C 3 h).

For both the learning experiments (Garcia effect and configural learning) a significant reduction in rasping at C 3 h compared to C pre was taken as evidence of intermediate-term memory (ITM, lasting up to 3 h) formation (Rivi et al., 2023a). The snails used for ShC in configural learning were tested again at 24 h to assess long-term memory (LTM, lasting 24 h) formation, as we wanted to test for enhanced memory using the ShC procedure; our thinking was that this might mimic a stronger predatory stimulus than the odour stimulus we have previously used for the configural learning procedure (Batabyal et al., 2021; C. Swinton, Swinton, Shymansky, et al., 2019), which gives rise to a 3 h memory only.

Transcriptional Effects of Different Stressors

For molecular analyses, we tested how a single exposure to the stressors altered various pathways in the organisms. We had seven snails exposed to ShC, eight to HS (30 °C for 1 h) and eight served as controls (exposed to 20 °C for 1 h). These groups did not undergo any experimental training but were only assessed for their molecular response to the individual stressors. Three hours after stressor exposure, all snails were euthanized by placement on ice for 10 min. Their central ring ganglia were then dissected and preserved in RNAlater (Qiagen, https://www.qiagen.com) and processed at the University of Modena and Reggio Emilia, Italy, as previously described elsewhere (Rivi, Batabyal, Wiley, et al., 2022). Sample sizes were determined based on a power analysis for detecting biologically meaningful differences in mRNA expression. Assuming a moderate effect size (Cohen's $d = 1.0$), a

significance level of $\alpha = 0.05$ and a desired statistical power of 80 % ($1 - \beta = 0.8$), the analysis indicated that 7–8 animals per group would be sufficient. Consistent with previous studies (Batabyal et al., 2021; Rivi, Batabyal, Wiley, et al., 2022; Rivi et al., 2022; Rivi, Pele, et al., 2025; Rivi, Rigillo, Batabyal, et al., 2024), biological variability in *L. stagnalis* for these assays is relatively low, further supporting the reliability of the chosen sample sizes. Power calculations were performed using G*Power (Faul et al., 2007).

Total RNA was extracted from individual ganglia using the GenElute Total RNA Miniprep Kit (Millipore Sigma, https://www.sigmaaldrich.com), followed by DNAse treatment with the DNASE70 On-Column DNase I Digestion Set (Merck Millipore, https://www.merckmillipore.com). A total of 200 ng of RNA per sample was used for cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher, https://www.thermofisher.com). Real-time quantitative PCR was carried out on 20 ng of cDNA using the Bio-Rad CFX Connect Real-Time PCR Detection System and SYBR Green Master Mix (Bio-Rad, https://www.bio-rad.com), with forward and reverse primers used at a final concentration of 300 nM. Cycle threshold (Ct) values were determined using CFX Maestro Software (Bio-Rad). We analysed the expression of several genes associated with stress response and neuroplasticity. These included orthologs of the heat shock proteins (Uplap et al., 2010) (LymHSP70, LymHSP40), the NMDA receptor subunit (LymGRIN1) and the transcription factor cAMP response element-binding protein (LymCREB1; Kagan et al., 2023; Rivi, Batabyal, et al., 2021; Rivi, Batabyal, Benatti, et al., 2025a). We also assessed genes related to the serotonergic system, including tryptophan hydroxylase (LymTPH) and the serotonin transporter (LymSERT), as well as the purinergic receptor LymP2X (Bavan et al., 2012; Rivi et al., 2023c). Finally, we examined four key enzymes involved in the endocannabinoid system: monoacylglycerol lipase (LymMAGL), diacylglycerol lipase (LymDAGL), N-acyl phosphatidylcholine phospholipase D (LymNAPE-PLD) and fatty acid amide hydrolase (LymFAAH; Rivi, Rigillo, et al., 2024).

For this purpose, specific forward and reverse primers were used at a final concentration of 300 nmol/L. Primer sequences were previously published: please see Rivi, Batabyal, Benatti, Tascedda, et al. (2023b) for LymHSP70, LymHSP40, LymGRIN1 and LymCREB1, Benatti et al. (2017) for LymTPH, LymSERT, Lym5HTR1, Lym5HTR2 and LymP2X, and Rivi, Rigillo, Batabyal, et al. (2024) for LymMAGL, LymDAGL, LymFAAH and LymNAPE-PLD. Single PCR products were subjected to a heat dissociation protocol (StepOne Real-Time PCR System, Applied Biosystems, https://www.thermofisher.com). The cycling parameters were: 95 °C for 2 min and 94 °C for 10 s, and 60 °C for 30 s, for 40 cycles. Cycle threshold (Ct) values were determined by CFX Maestro Software (Bio-Rad, https://www.bio-rad.com). Gene expression levels of the selected targets were normalized to the arithmetic mean of two reference genes: elongation factor 1α and tubulin (Rivi, Caruso, et al., 2024), as assessed using NormFinder (https://www.moma.dk/software/normfinder). Quantitative changes in gene expression were calculated using the comparative $2^{-\Delta\Delta C_t}$ method, with the control group of unstressed snails serving as the calibrator for each experiment.

Statistical Analyses

First, we analysed our data for normality assumption using the Kolmogorov–Smirnov one-sample test for normality (K–S distance, KS, and *P*). All targets displayed a normal distribution: Garcia effect with HS: KS = 0.15, $P = 0.95$ and KS = 0.16, $P = 0.9$; configural learning with HS: KS = 0.14, $P = 0.97$ and KS = 0.22, $P = 0.66$. LymHSP70: KS = 0.19, $P = 0.32$; LymHSP40: KS = 0.1, $P = 0.57$; LymTPH: KS = 0.15, $P = 0.44$; LymP2X: KS = 0.17, $P = 0.15$; LymSERT: KS = 0.12, $P = 0.82$; Lym5HTR1: KS = 0.1, $P = 0.95$; Lym5HTR2: KS = 0.14, $P = 0.71$; LymGRIN1: KS = 0.13, $P = 0.78$; LymCREB1: KS = 0.09, $P = 0.97$; LymDAGL: KS = 0.17, $P = 0.44$; LymMAGL, KS = 0.18465, $P = 0.37$; LymNAPE-PLD: KS = 0.14, $P = 0.72$; and LymFAAH: KS = 0.1, $P = 0.95$.

Behavioural data were analysed using a paired Student's *t* test comparing the number of rasps in C pre versus C 3 h. In case of more than two comparisons, a one-way ANOVA followed by Tukey's post hoc tests was performed.

For gene expression analyses, data were analysed with one-way ANOVA followed by Tukey's post hoc tests. All statistical analyses and graphs were performed using GraphPad Prism version 10.00e for Windows (GraphPad Software, Inc., La Jolla, CA, U.S.A., https://www.graphpad.com).

Ethical Note

In accordance with Canadian and European legislation governing the use of animals in scientific research (e.g. the Canadian Council on Animal Care guidelines; Directive 2010/63/EU of the European Parliament and of the Council), ethical approval is required only for research involving vertebrate animals and cephalopods. Pond snails, as invertebrates not covered by these frameworks, are therefore exempt from mandatory ethical review. Nevertheless, all procedures in this study were carried out in strict accordance with the Association for the Study of Animal Behaviour/Animal Behavior Society (ASAB/ABS) Guidelines for the Treatment of Animals in Behavioural Research and Teaching (ASAB Ethical Committee/ABS Animal Care Committee, 2020), and every effort was made to minimize adverse impacts and promote the animals' wellbeing. Animals were kept in clean, well-oxygenated pond water, housed at low densities to avoid overcrowding and provided with appropriate nutrition ad libitum. Stressors used in the study have previously been shown to have no lasting effects on *L. stagnalis* (K. Lukowiak, V. Rivi & A. Batabyal, personal observations) and their application followed established protocols (Dalesman & Lukowiak, 2010; De Caigny & Lukowiak, 2008; Rivi et al., 2022). Where killing the animals was necessary for molecular analyses, they were first anaesthetized on ice to minimize suffering. All other animals were returned to their holding tanks following behavioural testing and fully recovered from the applied stressors. No long-term negative effects were observed in any of the returned individuals. The number of animals used was kept to the minimum necessary to achieve the scientific objectives, as determined by an a priori power analysis, in accordance with the Reduction principle of the 3Rs framework (Russell & Burch, 1959).

RESULTS

Behavioural Results

We found that ShC did not induce the Garcia effect as there was no significant difference in the number of rasps between C pre and C 3 h, indicating that ShC alone is not sufficient to induce the sickness state required for the Garcia effect to be formed ($t_8 = 1.21$, $P = 0.26$, $N = 9$; Fig. 1a). In contrast, consistent with our previous findings, HS did induce a Garcia effect, as evidenced by a significant reduction in the rasping response to carrot slurry at 3 h post HS ($t_8 = 14.50$, $P < 0.0001$, $N = 9$; Fig. 1b). When ShC was paired with carrot slurry to test for configural learning, a significant reduction in rasping was observed at 3 h post exposure, indicating the formation of ITM for this higher form of learning (Fig. 1c), which is similar to a standard configural learning memory previously observed using an odour cue of the predator (Batabyal et al., 2021; C. Swinton, Swinton, Shymansky, et al., 2019). However,

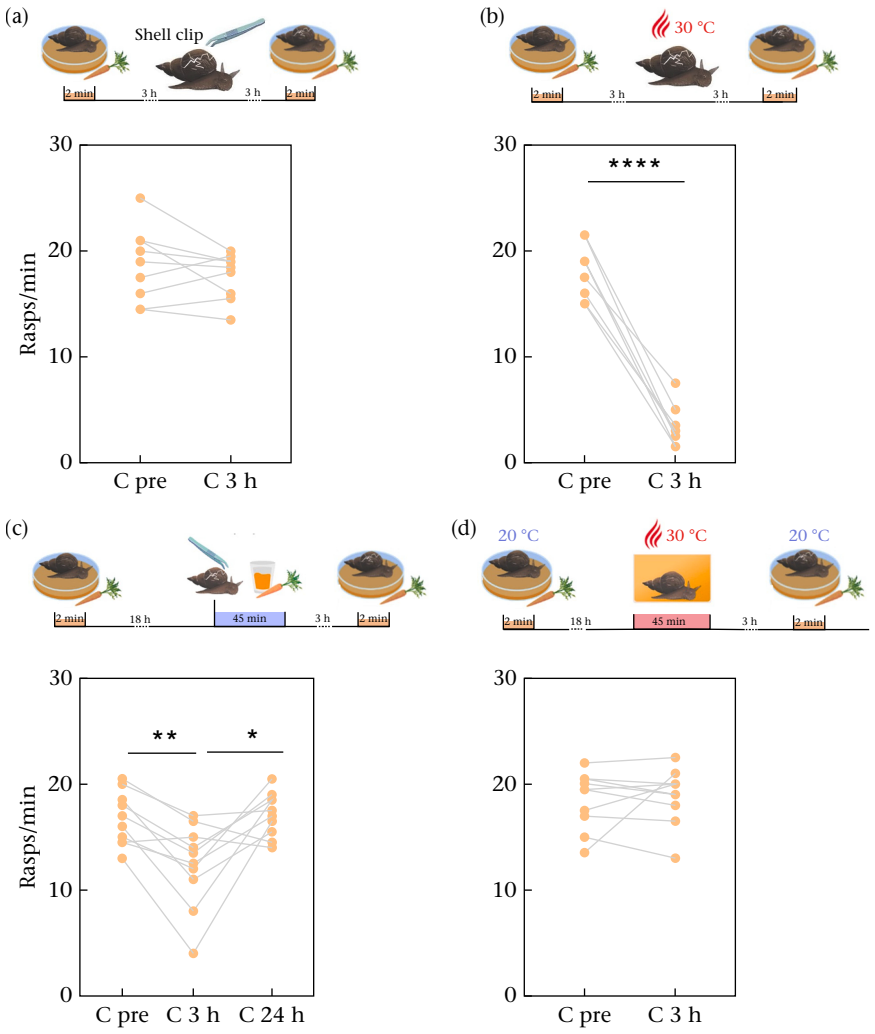

Figure 1. Shell clip and heat shock cannot be used interchangeably for inducing the Garcia effect and configural learning. (a, b) Garcia effect paradigm. The number of rasps elicited by the carrot slurry was recorded for 2 min during a pretest (C pre); 3 h later, snails were either (a) shell clipped (ShC) or (b) exposed to a 30 °C heat shock for 1 h (HS). Memory formation was assessed 3 h after treatment using a carrot slurry test (C 3 h). A significant reduction in rasping behaviour was interpreted as evidence of memory formation. (c, d) Configural learning paradigm. As above, rasping responses to the carrot were recorded at baseline (C pre); 18 h later, snails underwent either (c) shell clipping followed by immediate exposure to carrot slurry for 45 min, or (d) exposure to 30 °C carrot slurry for the same duration. Memory formation was assessed 3 h post treatment (C 3 h) and, in the case of successful learning (as in the C+ShC condition), long-term memory was also assessed 24 h later. Data in panels (a), (b) and (d) were analysed using paired *t* tests, while data in panel (c) were analysed using one-way ANOVA followed by Tukey's post hoc test; $N = 9–10$. The orange-coloured glass and carrot icon in all panels indicate the delivery of carrot slurry into the Petri dish. In (d), the orange square with the 30 °C icon indicates that snails were simultaneously exposed to the carrot slurry and heat shock. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{****}P < 0.0001$.

when the same animals were tested 24 h later to assess LTM, no significant reduction in rasping was found compared to C pre, suggesting that ShC is sufficient for ITM formation but not LTM under these conditions (RM ANOVA: $F_{1.494,13.45} = 9.68$, $P = 0.004$; Tukey's post hoc: C pre versus C 3 h: $P = 0.003$; C 3 h versus C24 h: $P = 0.03$; and C pre versus C 24 h: $P = 0.91$; $N = 10$). Conversely, simultaneous exposure to carrot slurry and HS did not produce configural learning as no significant reduction in rasping was observed at 3 h post treatment ($t_9 = 0.45$, $P = 0.66$, $N = 10$; Fig. 1d). Together, these results highlight the stressor-specific nature of memory formation in *L. stagnalis*: ShC causes configural learning but not the Garcia effect, whereas HS induces the Garcia effect but fails to cause configural learning.

Molecular Results

This experiment aimed to assess the transcriptional impact of two stressors (HS and ShC) in the central ring ganglia of naïve *L. stagnalis*. A main effect of stress exposure was observed on the expression levels of both LymHSP70 ($F_{2,20} = 107.4$, $P = 0.0001$; Fig. 2a) and LymHSP40 ($F_{2,20} = 16.43$, $P < 0.0001$; Fig. 2b). Post hoc analyses showed that both stressors significantly upregulated the mRNA levels of these genes relative to controls (LymHSP70: HS, $P = 0.0001$; ShC, $P = 0.026$; LymHSP40: HS, $P = 0.0001$; ShC, $P = 0.018$). Furthermore, LymHSP70 expression levels following HS were significantly higher than those induced by ShC ($P < 0.0001$), reflecting a more robust activation of the general stress response by HS.

Focusing on the serotonin-related targets, a main effect of stress exposure was also found for LymTPH ($F_{2,20} = 10.82$, $P = 0.0007$; Fig. 2c), LymSERT ($F_{2,20} = 12.38$, $P = 0.0003$; Fig. 2d), Lym5HTR1 ($F_{2,20} = 13.31$, $P = 0.0002$; Fig. 2e) and Lym5HTR2 ($F_{2,20} = 12.50$, $P = 0.0003$; Fig. 2f). Post hoc comparisons revealed that only ShC induced a significant upregulation of LymTPH and LymSERT compared to both the control and HS groups (LymTPH: ShC versus CTRL, $P = 0.0004$; ShC versus HS, $P = 0.03$; LymSERT:

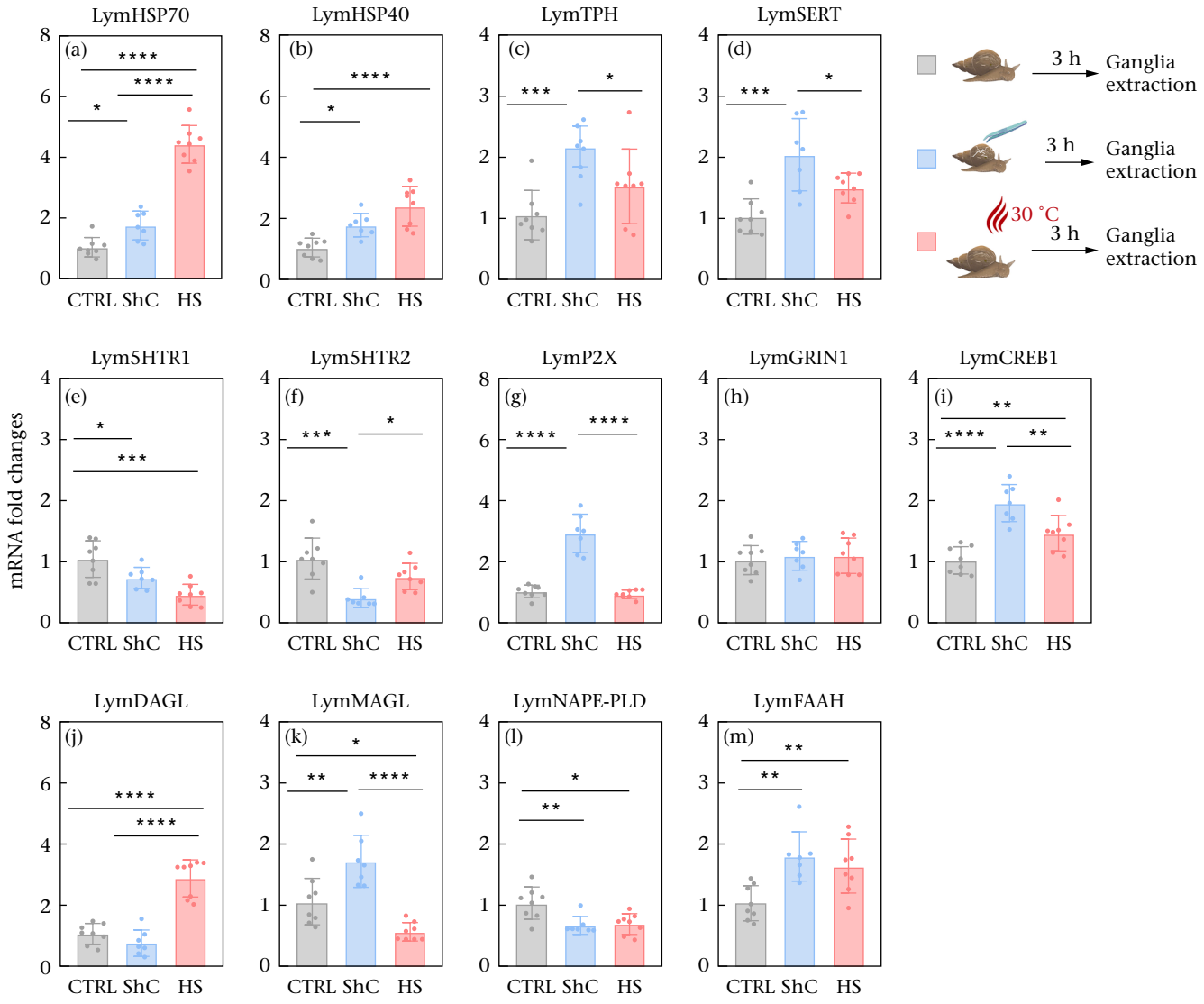

Figure 2. Transcriptional effects of shell clip and heat shock on the expression levels of key targets for stress, pain and neuroplasticity in snails' central ring ganglia. Relative mRNA levels of genes involved in stress, nociception and neuroplasticity were measured in the central ring ganglia of snails subjected to shell clip (light blue bars, ShC), heat shock at 30 °C for 1 h (red bars, HS) or maintained at room temperature (20 °C; grey bars, CTRL). Snails were killed 3 h post stressor exposure. Transcripts measured included the stress markers (a) LymHSP70 and (b) LymHSP40; serotonergic-related targets (c) LymTPH, (d) LymSERT, (e) Lym5HTR1 and (f) Lym5HTR2; the nociceptive/stress receptor (g) LymP2X; plasticity-related genes (h) LymGRIN1 and (i) LymCREB1; and components of the endocannabinoid system (j) LymDAGL, (k) LymMAGL, (l) LymNAPE-PLD and (m) LymFAAH. mRNA levels were quantified using real-time PCR. Data are presented as means (bars) ± SEM (whiskers), with $N = 7–8$ per group. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

ShC versus CTRL, $P = 0.0002$; ShC versus HS, $P = 0.036$). In contrast, both HS and ShC led to a significant downregulation of Lym5HTR1 compared to control (ShC versus CTRL, $P = 0.04$; HS versus CTRL, $P < 0.0001$), while only ShC significantly decreased the expression of Lym5HTR2 compared to control ($P = 0.002$) and HS ($P = 0.033$).

LymP2X expression levels were also modulated by stress exposure ($F_{2,20} = 66.80$, $P < 0.0001$; Fig. 2g). This target was significantly upregulated in the ShC group compared to both the control and HS groups ($P < 0.0001$). Although no significant effects were found for LymGRIN1 ($F_{2,20} = 0.21$, $P = 0.81$; Fig. 2h), a main effect of stress exposure was found for LymCREB1 [$F_{2,20} = 22.49$, $P < 0.0001$; Fig. 2i), with both HS and ShC upregulating its expression compared to control (ShC versus CTRL, $P < 0.0001$; HS versus CTRL, $P < 0.01$) and ShC inducing a significantly greater upregulation than HS ($P < 0.01$). Finally, a main effect of stress was found for LymDAGL ($F_{2,20} = 45.36$, $P < 0.0001$; Fig. 2j), LymMAGL ($F_{2,20} = 22.02$, $P < 0.0001$; Fig. 2k), LymNAPE-PLD ($F_{2,20} = 7.92$, $P = 0.003$; Fig. 2l) and LymFAAH ($F_{2,20} = 8.49$, $P = 0.002$; Fig. 2m). Specifically, HS exposure led to a strong upregulation of LymDAGL compared to both other groups ($P < 0.0001$), while LymMAGL was upregulated by ShC (versus CTRL, $P = 0.003$; versus HS, $P < 0.0001$) and downregulated by HS (versus CTRL, $P = 0.02$). LymFAAH was significantly upregulated by both stressors relative to controls (ShC, $P = 0.003$; HS, $P = 0.013$), whereas LymNAPE-PLD expression was downregulated in both HS and ShC groups (ShC, $P = 0.006$; HS, $P = 0.008$).

DISCUSSION

Our study highlights the stressor-specific modulation of learning and memory in *L. stagnalis*. We found that ShC causes configural learning but not the Garcia effect, whereas HS induces

the Garcia effect but not configural learning. These behavioural findings are mirrored by distinct transcriptional responses in the central nervous system, providing novel insights into how different stressors differentially engage neuromodulatory and plasticity-related gene networks (Fig. 3). This dissociation suggests that not all stressors are cognitively equivalent; rather, they establish distinct internal states or 'stress landscapes' that can enable or preclude specific forms of learning (Mendl, 1999). We frame this distinction through the conceptual lens of landscapes of fear versus landscapes of sickness: internal neurochemical milieus that prioritize vigilance and sensory integration or malaise-driven withdrawal, respectively. Specifically, the Garcia effect induced by HS probably reflects a sickness-associated motivational shift in which food stimuli are devalued due to malaise or systemic stress (Hasday & Singh, 2000; Rivi et al., 2023b). Conversely, ShC evokes a fear-like internal state, where a salient nociceptive cue is paired with a positive food stimulus (carrot), causing configural learning (Rivi, Sarti, et al., 2025) that leads to avoidance of the food cue. These results contribute to a growing body of research demonstrating that 'stress' is not a generalizable construct, but rather a diverse set of physiological states with unique cognitive outcomes. Additionally, they offer a tractable model to dissect how specific stressor modalities sculpt memory systems, providing insights with direct relevance to understanding the conserved mechanisms underlying how stress affects memory in psychiatric conditions such as PTSD, anxiety disorders and stress-induced cognitive impairments (Blom et al., 2022; Bremner, 2006; Mendl, 1999; Samuelson, 2011; Scarponi et al., 2023).

At the transcriptional level, we found that both HS and ShC significantly increased the expression levels of LymHSP70 and LymHSP40, canonical markers of cellular stress (Clark et al., 2008; Clark & Peck, 2009; Fawcett et al., 1997; Foster et al., 2015; Hamdoun et al., 2003). The higher induction of LymHSP70 elicited by the HS is consistent with systemic heat-induced protein unfolding and chaperone activation. We previously demonstrated that blocking the HS-induced upregulation of HSPs by treating snails with an HSP-blocker (i.e. the flavonoid quercetin; Rivi, Batabyal, Benatti, et al., 2025b) prevented the formation of the Garcia effect (Rivi, Batabyal, Benatti, Tascedda, et al., 2023a; Rivi, Batabyal, & Lukowiak, 2024b), and our present results further support these findings, underscoring the importance of LymHSPs as reliable indicators of organismal stress load (Desai et al., 2010).

On the other hand, ShC uniquely upregulated LymTPH and LymSERT, while downregulating Lym5HTR1 and Lym5HTR2 (Il-Han et al., 2010; Rivi et al., 2023c). This transcriptional profile suggests increased serotonin synthesis and transport capacity, coupled with postsynaptic receptor desensitization, consistent with a homeostatic feedback mechanism seen in mammals under acute or repeated stress, where elevated extracellular serotonin leads to receptor internalization or transcriptional suppression, thereby preventing overstimulation. These results closely mirror our previous studies on the transcriptional effects induced by another predator cue, crayfish effluent (CE; Rivi et al., 2023c), an odour cue, reinforcing the idea that predator-related stressors activate the serotonergic system, possibly to support associative flexibility, increase vigilance and reinforce threat-related learning. Notably, our earlier work demonstrated that exposure to predator-derived olfactory cues (CE) also induces configural learning and serotonin pathway activation in *L. stagnalis* (Dalesman et al., 2006; Il-Han et al., 2010; Rivi et al., 2023c). The fact that both CE and ShC (i.e. predator cues) produce similar behavioural and molecular effects strengthens the idea that configural learning in this species

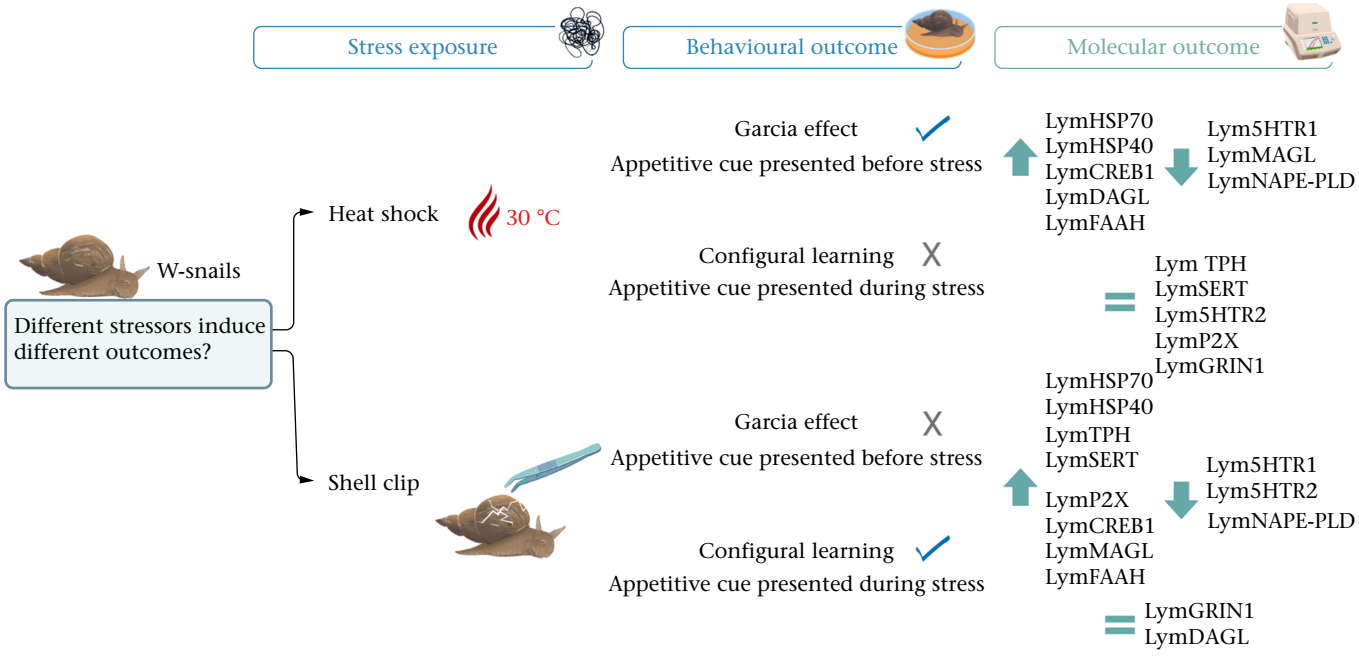

Figure 3. Summary of the behavioural and transcriptional results. Lab-inbred W-strain snails were exposed to two ecologically relevant stressors: predator attack-mimicking shell clip and acute heat shock (30 °C for 1 h), and subjected to two distinct behavioural paradigms. Garcia effect procedure: in this paradigm, snails experienced a novel appetitive taste followed by exposure to either heat shock or shell clip. If the Garcia effect (a special form of conditioned taste aversion that requires animals to become sick) is established, snails will subsequently avoid the taste paired with the aversive stimulus. Only heat shock induced a Garcia effect in W-strain snails, suggesting a selective association between thermal stress and taste aversion learning. Configural learning procedure: snails were simultaneously exposed to both the appetitive taste and one of the stressors. If configural learning occurs, subsequent exposure to the appetitive taste alone will fail to elicit feeding behaviour.
Snails forming the Garcia effect following HS exposure showed significant upregulation of LymHSP70, LymHSP40, LymCREB1, LymDAGL and LymFAAH, along with downregulation of Lym5HTR1, LymMAGL and LymNAPE-PLD. In contrast, snails forming configural learning after the ShC procedure exhibited upregulation of LymHSP70, LymHSP40, LymTPH, LymSERT, LymP2X, LymCREB1, LymMAGL and LymFAAH, together with downregulation of Lym5HTR1, Lym5HTR2 and LymNAPE-PLD.

is tightly linked to stress-induced serotonergic activation, which primes the nervous system for processing predator-associated affective states and also showcases that multiple sensory cues (olfactory or tactile) are processed similarly if they invoke the same threat association.

Intriguingly, Lym5HTR1 was also significantly downregulated by HS exposure, despite the absence of a broader serotonergic activation profile (i.e. no upregulation of TPH or SERT). This suggests a qualitatively different form of stress response. In mammals, 5-HT1A receptor downregulation is associated with sustained or overwhelming stress and is often linked to blunted affect or behavioural shutdown (Banerjee et al., 2007; Holmes et al., 2003). Thus, the selective downregulation of Lym5HTR1 caused by the HS may reflect an organism-wide suppression of excitability regulation in response to systemic malaise. In other words, unlike predator cues, which require sensory vigilance and learning, thermal stress may elicit a more generalized protective state, characterized by metabolic suppression (Rivi et al., 2022).

Moving to neuroplasticity-related targets, while both stressors upregulated LymCREB1, ShC induced a more pronounced effect (Barco et al., 2003; Finkbeiner et al., 1997). CREB1 is a central regulator of long-term plasticity and memory formation across taxa (Barco et al., 2003; Josselyn & Nguyen, 2005). Its upregulation in untrained animals suggests that stress exposure may precondition the neural circuitry for enhanced learning, potentially lowering the threshold for memory consolidation. This supports the idea that stress, in certain contexts, may prime the system for more efficient memory encoding in response to future stimuli (Kim & Kim, 2023). In contrast, LymGRIN1, which encodes an NMDA receptor subunit central to synaptic remodelling and associative learning (Ha et al., 2006), did not show significant changes under either stress condition. This may reflect the early time point of sampling (3 h post stressor), which could precede NMDA-dependent plasticity events. Alternatively, the absence of reinforcement during the stress exposures may have failed to engage the Hebbian mechanisms necessary to induce GRIN1 transcription, consistent with its activity-dependent nature (Forest et al., 2016; Teigen, 1994). Notably, LymP2X, a purinergic receptor involved in nociceptive processing and stress signalling, was significantly upregulated following ShC but not HS (Bavan et al., 2012; Burnstock & Wood, 1996). This selective regulation aligns with findings from mammalian models where P2X receptors contribute to fear learning and pain sensitization (Kuan & Shyu, 2016). Its upregulation in response to predator-associated cues suggests that LymP2X may participate in sensory gating or threat-induced arousal states that facilitate associative learning. Conversely, the downregulation of LymP2X following HS may indicate a suppression of nociceptive signalling under conditions of systemic thermal stress, potentially as a neuroprotective strategy or to reallocate cellular resources away from pain processing towards core survival pathways. Alternatively, HS may trigger an antinociceptive or inhibitory feedback mechanism, dampening excitatory signalling to prevent excitotoxicity during heat-induced cellular stress. One of the most compelling findings was the differential engagement of the endocannabinoid system (ECS) across various stressors. In both ShC and HS conditions, expression changes in ECS enzymes indicated a stressor-specific modulation of cannabinoid tone, a dynamic and tightly regulated system implicated in stress resilience, emotional regulation and memory updating (Bariani et al., 2015; Elphick, 2012; Hryhorowicz et al., 2021).

The ECS operates through two key lipid signalling molecules: anandamide (AEA) and 2-arachidonoylglycerol (2-AG), which are synthesized and degraded on demand: AEA via NAPE-PLD (synthesis) and FAAH (degradation), and 2-AG via DAGL (synthesis) and MAGL (degradation). This tight enzymatic regulation enables the ECS to fine-tune the central nervous system's function in response to changing environmental and internal demands. Importantly, while AEA is often linked to tonic modulation of stress and mood, 2-AG supports rapid, activity-dependent synaptic suppression, particularly under acute stress (Elphick, 2012; Maldonado et al., 2020). Therefore, shifts in their respective enzymatic pathways offer critical insights into how the nervous system adapts its functional state to different stress contexts (Katona & Freund, 2012). Our present study provides evidence that in *L. stagnalis*, as in mammals, ECS is not uniformly responsive to stress, but is instead tailored to the specific landscapes imposed by different stressors. In particular, we found that, consistent with Rivi, Rigillo, Batabyal, et al. (2024), HS induced a strong upregulation of LymDAGL and LymFAAH, coupled with decreased LymMAGL and LymNAPE-PLD mRNA levels. This profile probably shifts the endocannabinoid tone towards 2-AG dominance and AEA depletion, mimicking vertebrate responses to acute stress that involve ECS-mediated synaptic dampening and restoration of homeostasis (Gunduz-Cinar et al., 2013). In contrast, ShC (and CE; Rivi, Rigillo, Batabyal, et al., 2024) produced an inverse pattern: increased expression levels of MAGL and FAAH, with decreased DAGL and NAPE-PLD. This suggests an overall suppression of ECS tone, associated with increased arousal, vigilance and memory encoding. To further strengthen the validity of this model, these results are consistent with our previous studies showing the same transcriptional effects induced by CE, another predator-related cue (Rivi, Rigillo, Batabyal, et al., 2024). Such duality also mirrors results from rodent models where ECS suppression enhances fear memory consolidation and ECS potentiation is anxiolytic (Ibarra-Lecue et al., 2018; Maldonado et al., 2020). Overall, our results highlight that ECS modulation is a finely tuned response reflective of the organism's internal cognitive and affective state. The dissociation between fear-induced configural learning and sickness-induced Garcia effect provides a powerful comparative model for studying the complex interaction between stress and memory, also resonating with current psychiatric frameworks, which distinguish fear-based pathologies (e.g. phobias, hypervigilance) from anhedonic and malaise-driven states (e.g. depression, learned helplessness; Conoscenti & Fanselow, 2019; Dantzer, 2009). By identifying molecular fingerprints of these internal states in a relatively simple system such as *L. stagnalis*, we lay the groundwork for the mechanistic dissection of the stressor-induced landscape across species.

Open Questions and Future Perspectives

Although this study provides a rich framework, it also raises new questions that need to be addressed in future studies. First, the transcriptional data focused on a single 3 h post-stress timepoint in naïve (untrained) animals. Future studies should employ time course analyses during learning and post-recall phases to better map causality. Second, while gene expression profiles suggest affective-like processing (e.g. sickness or fear), these states require additional analyses, including electrophysiological recordings, to clarify how molecular changes translate into real-time neural activity and affective modulation during learning. Third, the timing of the stressors in the learning paradigms used in this study highlights the importance of understanding how each stressor may operate within a critical time window that requires further testing. For example, ShC could only invoke configural learning memory when the appetitive cue and stressor occurred together. However, when the cue and the stressor occurred over a time gap, as in the Garcia effect, it could not form the association and thus resulted in no memory phenotype for the Garcia effect. While this offers a powerful system for probing conserved stress-response

mechanisms, causal validation is essential. Future studies should employ targeted pharmacological manipulations to test whether genes such as LymCREB1, LymTPH and LymP2X are necessary and/or sufficient for stressor-specific learning and plasticity. For example, blocking LymTPH with p-chlorophenylalanine, a serotonin synthesis inhibitor, could confirm whether serotonergic signalling is essential for memory formation under either stressor. In contrast, LymP2X function could be modulated with specific P2X receptor antagonists, such as PPADS or A-317491, to explore its role in nociception and predator cue processing. Establishing whether these manipulations mimic or block known behavioural outcomes would provide direct evidence for their functional roles.

Lastly, stressors in natural environments are rarely encountered in isolation. Investigating multimodal stressor interactions, such as simultaneous exposure to predator cues and environmental toxins (e.g. heavy metals like cadmium or neonicotinoid insecticides) or under severe food deprivation, overcrowding or heat and cold shock (Fernell et al., 2021; Kagan et al., 2023), could reveal additive, synergistic or antagonistic effects on molecular pathways and behaviour. For instance, combining predator cues with a sublethal toxin dose might amplify LymHSPs or LymP2X expression or, conversely, impair memory formation due to resource competition or conflicting physiological priorities. Exploring such interactions would not only increase ecological realism but also provide insight into how organisms integrate complex stress landscapes, a crucial step for improving the translational fidelity of invertebrate models in neurobiology and ecotoxicology.

Conclusions

This study provides strong evidence that distinct stressors elicit dissociable behavioural and molecular responses in *L. stagnalis*, reinforcing its value as a powerful model for translational neuroscience. The clear dissociation between 'landscapes of fear' and 'landscapes of sickness' induced by ShC and HS, respectively, underscores that stress is not a unitary experience but rather a spectrum of internal states, each with distinct neuromodulatory and transcriptional signatures. These findings align with conserved mechanisms observed across invertebrate and vertebrate systems, bridging simple and complex models and offering valuable insight into stressor-specific memory modulation, a phenomenon highly relevant to human neuropsychiatric conditions. This work lays critical groundwork for future research involving targeted manipulations to test causal links between molecular changes and behavioural outcomes, establishing a translational framework for uncovering the shared principles that govern how stress shapes cognition. Moreover, *L. stagnalis*' well-characterized neural circuits, conserved molecular pathways and ecological relevance uniquely position it to explore how diverse environmental stressors influence cognitive and affective states.

Author Contributions

Veronica Rivi: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Anuradha Batabyal:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Jasper Hollings:** Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **Jillian Kitt:** Writing – review & editing, Formal analysis, Data curation. **Fabio Tascedda:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Johanna Maria Catharina Blom:** Writing – review & editing, Supervision, Resources. **Cristina Benatti:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Ken Lukowiak:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Data Availability

The raw behavioural and gene expression data supporting the findings of this study are provided as Supplementary Material.

Declaration of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Material

Supplementary material associated with this article is available at https://doi.org/10.1016/j.anbehav.2026.123481.

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