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Behavioral and Transcriptional Effects of Short or Prolonged Fasting on the Memory Performances of *Lymnaea stagnalis*

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Fasting · Garcia effect · Insulin · Invertebrates · Neuroplasticity

Abstract
Introduction: The Garcia effect, a solid learning paradigm, was used to investigate the molecular and behavioral effects induced by different lengths of fasting on the cognitive functions in the pond snail *Lymnaea stagnalis,* a valid model system. ***Methods:*** Three experimental groups were used: moderately hungry snails, food-deprived for 1 day (D1 snails), severely hungry snails (D5 snails), fasting for 5 days, and satiated snails with ad libitum access to food (AL snails). In the Garcia effect, a single pairing of an appetitive stimulus with a heat stressor results in a learned taste-specific negative hedonic shift. D5 snails were injected with bovine insulin and D1 snails with the insulin receptor antibody (Ab). As a control group, AL snails were injected with saline. Gene expression analyses were performed by real-time PCR in snails' central nervous system (CNS). ***Results:*** AL snails are "average learners," D1 snails are the best performers, whereas the D5 ones do not show the Garcia effect. Severely fasting snails injected with insulin 3 h before the training procedure show the Garcia effect, whereas injecting 1-day fasting snails with insulin receptor Ab blocks their ability to express memory. The differences in memory performances are associated with changes in the expression levels of selected targets involved in neuronal plasticity, energy homeostasis, and stress response. ***Discussion:*** Our results suggest that short-term fasting creates an optimal internal state in *L. stagnalis*' CNS, allowing a spike in insulin release and an upregulation of genes involved in neuroplasticity. Long-term fasting, instead, upregulates genes involved in energy homeostasis and animal survival.

Introduction

A growing interest is now emerging regarding the benefits associated with short-term fasting in both humans and animal models [1–7]. Recent reports indicate that short periods of fasting increase neuronal resilience to injuries, inflammation, and degeneration while improving brain plasticity, as well as learning and motor perfor-

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mances [8–12]. On the other hand, prolonged fasting results in severe consequences on brain functions, including memory impairment [8, 13–16].

To date, the molecular mechanisms underlying the contrasting effects of short- and long-term starvation on cognitive functions remain almost unexplored. Among the challenges of studying the effects of different lengths of fasting on learning and memory, there is the difficulty of reproducing prolonged starvation without affecting the organisms' survival or inducing defensive responses and anxious behaviors [17, 18]. Moreover, because of the complexity of mammalian brains and behaviors, the literature on the role that fasting plays in learning and memory formation lacks coherence [4, 19, 20]. Thus, in the present study, we tackled these challenges at both the behavioral and molecular levels by adopting a simpler model system, the pond snail *Lymnaea stagnalis* (Linnaeus, 1758), and a solid and highly reproducible learning paradigm, the Garcia effect [21].

L. stagnalis represents an attractive and suitable model system to study the consequences of fasting on learning and memory [22, 23]. Its central nervous system (CNS) – in fact – consists of large accessible neurons and its feeding behavior has been widely used to investigate both classical appetitive and aversive conditioning [24–27]. In a recent study, we demonstrated that *L. stagnalis* displays the Garcia effect, a "special" form of conditioning taste aversion (CTA), whose acquisition depends on animals experiencing visceral sickness (i.e., nausea) [28]. In fact, the presentation of a novel food taste (i.e., carrot slurry – C) paired with a nausea-inducing stimulus (i.e., heat-shock stressor – HS) results in a taste-specific negative hedonic shift lasting for at least 24 h, labeled as long-term memory in laboratory-reared snails fed ad libitum [21, 29]. Given that snails subjected to different lengths of fasting and then trained for CTA have different memory performances [30–38], we hypothesized that, when tested for the Garcia effect, snails experiencing short- and long-term fasting show different memory performances.

In fact, previous work by Ito's laboratory demonstrated that animals fasting for 1 day before training became better learners than the ad libitum-fed ones, whereas 5 days of fasting impaired the expression of the formed memory [35, 39]. The molecular mechanism behind these behavioral effects has not been fully understood. What is known is that severely fasting snails have an increased content of serotonin (5-hydroxytryptamine: 5HT) and lower levels of insulin compared to the 1-day fasting ones [40].

To further elucidate the role played by the serotoninergic and the insulinergic pathways in the CNS of *L. stagnalis* subjected to different fasting regimens, we then assessed transcriptional changes of the following targets involved in the regulation of energy homeostasis and/or in neuroplasticity in our experimental conditions: the molluscan insulin peptide II (*Lym*MIPII) and its receptor (*Lym*MIPR), the glutamate ionotropic receptor NMDA type subunit 1 (*Lym*GRIN1), the transcription factor cAMP response element-binding protein 1 (*Lym*CREB1), neuropeptide Y (*Lym*NPY), the tumor suppressor gene multiple endocrine neoplasia type 1 (*Lym*MEN1), tryptophan hydroxylase (*Lym*TPH), the transporter of 5HT, (*Lym*SERT), and heat shock protein 70 (*Lym*HSP70). In particular, we investigated whether short- and long-term fasting differently affects the transcriptional regulation of the insulin system, *Lym*MIPII, and *Lym*MIPR, in the CNS of *Lymnaea*. Our working hypothesis is that 1 day of fasting along with the activation of the insulin pathway induces the synthesis of new proteins involved in promoting the effects of short-term fasting on memory consolidation and recall, like *Lym*GRIN1 and *Lym*CREB1 [41, 42].

Along with their key role in synaptic plasticity [43], studies from rodents showed an upregulation of these targets induced by prolonged starvation [44–46]. CREB is phosphorylated in response to a wide variety of signals and, working in concert with cytoplasmic co-activators, regulates the effects of fasting and feeding signals in insulin-sensitive tissues [47]. Furthermore, glutamatergic synaptic transmission and its modulation by NMDA receptors play a central role in determining the cellular and behavioral response to fasting [48].

Also, NPY, a potent orexigenic peptide involved in modulating hunger and energy balance, has been proposed to mediate neuroplasticity in animal models. Its expression is thought to be regulated by peripheral factors that signal energy status, including insulin, as well as by targets that promote animal survival and neuroplasticity [49, 50].

We also analyzed *Lym*MEN1 which plays an essential role in synapse formation in *L. stagnalis* as well as in vertebrates and mammals, acting as a co-activator of molecular cascades involved in glucose metabolism [51, 52]. Given that insulin promoter activity and secretion are inhibited in mammals by menin, the product of MEN1, we hypothesized that this target and its role are conserved in *L. stagnalis* and mediate the insulin pathway in severely fasting snails [53].

As 5HT regulates both feeding behavior and food satiety in *L. stagnalis* and mediates stress-induced arousal and vigilance behaviors [37, 54, 55], we thought it important to study the differences in the expression levels of these targets in both untrained and trained snails subjected to the different fasting regimens. Therefore, we included two molecular targets of the serotoninergic system: the rate-limiting enzyme for the synthesis of 5HT, *Lym*TPH, and *Lym*SERT.

The last target we investigated has been *Lym*HSP70, as we previously demonstrated that its upregulation induced by the HS plays a necessary role in long-term memory formation following the Garcia effect procedure [21]. In brief, the hypothesis underlying our study is that moderately fasting snails are "smarter" than the severely food-deprived ones and that the insulin pathway plays a key role in modulating both energy homeostasis and learning and memory formation. To test our overall hypothesis, we performed behavioral tests and gene expression analyses and asked the following specific questions:

- What are the transcriptional effects induced by the different fasting regimens and/or the HS procedure in D1, D5, and AL snails?
- Is there a relationship between the fasting regimens and learning induced by the Garcia effect? If so, what are the transcriptional effects induced by the training procedure in ad libitum-fed snails and those food deprived for 1 or 5 days?
- Does insulin modulate fasting and/or memory induced by the Garcia effect? What happens at the behavioral and molecular level if we inject 5-day fasting snails with insulin and 1-day fasting snails with the antibody (Ab) against the insulin receptor?

Given the variety of these questions, the study was divided into 3 experiments: experiment 1 focussed on the transcriptional effects induced by the HS, the nausea-inducing stimulus used in the Garcia effect procedure, in untrained snails subjected to different fasting regimens. In experiment 2, we assessed the learning and memory performances and the transcriptional effects on the abovementioned targets between satiated and moderately and severely fasting snails subjected to the Garcia effect procedure. Finally, in experiment 3, we treated severely and moderately fasting snails with either insulin or insulin receptor Ab to investigate the role of the insulin system in mediating the memory performances of these animals.

Materials and Methods

Study Animal

The populations of *L. stagnalis* used in the current study were established at the University of Modena and Reggio Emilia (Italy) and were kindly provided by the Vrije Universiteit Amsterdam. Adult animals (6–8 months old) having shell lengths of approximately 20–25 mm were used in these experiments. Snails were kept in 12 L tanks supplied with well-aerated water and maintained in standard laboratory conditions (21–23°C, 12:12 light-dark cycle). Three times a week, animals were fed pesticide-free romaine lettuce and goldfish pellets. The snails were given a 3 min acclimation period to the carrot slurry (i.e., the novel appetitive stimulus) in each behavioral session.

Experiment 1: Transcriptional Effects of Different Fasting Regimens in Snails Exposed or Not to a Heat-Shock Stressor (HS)

Six cohorts of snails were used in this experiment ($n = 7$ for each cohort):

1. Snails that had been given ad libitum access to food and did not experience the HS (AL);
2. Snails that had been fasting for 1 day and did not experience the HS (D1);
3. Snails that had been fasting for 5 days and did not experience the HS (D5);
4. Snails that had been given ad libitum access to food when were exposed to the HS (AL + HS);
5. Snails that had been fasting for 1 day before being exposed to the HS (D1+HS);
6. Snails that had been fasting for 5 days and then experienced the HS (D5+HS).

All fasting regimens have been successfully adopted in previous studies [34, 40], which led us to define snails food deprived for 1 day as "moderately hungry," whereas those fasting for 5 days as "severely hungry snails." For the HS procedure, snails were placed for 1 h in a 1 L beaker filled with 500 mL of water from their aquaria which was maintained in a water bath at 30°C. Unexposed control snails were moved from their home aquarium and were placed for 1 h in a beaker containing clean, room-temperature (20–22°C) water from the snails' aquaria. Three hours later, HS-exposed snails and their unexposed controls were euthanized in ice for 10 min, and the central ring ganglia were dissected (buccal ganglia were excluded) and stored at −80°C prior to analysis.

Experiment 2: Impact of Different Fasting Regimens on the Behavioral and Molecular Outcomes of a Garcia Effect Procedure

To compare the memory performances among AL, D1, and D5 snails for the Garcia effect procedure, we divided snails ($n = 18$) into 3 cohorts ($n = 6$ each) and subjected them to different fasting regimens (i.e., AL, D1, and D5). Then, we examined whether their feeding response to a carrot slurry (C), a new appetitive stimulus, which elicits a robust rasping behavior, before (C-pre) and 3 h after (C-post 3 h) HS exposure was significantly reduced, resulting in a Garcia effect. Thus, AL, D1, and D5 snails were exposed to C [56, 57], 1 h before and 3 h after the exposure to the HS as previously described.

The carrot slurry was prepared by blending two fresh medium carrots in 500 mL of water from snails' aquaria; the solution was sequent strained to obtain a C without any observable pieces of carrot. Importantly, the snails used in these experiments never expe-

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

Gene bank accession	Target	Product length, bp	Type sequence
X59302.1	*Lym*MIPII	186 bp (152–338)	5′ – CCAATCATCTTGCAGTTTA – 3′ 5′ – GTCGTCCAGATCTGTTTCT – 3′
X84994.1	*Lym*MIPR	78 bp (4,137–4,215)	5′ – ATTGGAGACTTTGGTATGAC – 3′ 5′ – ACACTCCATCTTTGAGAGAC – 3′
AJ238276.1	*Lym*NPY	188 bp (432–620)	5′ – ACTCTTGGTGTCACTGCTCG – 3′ 5′ – CTTGCGCCGTTTCTCTTTCC – 3′
AY571900.1	*Lym*GRIN1	140 bp (831–917)	5′ – AGAGGATGCATCTACAATTT – 3′ 5′ – CCATTTACTAGGTGAACTCC – 3′
AB041522.1	*Lym*CREB1	180 bp (49–229)	5′ – GTCAGCAGGGAATGGTCCTG – 3′ 5′ – AACCGCAGCAACCCTAACAA – 3′
AF395538	*Lym*MEN1	160 bp (551–711)	5′ – TGTATTGGGTTGTATTGAAA – 3′ 5′ – TAAGATCCACAGAACCTTTT – 3′
AF129815.1	*Lym*TPH	179 bp (238–417)	5′ – AGGATACAGTCTACCGACAG – 3′ 5′ – TGAGTTCACGGAAAACTATT – 3′
FX185022	*Lym*SERT	177 bp (726–903)	5′ – ATACCGTACCTTGTCATGTT – 3′ 5′ – TGTTGTAGTACCAGGAGACA – 3′
DQ206432.1	*Lym*HSP70	199 bp (134–333)	5′ – AGGCAGAGATTGGCAGGAT – 3′ 5′ – CCATTTCATTGTGTCGTTGC – 3′
X15542.1	Snail, beta-tubulin, *Lym*TUB	100 bp (92–192)	5′ – GAAATAGCACCGCCATCC – 3′ 5′ – CGCCTCTGTGAACTCCATCT – 3′
DQ278441.1	*Lym*EF1α	150 bp (7–157)	5′ – GTGTAAGCAGCCCTCGAACT – 3′ 5′ – TTCGCTCATCAATACCACCA – 3′

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

rienced this carrot taste, as we previously demonstrated that food novelty is a requisite for the Garcia effect to be formed [21]. Animals were placed into a 14 cm diameter Petri dish with enough C for the snails to be partially submerged. To observe the rasping behavior, the Petri dishes were put on a clear Plexiglas stand raised 10 cm above a mirror. Snails were first acclimated for 3 min after which the experimental session began. The number of rasps was counted for 2 min in the C-pre condition. One hour later, snails were exposed to the HS for 1 h. The rasping behavior was again determined for 2 min in C 3 h after the HS (C-post 3 h). During the 3 h interval between HS exposure and C re-exposure, snails were kept in their home tanks without access to food. The behavioral experiments were performed in the morning because learning scores are better in this period than at other times during the day [58].

To study the difference between the response of AL, D1, and D5 snails to C-pre and 3 h post-HS, snails were marked according to the following scheme: "A" for a greater than 50% reduction; "B" for a 35–49.99% reduction; "C" for a 20–34.99% decrease; and an "F" when the decrease was less than 20%, as previously described [21, 59]. Once we tested the memory formation for the Garcia effect (C-post 3h), trained AL, D1, and D5 snails were euthanized in ice for 10 min, and the central ring ganglia were dissected (buccal ganglia were excluded) and stored at −80°C prior to analysis.

Experiment 3: Behavioral and Transcriptional Effects Induced by Insulin or Insulin Receptor Ab in Moderately and Severely Fasting Snails

Severely food-deprived snails (i.e., D5, $n = 8$) were injected with 40 µL bovine insulin (Merck Millipore) mixed in *Lymnaea* saline (NaCl 50 mM, KCl 1.6 mM, $MgCl_2$ 2.0 mM, $CaCl_2$ 3.5 mM, and HEPES 10 mM – pH 7.9) as previously described [33]. The estimated final concentration of insulin was 100 nM as the calculated volume of hemolymph in a snail with a 20-mm shell length is ~400 µL.

To block the action of insulin, D1 snails ($n = 8$) were injected with mouse monoclonal Ab to the a-subunit of the human insulin receptor (MAB1137Z Merck Millipore), which recognizes and binds the extracellular domain of the insulin receptor, acting as an antagonist. As previously reported, the estimated final concentration of the insulin receptor Ab in the body was 17.5 nM [33]. AL snails (control group) were injected with *Lymnaea* saline ($n = 8$). Pilot studies demonstrated that injection with *Lymnaea* saline did not affect the expression levels of the targets evaluated (data not shown). Three hours after the treatment, snails were subjected to the Garcia effect procedure (i.e., C-HS) as previously described. Immediately after the C 3 h re-exposure, snails were euthanized in ice for 10 min; the central ring ganglia were dissected (buccal ganglia were excluded) and stored at −80°C prior to analysis.

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

Total RNA extraction and DNAse treatment were performed on snails from experiments 1, 2, and 3 using GenElute™ Total RNA Miniprep Kit and DNASE70-On-Column DNase I Digestion Set (Merck Millipore) as previously described [60, 61]. A single central ring ganglion was used for total RNA extraction. 6–8 samples were analyzed for each group. A 200 ng sample of total RNA was reverse transcribed with a High-Capacity cDNA Reverse

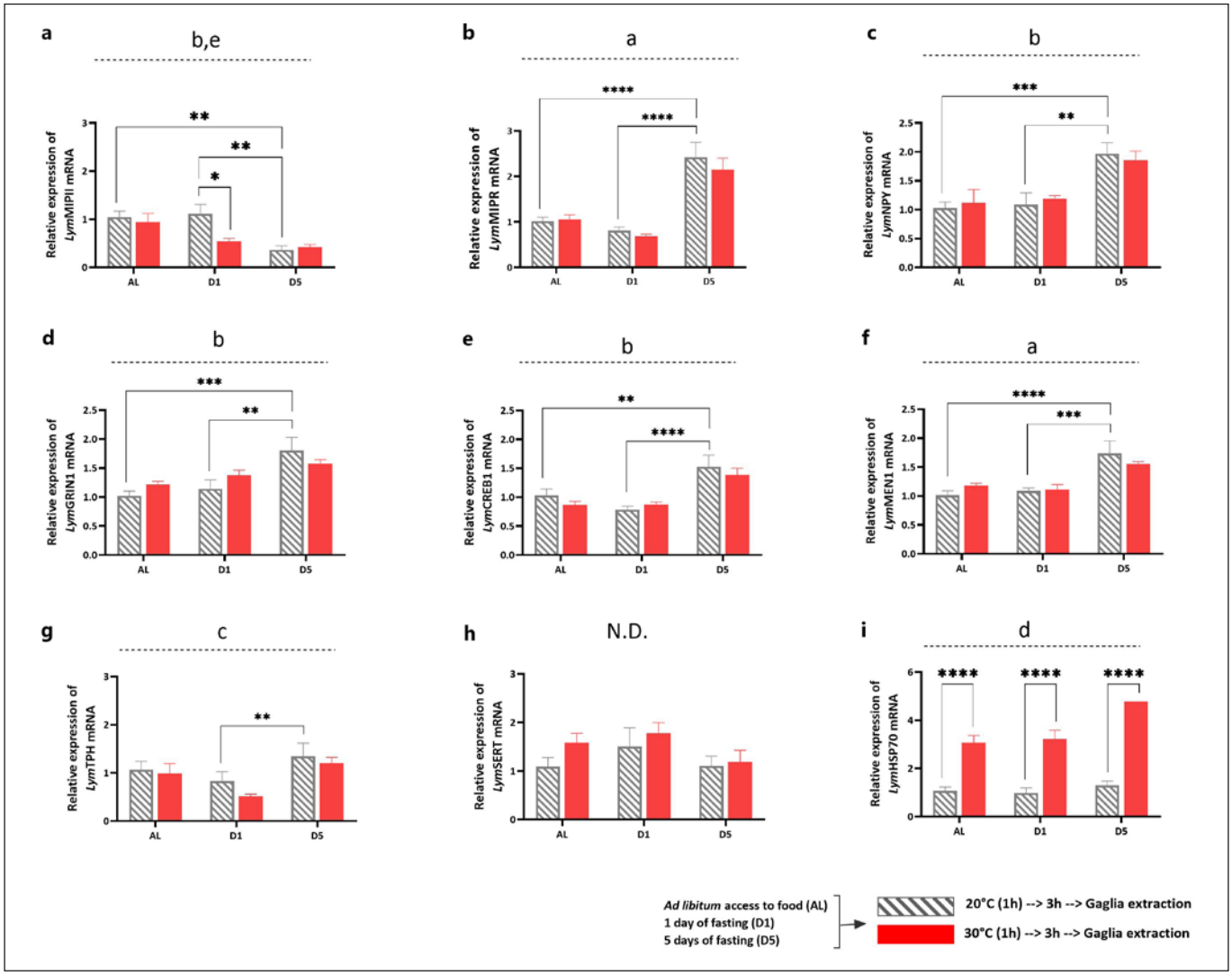
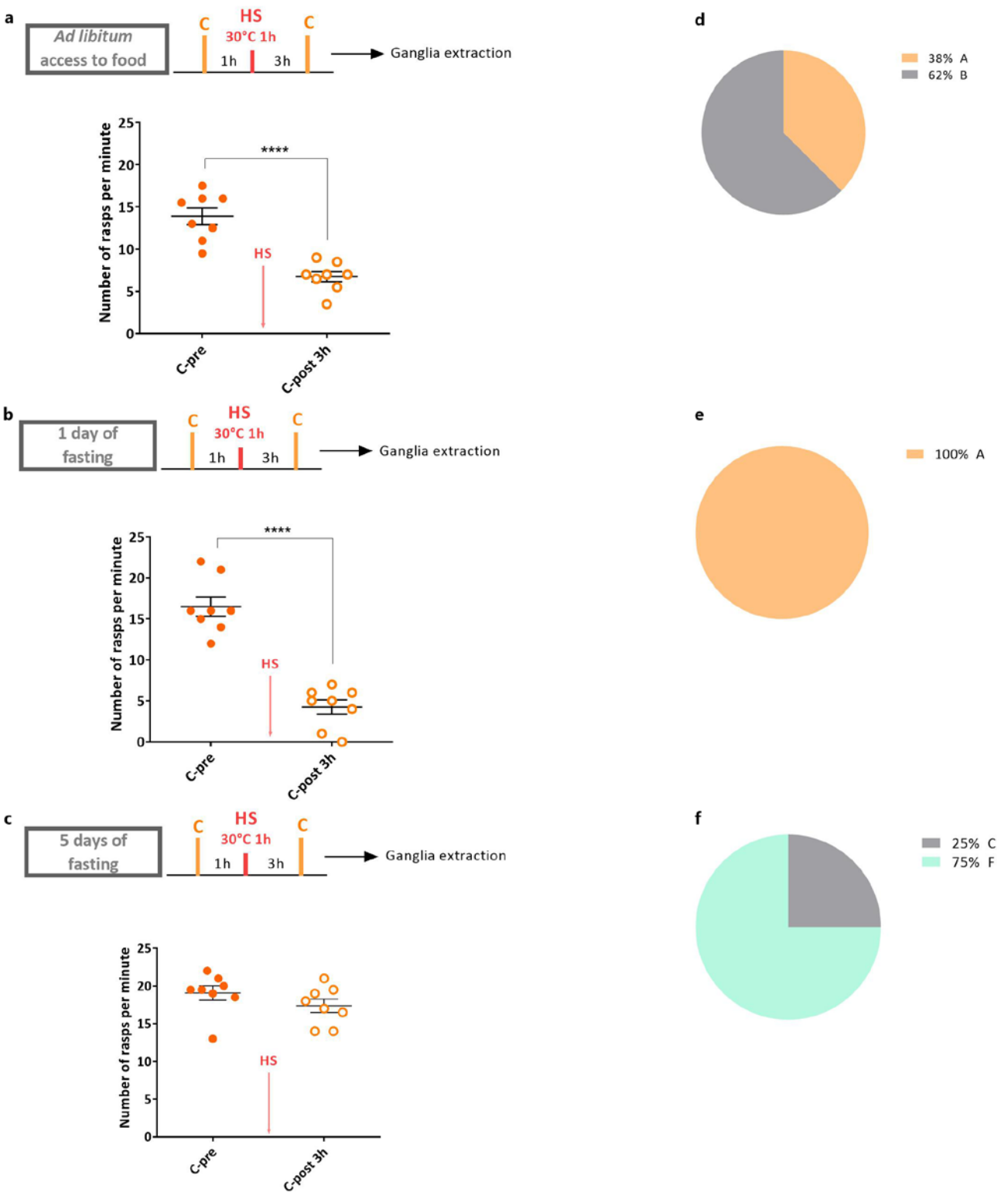


Fig. 1. Transcriptional effects of different fasting regimens in snails exposed or not to a heat-shock stressor. The expression of *Lym*MIPII (**a**), *Lym*MIPR (**b**), *Lym*NPY (**c**), *Lym*GRIN1 (**d**), *Lym*CREB1 (**e**), *Lym*MEN1 (**f**), *Lym*TPH (**g**), *Lym*SERT (**h**), and *Lym*HSP70 (**i**) has been measured in the central ring ganglia of (1) ad libitum (AL) not experiencing the HS (NO HS); (2) ad libitum subjected to the HS procedure; (3) fasting for 1 day (D1) NO HS; (4) fasting for 1 day before the HS presentation; (5) fasting for 5 days (D5) NO HS, (6) fasting for 5 days that experienced the HS procedure. The mRNA levels were analyzed by real-time PCR. Data are represented as means ± SEM and were analyzed with two-way ANOVA followed by Tukey post hoc analyses. a: $p < 0.001$, b: $p < 0.001$, c: $p < 0.05$ main effect of the fasting regimens; d: $p < 0.0001$ main effect of the heat-shock procedure; e: $p < 0.5$ main interaction between the two terms. Post hoc $^{****}p < 0.0001$, $^{***}p < 0.001$, $^{**}p < 0.01$. The diagonal bars refer to AL, D1, and D5 snails unexposed to the HS procedure, while the full ones represent AL, D1, and D5 snails exposed to the HS. $n = 7$ for each group.

Fig. 2. Impact of different fasting regimens on the behavioral and molecular outcomes of a Garcia effect procedure. The timeline of each experiment is presented above the data. The number of rasps in C (C-pre – closed circles) performed by ad libitum fed, (AL) (**a**), 1-day fasting (D1) (**b**), and 5-day fasting (**c**) snails were counted for 2 min. One hour later, these snails experienced the heat-shock stressor (HS) for 1 h and 3 h later were re-exposed to C (C-post 3 h – open circles) for 2 min. The C-HS procedure resulted in a Garcia effect in AL and D1 snails, whereas the feeding response to C was not suppressed in D5 snails. Data were analyzed using a paired *t* test. The solid line is the mean, and the error bars are the SEM. Pie charts (**d–f**). The same data have been replotted using a marks scheme, which allowed us to grade snails based on their performances. $^{****}p < 0.0001$, $^{**}p < 0.001$.

(For figure see next page.)



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Transcription Kit (ThermoFisher). Real-time quantitative PCR was carried out on 20 ng mRNA using a Bio-Rad® CFX Connect™ Real-Time PCR Detection System with SYBR Green Master Mix (Bio-Rad). The cycling parameters were 95°C for 2 min and 95°C for 10 s, 60°C for 30 s for 40 cycles, and a dissociation curve analysis followed the amplification. Each sample was run in simplicate (i.e., was analyzed once).

Cycle threshold (Ct) values were determined by CFX Maestro™ Software (Bio-Rad). Specific forward and reverse primers were designed with NCBI Primer-BLAST software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and synthesized by Merck KGaA (Darmstadt, Germany). Primers, with a length of 19–23 nucleotides, a melting temperature between 58 and 62°C, and a GC content between 40% and 60%, generating an amplicon between 75 and 200 bp were used at the final concentration of 300 nM (Table 1). Dissociation curve analysis and electrophoresis in 2% agarose gel excluded the presence of different PCR products or primer dimers. The mRNA levels of each target were normalized to two reference genes, elongation factor 1α, and tubulin. The stability of mRNA expression of these endogenous controls was assessed using Normfinder®, considering intra- and intergroup variations [62]. The mean between the two endogenous genes was found to be the most stable gene across groups and was used for gene normalization. The endogenous control mRNA levels were not affected by any procedure (one-way analysis of variance [ANOVA]) and the amplification efficiency of the target genes and endogenous control genes was approximately equal. For quantitative evaluation of changes, the comparative $2^{-\Delta\Delta Ct}$ method was performed using as a calibrator the average levels of expression of control animals (i.e., AL-fed snails for experiments 1 and 2 and AL-fed snails injected with *Lymnaea* saline for experiment 3).

Statistical Analysis

Behavioral data were analyzed using a paired Student's *t* test comparing the number of rasps before and after the HS exposure (C-pre vs. C-post 3 h). For gene expression analyses, first, we analyzed our data for normality assumption using Kolmogorov-Smirnov one-sample test for normality (K-S distance and P): all targets displayed a normal distribution. A two-way ANOVA for the main effects of fasting, HS, or interaction between the two factors was performed. For post hoc analyses, where necessary, we performed planned pairwise comparisons to follow up on significant interactions or main effects using one-way ANOVA followed by Tukey's post hoc tests. One-way ANOVA was used to compare the expression levels of each target in AL, D1, and D5 snails trained for the Garcia effect procedure and sacrificed immediately after the memory test (3 post-3 h). Significant changes were determined by Tukey's post hoc test.

All tests were defined as significant at $p < 0.05$. Data were presented as mean ± standard error (SEM). All statistical analyses were performed using 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

Experiment 1: Transcriptional Effects of Different Fasting Regimens in Snails Exposed or Not to a Heat-Shock Stressor

The aim of experiment 1 was to answer the following question: what are the transcriptional effects induced by different fasting regimens and/or the HS procedure in AL, D1, and D5 snails? Thus, we investigated whether (1) the different fasting regimens (i.e., short or prolonged starvation), (2) the HS used as the sickness-inducing stimulus in the Garcia effect procedure, and (3) the interaction between 1 and 2 would affect the expression levels of selected targets involved in energy homeostasis, response to stressors, and memory formation in the CNS of *L. stagnalis*.

First, we investigated the effects of different lengths of fasting and/or those induced by the HS on the expression levels of specific targets involved in modulating energy balance, response to stressors, and learning and memory. A main effect of the fasting regimen ($F_{2,36} = 11$, $p = 0.0001$) and an interaction between the fasting regimen and HS ($F_{2,36} = 3.28$, $p = 0.049$) was observed for *Lym*MIPII mRNA levels (Fig. 1a). Tukey's multiple comparisons test showed significant downregulation of the expression levels of *Lym*MIPII in severely hungry snails (D5) compared to the fed (AL) and moderately hungry ones (D1) ($p = 0.002$ and $p = 0.0006$, respectively). Moreover, the HS procedure induced a significant reduction in the expression levels of *Lym*MIPII in D1 snails compared to their HS-unexposed counterparts (D1 vs. D1 + HS: $p = 0.009$). We also observed a main effect of the fasting regimen regarding the expression levels of *Lym*MIPR ($F_{2,36} = 41.41$, $p < 0.0001$) (Fig. 1b). Post hoc analysis showed that 5 days of fasting significantly upregulated *Lym*MIPR mRNA levels with respect to the other fasting regimens (D5 vs. AL: $p < 0.0001$ and D5 vs. D1: $p < 0.0001$).

The two-way ANOVA also evidenced a main effect of fasting on the expression levels of *Lym*NPY ($F_{2,36} = 15.57$, $p < 0.0001$) (Fig. 1c), *Lym*GRIN1 ($F_{2,36} = 10.89$, $p < 0.0001$) (Fig. 1d), and *Lym*CREB1 ($F_{2,36} = 18.03$, $p < 0.0001$) (Fig. 1e). Post hoc analysis showed a significant upregulation of these targets in severely hungry D5 snails compared to their AL (*Lym*NPY: $p < 0.0001$; *Lym*GRIN1: $p = 0.0002$; *Lym*CREB1: $p = 0.0003$) and D1 counterparts (*Lym*NPY: $p = 0.0001$; *Lym*GRIN1: $p = 0.0051$; *Lym*CREB1: $p < 0.0001$). Similarly, severe fasting caused a significant upregulation of *Lym*MEN1 ($F_{2,36} = 18.34$, $p < 0.0001$) (Fig. 1f) with respect to the other fasting regimens ($p < 0.0001$ for both).

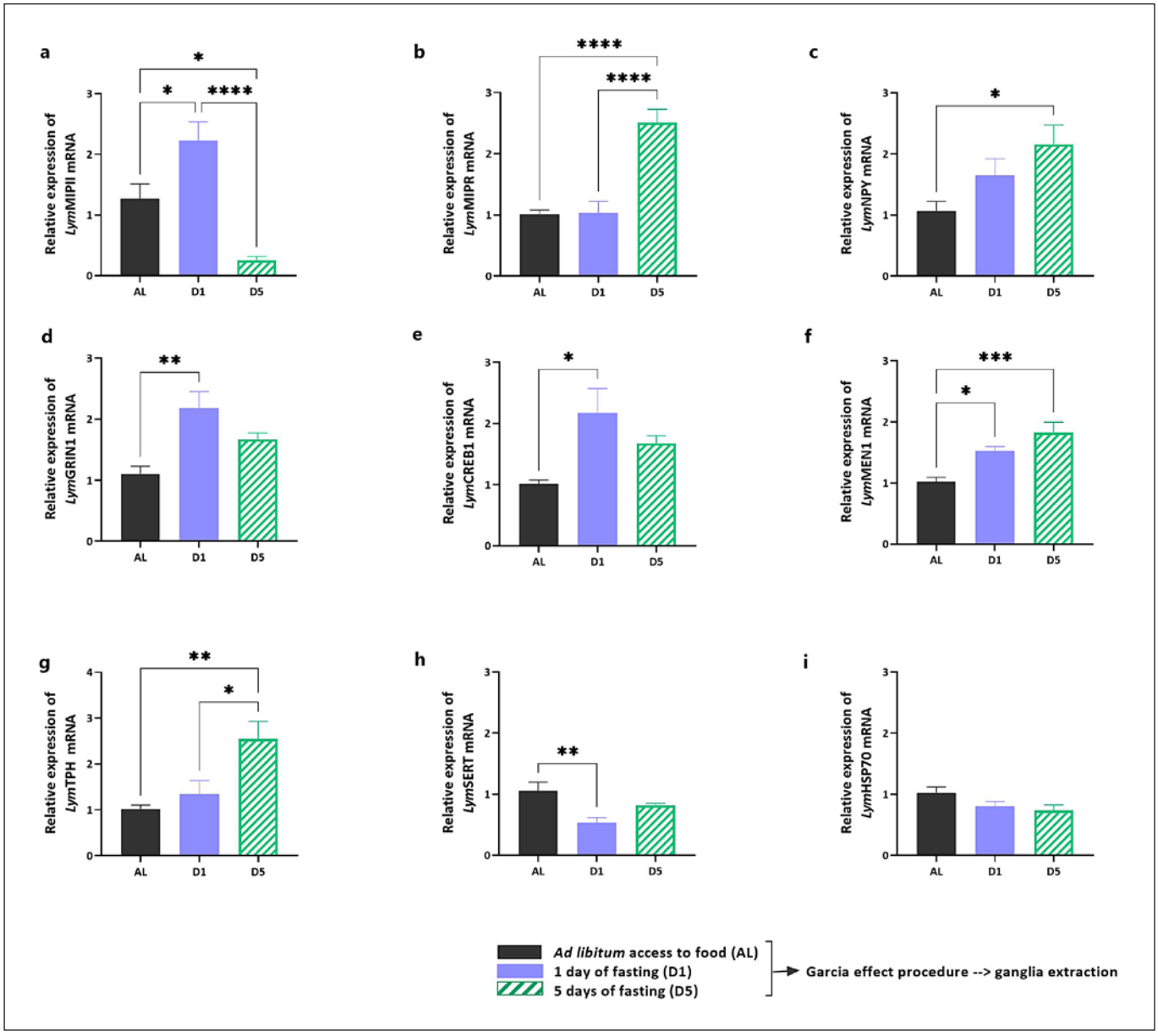

Fig. 3. Transcriptional effects induced by the Garcia effect procedure in snails subjected to different fasting regimens. The expression levels of *Lym*MIPII (**a**) and *Lym*MIPR (**b**), *Lym*NPY (**c**), *Lym*GRIN1 (**d**), *Lym*CREB1 (**e**), *Lym*MEN1 (**f**), *Lym*TPH (**g**), *Lym*SERT (**h**), and *Lym*HSP70 (**i**) have been measured in the central ring ganglia of snails fed ad libitum, fasting for 1 day (D1), and fasting for 5 days (D5) before being trained for the Garcia effect (n = 6 each group). Data are represented as means ± SEM and were analyzed with one-way ANOVA followed by Tukey post hoc analyses. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. Solid bars (AL and D1 snails), Garcia effect induced; Diagonal bars (D5 snails), Garcia effect not formed.

As for the serotonin system, prolonged fasting upregulated the expression levels of *Lym*TPH in comparison to D1 short-term starvation ($F_{2,35} = 5.18$, $p < 0.011$; D1 vs. D5 snails: $p = 0.003$) (Fig. 1g), whereas no main effects regarding the expression levels of *Lym*SERT were found (Fig. 1h). No main effects of the HS procedure were observed for any of the targets evaluated with the sole exception of the *Lym*HSP70 ($F_{2,36} = 184.2$, $p < 0.0001$) (Fig. 1h), whose mRNA levels were upregulated in all the snails exposed to the thermal stressor with respect to their unexposed matching fasting regimen counterparts ($p < 0.0001$ for all).

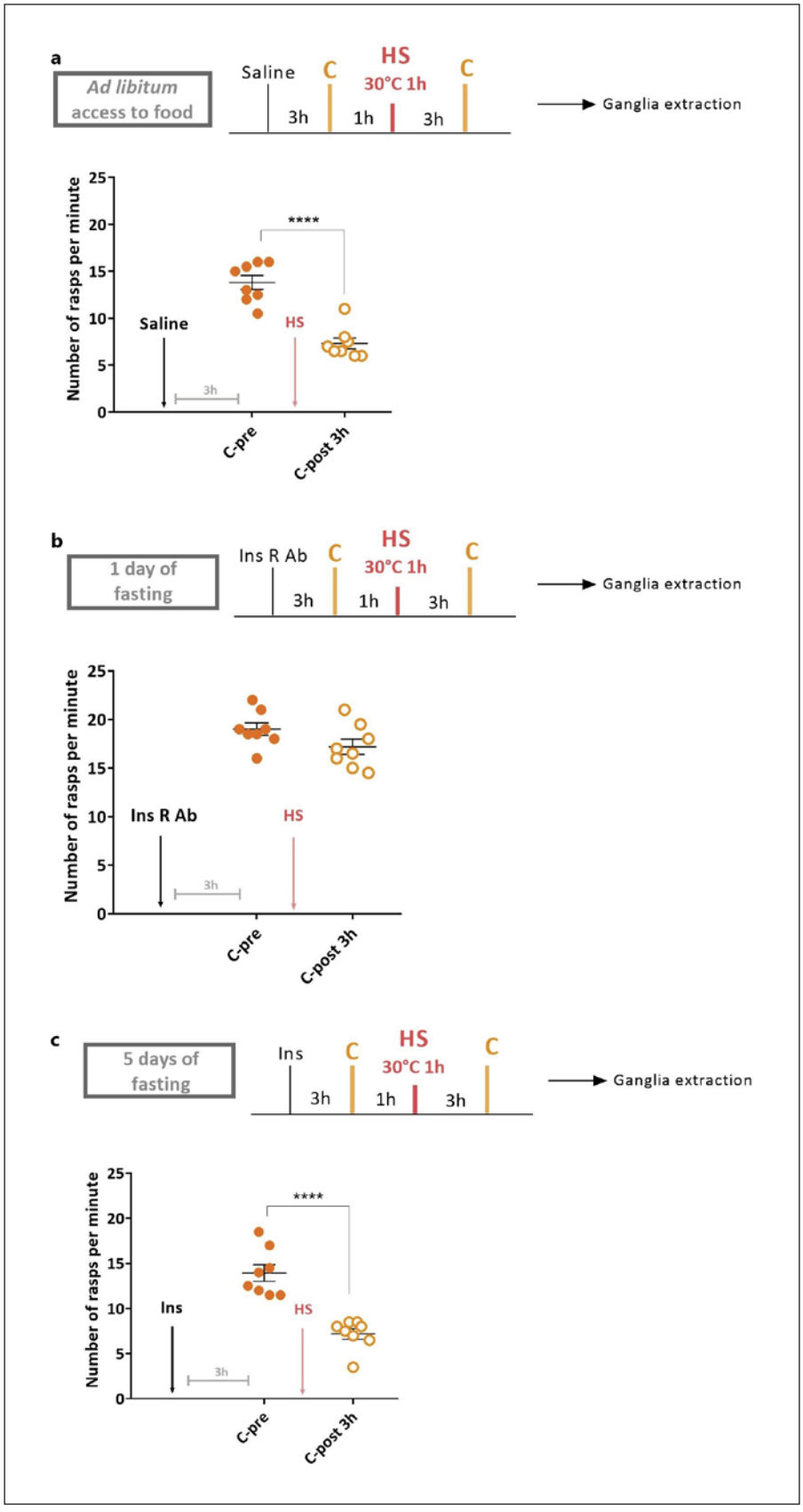

Fig. 4. Behavioral effects induced by insulin or insulin receptor Ab in moderately and severely fasting snails. The timeline of each experiment is presented above the data. 3 cohorts of snails (n = 8 for each cohort) were employed. (1) Ad libitum injected with saline (AL Sal), (2) fasting for 1 day and injected with insulin receptor Ab (D1 Ins R Ab), (3) fasting for 5 days and then injected with insulin (D5 Ins). The number of rasps elicited by C was counted for 2 min (C-pre – closed circles). Snails subsequently experienced the heat-shock stressor (HS) for 1 h and 3 h later have been re-exposed to C (C-post 3 h – open circles) for 2 min. The C-HS procedure resulted in a Garcia effect in AL snails injected with saline (**a**) D5 snails injected with insulin (**c**), whereas the feeding response to C was not suppressed in D1 snails injected with insulin receptor Ab (**b**). Data were analyzed using a paired t test. The solid line is the mean, and the error bars are the SEM. ****p < 0.0001.

Experiment 2: Impact of Different Fasting Regimens on the Behavioral and Molecular Outcomes of a Garcia Effect Procedure

This experiment investigated the possible relationship between the fasting regimens and memory performances and eventually studied the transcriptional effects induced by the training procedure in AL, D1, and D5 snails. We hypothesized that consistent with previous studies from mammals and *Lymnaea*, prolonged fasting would alter the snails' abilities to learn and form memory, whereas 1 day of fasting would result in better memory performances. Thus, we compared the learning abilities and memory performances of AL, D1, and D5 snails under the Garcia effect behavioral procedure.

First, the capacity of AL, D1, and D5 snails to mount a Garcia-like effect of their feeding response to C before (C-pre) and 3 h after (C-post 3 h) the HS exposure was measured. A significant reduction of the rasping behavior 3 h after the HS procedure was considered a Garcia effect. A Garcia effect was observed in both AL (Fig. 2a) and D1 snails (Fig. 2b): snails exposed to C 3 h after the HS (C-post 3 h) displayed significantly a suppressed feeding response (AL: $t = 12.4$, df = 5; $p < 0.0001$; D1: $t = 15.30$, df = 5; $p < 0.0001$). Thus, following the HS, C no longer acted as a strong appetitive stimulus. On the other hand, in D5 snails the number of rasps at C 3 h post-HS was not different from that recorded in C-pre (Fig. 2c). In other words, the homeostatic drive to eat prevailed over the negative hedonic shift to C brought about by the pairing of C with the HS.

To compare the memory performances between the 3 cohorts of snails more easily, we employed the marks scheme as reported in "Methods." While 38% of AL snails were graded "A" and 62% of subjects were marked B (Fig. 2d), 100% of D1 snails were marked "A" (Fig. 2e). Moreover, 75% of D5 snails were marked F and 25% C (Fig. 2f). Thus, this grading system revealed that the "best" performers were D1 snails, whereas D5 snails were the "worst" performers.

Having demonstrated that different fasting regimens are associated with different memory performances, we sacrificed those animals immediately after the memory test (i.e., C-post 3 h) to assess mRNA expression levels of *Lym*MIPII, *Lym*MIPR, *Lym*NPY, *Lym*GRIN1, *Lym*CREB1, *Lym*MEN1, *Lym*TPH, *Lym*SERT, and *Lym*HSP70 in their CNS (Fig. 3). A one-way ANOVA followed by Tukey's post hoc test showed a main effect of the behavioral procedure on the expression levels of *Lym*MIPII ($F_{2,17} = 28.87$, $p < 0.0001$) (Fig. 3a) and *Lym*MIPR ($F_{2,17} = 13.12$, $p = 0.001$) (Fig. 3b). We found an upregulation of *Lym*MIPII in D1 snails compared to AL and D5 snails ($p = 0.04$; $p < 0.0001$, respectively). As observed in untrained animals, the mRNA levels of *Lym*MIPII were still significantly lower in severely fasting snails in comparison with the satiated ones (AL vs. D5: $p = 0.027$). The expression levels of *Lym*MIPR were upregulated in D5 snails with respect to their AL and D1 counterparts even following the Garcia effect procedure ($p < 0.0001$ for all), while no differences were found between AL and D1 snails.

Moreover, one-way ANOVA showed a main effect of the behavioral procedure on the expression levels of *Lym*NPY ($F_{2,17} = 5.46$, $p = 0.017$) (Fig. 3c), *Lym*GRIN1 ($F_{2,17} = 6.67$, $p = 0.002$) (Fig. 3d), *Lym*CREB1 ($F_{2,17} = 10.06$, $p = 0.002$) (Fig. 3e), and *Lym*MEN1 ($F_{2,17} = 12.97$, $p = 0.001$) (Fig. 3f). In particular, post hoc analyses revealed that *Lym*GRIN1, *Lym*CREB1, and *Lym*MEN1 were upregulated in D1 snails compared to their AL counterparts (*Lym*GRIN1: $p = 0.005$; *Lym*CREB1: $p = 0.01$; *Lym*MEN1: $p = 0.02$). As for the expression levels of *Lym*NPY, significant upregulation was observed in D5 snails compared to the AL ones ($p = 0.03$).

Our behavioral paradigm also induced a main effect on the expression levels of *Lym*TPH ($F_{2,17} = 16.23$, $p < 0.0001$) (Fig. 3g) and *Lym*SERT ($F_{2,17} = 7.39$, $p = 0.006$) (Fig. 3h). Post hoc analyses showed a significant upregulation of *Lym*TPH and *Lym*MEN in severely fasting D5 snails compared to their AL and D1 counterparts ($p =$ 0,005 and $p = 0.03$, respectively) following the training procedure. On the other hand, *Lym*SERT was significantly downregulated in D1 snails compared to the AL-fed ones ($p = 0.004$). No differences in the expression levels of *Lym*HSP70 were observed after training among the three cohorts of snails ($F_{2,17} = 2.9$, $p = 0.08$) (Fig. 3i).

Experiment 3: Behavioral and Transcriptional Effects Induced by Insulin or Insulin Receptor Ab in Moderately and Severely Fasting Snails

Based on data from experiments 1 and 2, we further explored the role of insulin in mediating memory performances. We hypothesized that the "right amount of fasting" would result in better memory performances. Thus, to test our hypothesis in experiment 3 we investigated whether injecting D1 snails with insulin receptor Ab (i.e., D1 Ins R Ab) would block their ability to show the Garcia effect and similarly whether D5 snails treated with bovine insulin (i.e., D5 Ins) would display the memory phenotype. We found that injecting AL-fed snails with saline still resulted in a Garcia-like effect ($t = 9.11$, df = 7, $p <$ 0.0001 – Fig. 4a), whereas D1 snails – previously classified as the "best performers" in experiment 2 (Fig. 2b) – when

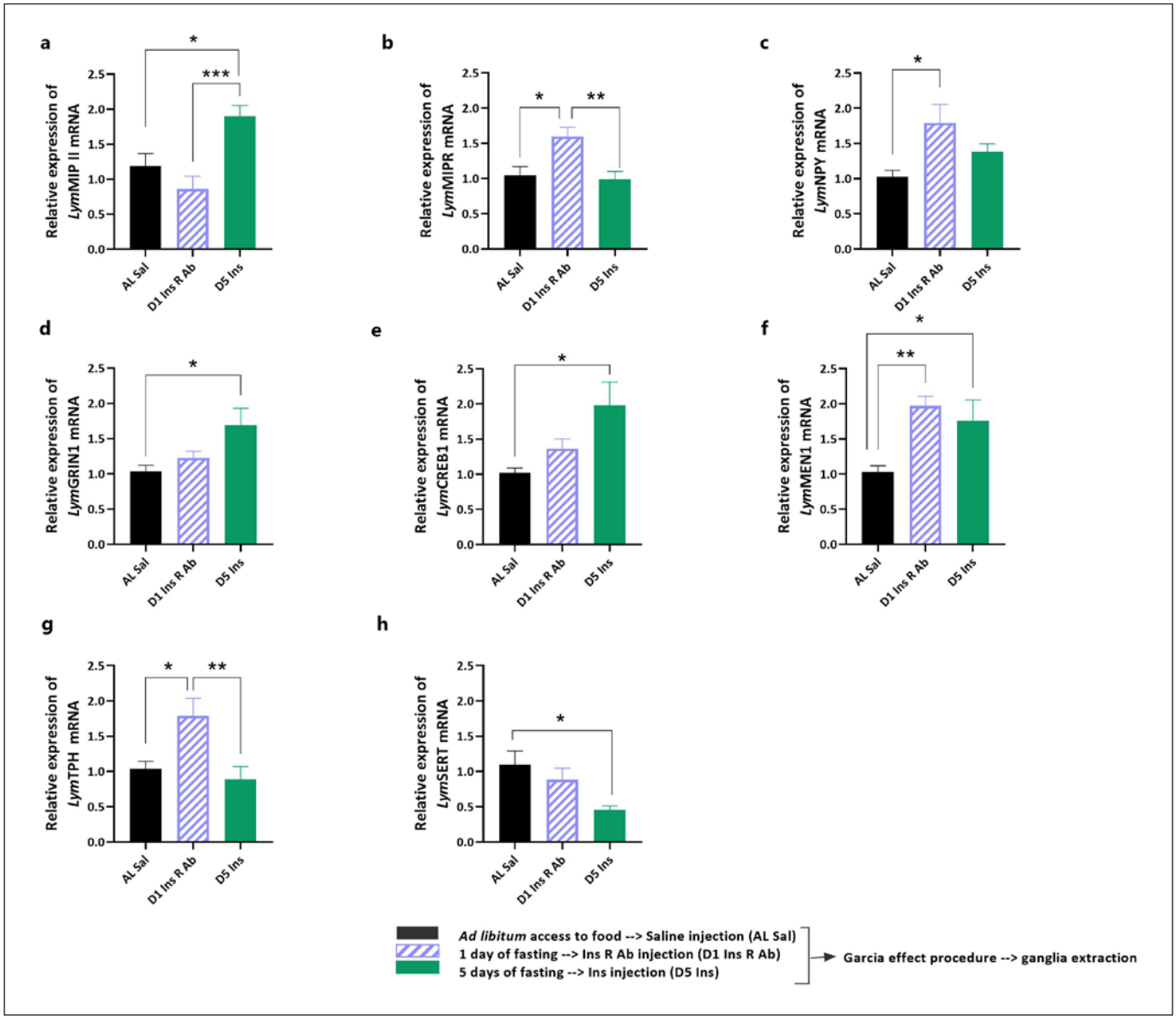

Fig. 5. Transcriptional effects induced by insulin or insulin receptor Ab in moderately and severely fasting snails. The expression levels of *Lym*MIPII (**a**) and *Lym*MIPR (**b**), *Lym*NPY (**c**), *Lym*GRIN1 (**d**), *Lym*CREB1 (**e**), *Lym*MEN1 (**f**), *Lym*TPH (**g**), and *Lym*SERT (**h**) have been measured in the central ring ganglia of (1) snails fed ad libitum and injected with saline (AL Sal), (2) fasting for 1 day and then injected with insulin receptor Ab (D1 Ins R Ab), or (3) fasting for 5 days and injected with insulin (D5 Ins) ($n = 8$) trained for the Garcia effect. Data are represented as means ± SEM and were analyzed with one-way ANOVA followed by Tukey post hoc analyses. ***$p < 0.001$, **$p < 0.01$, *$p < 0.005$. Solid bars (AL snails injected with saline and D5 snails injected with Ins), Garcia effect induced; Diagonal bars (D1 snails injected with Ins R Ab), Garcia effect not formed.

treated with the Ab for the insulin receptor (D1 Ins R Ab) did not mount a Garcia-like response ($t = 1.98$, df = 7, $p = 0.08$ – Fig. 4b).

Interestingly, injection with insulin before the training (D5 Ins) resulted in a Garcia effect memory ($t = 14.79$, df = 7, $p < 0.0001$ – Fig. 4c). As the Garcia effect was formed in both AL Sal and D5 Ins snails, but not in D1 Ins R Ab snails, an external interference impinged on the insulin system seems to "switch" the memory phenotypes of D5 and D1 snails.

Finally, we investigated the molecular effects underlying these changes in memory phenotypes in the same co-

horts of animals. Immediately after the memory test (i.e., C-post 3 h), AL Sal, D1 Ins R Ab, and D5 Ins snails were sacrificed and the mRNA expression levels of *Lym*MIPII, *Lym*MIPR, *Lym*NPY, *Lym*GRIN1, *Lym*CREB1, *Lym*MEN1, *Lym*TPH, and *Lym*SERT were measured in their CNS (Fig. 5). Because in experiment 2 we demonstrated that the Garcia effect procedure did not affect the mRNA levels of HSP70 (Fig. 3i), in experiment 3 we excluded this target from the gene expression analysis. A one-way ANOVA followed by Tukey's post hoc test showed a main effect of treatment on the expression levels of *Lym*MIPII ($F_{2,21}$ = 9.78, p = 0.001) (Fig. 5a) and *Lym*MIPR ($F_{2,21}$ = 7.62, p = 0.003) (Fig. 5b). In particular, the mRNA levels of *Lym*MIPII were upregulated in D5 Ins snails with respect to the AL Sal and D1 Ins R Ab ones (p = 0.023 and p = 0.0009, respectively). On the other hand, the expression levels of *Lym*MIPR were significantly upregulated in D1 Ins R Ab snails compared to their AL Sal and D5 Ins counterparts (p = 0.01 and p = 0.006, respectively). Moreover, a main effect of treatment on the expression levels of *Lym*NPY ($F_{2,21}$ = 4.97, p = 0.02) (Fig. 5c), *Lym*GRIN1 ($F_{2,21}$ = 4.54, p = 0.023) (Fig. 5d), *Lym*CREB1 ($F_{2,21}$ = 5.29, p = 0.014) (Fig. 5e), *Lym*MEN1 ($F_{2,21}$ = 7.44, p = 0.004) (Fig. 5f), *Lym*TPH ($F_{2,21}$ = 6.66, p = 0.006) (Fig. 5g), and *Lym*SERT ($F_{2,21}$ = 4.69, p = 0.021) (Fig. 5h) was found. Post hoc analyses showed that the mRNA levels of *Lym*GRIN1, *Lym*CREB1, and *Lym*MEN1 were upregulated in D5 Ins snails compared to their AL Sal counterparts (*Lym*GRIN1: p = 0.0024; *Lym*CREB1: p = 0.011; *Lym*MEN1: p = 0.04), whereas *Lym*NPY, *Lym*MEN1, and *Lym*TPH were significantly higher in D1 Ins R Ab snails than in the AL Sal ones (*Lym*NPY: p = 0.014; *Lym*MEN1: p = 0.004; *Lym*TPH: p = 0.03). *Lym*MEN1 levels, instead, were significantly higher in D5 Ins snails than in AL Sal snails (p = 0.04), whereas *Lym*TPH levels were significantly downregulated in D5 Ins snails compared to D1 Ins R Ab ones (p = 0.008). Finally, we found that the mRNA levels of *Lym*SERT were significantly reduced in D5 Ins snails compared to AL Sal snails (p = 0.02).

Discussion

In the current study, we used a valid model system, the pond snail *L. stagnalis*, and a solid and highly conserved conditioning paradigm, the Garcia effect, to demonstrate that short-term and long-term fasting results in different molecular cascades and memory performances. We found that the Garcia effect is not apparent in severely fasting snails, whereas snails fasting for 1 day before the training procedure are "better memory performers" than the fed ones.

This study also investigated the molecular effects induced by different lengths of fasting, the aversive stimulus used in the Garcia effect training (i.e., HS), and the conditioning procedure. We demonstrated that severe fasting is associated with a downregulation of *Lym*MIPII and an upregulation of its receptor (*Lym*MIPR). These effects, albeit only at the transcriptional levels, support the existence of a negative feedback loop necessary for the maintenance of glucose levels within a narrow range [63].

On the other hand, although moderate fasting per se does not affect the expression levels of *Lym*MIPII (Fig. 1a), its combination with the conditioning procedure for the Garcia effect results in an insulin expression upregulation possibly leading to an insulin spike and, behaviorally, to better memory performances. These data, consistent with previous studies, strongly suggest that insulin plays a role in enhancing memory performances in trained snails [64, 65]. Moreover, the HS used as the nausea-inducing stimulus in the training procedure failed to affect the transcriptional regulation of *Lym*MIPII and *Lym*MIPR. Especially, with respect to the dramatic upregulation HS exerts on *Lym*HSP70, this suggests that the effects of the fasting regimen prevail over that of heat shock.

In this study, we also demonstrated that 5 days of fasting upregulated the expression levels of *Lym*NPY, *Lym*GRIN1, *Lym*CREB1, and *Lym*MEN1. Our results indicate that these targets, in *Lymnaea* as in mammals, may participate in the organisms' survival and homeostatic-energy economy. Previous studies from rodents indicated that fasting induces a hypothalamic upregulation of NPY which, in turn, promotes food intake and energy conservation [49, 66]. Furthermore, a putative role of CREB in regulating NPY action and mediating the signals of hunger and satiety has been suggested in other models [47, 50, 66, 67]. Finally, NMDA receptors have been reported to take part in the central regulation of appetite [68, 69], whereas MEN1 acts as a co-activator for peroxisome proliferator-activated receptor-γ and the vitamin D receptor, which are involved in glucose metabolism [70].

In presence of a training procedure, the mRNA levels of *Lym*NPY and those of *Lym*GRIN1, *Lym*CREB1, and *Lym*MEN1 in D5 snails remained higher – even though not significantly – than in the AL-fed ones. On the other hand, a more pronounced upregulation of these targets was observed in trained D1 snails. This upregulation may underlie the improvement of the learning abilities of these snails: in fact, 1-day fasting snails got the highest grades for their capacity to learn while the AL ones grad-

ed average. GRIN1, CREB1, and MEN1, in fact, are "best known" for their role in promoting and modulating neuroplasticity in *L. stagnalis* as well as in vertebrates [51, 52, 61, 71–74]. In short, our data suggest a double role of these targets (1) regarding the best memory performance of moderately fasting snails and (2) in preserving energy homeostasis of severely fasting animals.

Furthermore, we observed a different regulation of the serotoninergic system. Long-term starvation upregulated the expression levels of the rate-limiting enzyme for the synthesis of 5HT for short-term fasting (i.e., *Lym*TPH) in both trained and untrained snails. An increase in *Lym*TPH suggests an increase in the serotoninergic tone in the presence of severe fasting. In line with this hypothesis, recent studies demonstrated a higher concentration of tryptophan, the 5HT precursor, in the CNS of 5-day fasting snails compared to the nonfasting or 1-day fasting ones [75]. Moreover, we found a significant downregulation of *Lym*SERT in moderately fasting snails sacrificed after the Garcia effect procedure compared to AL-fed snails. As this target is constitutively expressed in serotonin-containing neurons [76], its mRNA levels possibly reflect the proportional activity of the serotoninergic neurons in *L. stagnalis* CNS.

Our data further strengthen the studies performed by Ito and collaborators indicating that 1-day fasting snails have superior CTA memory formation and a low monoamine content (e.g., 5HT, dopamine, and octopamine) in their CNS, whereas the 5-day fasting ones have poorer CTA memory and a higher monoamine content. To better understand this, additional studies are currently underway in our laboratory to study the mRNA and protein levels of selected targets of the monoaminergic system.

For now and until proven differently, we can only consider that 5HT might be necessary for plasticity in the neural network controlling feeding behavior and the Garcia effect. In fact, according to Kandel's "presynaptic hypothesis of learning and memory," 5HT induces a cAMP increase in the presynaptic sensory neurons, which, in turn, activates protein kinase A (PKA), which in turn phosphorylates CREB1, enhancing neurotransmitter release (i.e., glutamate) for memory consolidation [72]. Moreover, 5HT is also involved in feeding regulation and response to stress [77].

Thus, the different memory phenotypes observed in D1 and D5 snails seem to be due to the different lengths of fasting which, in turn, activate and modulate specific pathways. One day of fasting results in better memory performances and is associated with an upregulation of *Lym*MIPII and the targets of the "neuroplastic" pathway (i.e., *Lym*GRIN1, *Lym*CREB1, and *Lym*MEN1) and downregulation of the serotoninergic targets. Instead, 5 days of fasting result in the absence of the Garcia effect and is associated with a downregulation of *Lym*MIPII and an upregulation of the targets of the serotoninergic and "survival" pathway through *Lym*TPH and *Lym*SERT and *Lym*NPY and *Lym*MEN1, respectively. Given our previous results, we hypothesize a possible role for insulin in orchestrating the activation of the "neuroplastic" versus the "survival" pathway in 1-day fasting snails and vice versa in 5-day fasting animals. The discovery of insulin and its receptors in the mammalian brain revolutionized the concept of insulin signaling in the nervous system and a growing number of studies from both mammals and invertebrates indicates that this pleiotropic hormone directs metabolic, neurotrophic, neuromodulatory, and neuroendocrine processes as necessary elements for learning and memory [11].

To investigate the role played by insulin in driving "the neuroplastic" or "the survival pathways and the snails" memory phenotype, we treated severely fasting animals with bovine insulin and the moderately fasting ones with insulin receptor Ab. The relatively simple nervous system of *L. stagnalis*, which does not possess a blood-brain barrier, and its open circulatory system, allowed us to treat the animals peripherally and easily reach the nervous system [40, 78–80].

Our results indicate that treating D1 snails with Ins R Ab resulted in no Garcia effect, whereas the insulin injection in D5 snails led to memory formation. This suggests a key role for the level of insulin at the moment the conditioning procedure occurs: Snails did not express the memory phenotype if they were extremely hungry (i.e., a condition associated with severely low levels of *Lym*MIPII) or if they were injected with Ins R Ab following short-term starvation. Apart from indicating the existence of a specific energetic state needed for the formation of the Garcia, our data also underscore the hypothesis that insulin plays a central, and maybe necessary, role in determining the final memory phenotype. More precisely, in *Lymnaea*, as in mammals, energy homeostasis is fundamental for learning and memory and is regulated by a complex interplay of nutritional, neuronal, and hormonal inputs that are integrated at the level of the CNS via insulin-dependent and insulin-independent pathways [81].

Gene expression analyses revealed that the insulin injection in D5 snails not only upregulated the expression levels of *Lym*MIPII but was also accompanied by an upregulation of *Lym*CREB1, *Lym*GRIN1, and *Lym*MEN1 as well as by a reduction in SERT levels. This strengthens the

hypothesis that insulin levels in D5 Ins snails, like that in the D1 ones, may activate molecular cascades involved in neuroplasticity. On the other hand, injection of the best performers, i.e., D1 snails, with insulin receptor Ab, was associated with a downregulation of *Lym*MIPII and an upregulation of *Lym*MIPR, *Lym*NPY, and *Lym*TPH, suggesting that this treatment mimics what occurred in D5 snails, "switching" the neuroplastic pathway into survival mode. These opposite effects induced by short and prolonged periods of fasting can be explained in the context of the so-called Yerkes-Dodson/Hebb law [82–84], according to which just the right amount of stress (in this case provoked by hunger) has a memory-enhancing effect: short-term fasting corresponds to a level of stress that the individuals can cope with acting as a motivator for learning and memory [38, 85]. Therefore, moderately fasting animals show higher memory performances when compared to those who have full access to food. On the other hand, prolonged starvation is associated with the necessity to maintain energetic homeostasis and preserve animal survival. We know that the memory score is dependent on the level of stress the animal is encountering [83]. In this view, when tested for effect-induced memory formation, D1 and D5 Ins snails are at optimal stress levels which results in an "insulin spike" (and the following upregulation of GRIN1, CREB1, and MEN1), downregulation of the 5HTergic system (i.e., SERT downregulation), and good memory performances. On the other hand, D5 and D1 Ins R Ab snails are highly hungry and thus stressed and this stressful condition is associated with an upregulation of the serotonin system and a downregulation of the insulinergic one (i.e., MIPII downregulation and MIPR upregulation), resulting in the inability of the animals to recall a formed memory.

Finally, the results obtained in this study raise several questions. First, it is unknown whether prolonged fasting overwhelms the ability of snails to learn and form memory or if in these animals learning occurs (i.e., they associated the appetitive novel taste with the visceral sickness induced by the HS), but the memory phenotype (i.e., feeding suppression) is not exhibited when they are re-exposed to the carrot slurry because they must eat to survive. Thus, future experiments will be performed to answer this question. In particular, we hypothesized that if learning occurs, D5 snails would express memory if the memory test is performed following a period of ad libitum access to food, which, in turn, is necessary to increase insulin levels. Second, as *Lymnaea* possesses an open circulatory system, an injection of Ins or Ins R Ab can also affect the peripheral nervous system as well as other organs. Thus, in the next series of experiments, we plan to compare the expression levels of selected targets among several tissues. Furthermore, based on the results of this study, we will next focus on the role of the serotoninergic and cannabinoid systems, as well as NPY, in mediating both fasting and neuroplasticity in *L. stagnalis*. Third, as previously mentioned, proteomic and metabolomic analyses will be conducted to correlate the effects of fasting and the conditioning procedure on the homeostatic functions in *Lymnaea* as well as on neuroplasticity.

Conclusions

Together, our results suggested that 1 day of fasting created an optimal internal state in the CNS of *L. stagnalis*, allowing a spike in insulin release and an upregulation of genes involved in neuroplasticity (i.e., NMDA, CREB1, and MEN1) when animals are trained for the Garcia effect procedure. Consequently, animals subjected to short-term fasting showed better memory performance. On the other hand, severe fasting upregulated the 5HTergic system and increased the mRNA expression levels of the orexigenic peptide NPY, as well as of MIP-R, GRIN1, CREB1, and MEN1, which may all participate in the molecular machinery necessary for maintaining energy homeostasis in the CNS. Although evolutionarily quite distant from humans, *L. stagnalis* shows molecular and behavioral properties that make it a versatile platform to study the relationship between different lengths of fasting and memory performance, paving the way for future studies in mammals. The use of snail models will limit as much as possible the use of mammalian models and allow mammals to be involved only for the validation of the results obtained from invertebrates. This will reduce by several orders of magnitude the costs of numerous studies. Thus, *L. stagnalis* as a model system provides an important experimental tool and offers a translational approach that may help gain important knowledge and comprehension in the field of neuroendocrinology.

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Statement of Ethics

Lymnaea stagnalis are invertebrate animals; thus, the approval of IACUC (Institutional Animal Care and Use Committee) was not required (Italian Legislative Decree D.L. 4 marzo 2014, n. 26 "Attuazione della Direttiva n. 2010/63/UE sulla protezione degli animali utilizzati a fini scientifici"). However, every effort was made to minimize the number of animals used, ensuring adequate food, clean oxygenated water, and low-density conditions. Guided by previous studies and pilot data in our laboratory, we considered 5 days of starvation as a long enough period to be considered prolonged fasting.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Veronica Rivi and Cristina Benatti were responsible for the study concept. Veronica Rivi, Cristina Benatti, and Pietro Actis performed the behavioral and molecular experiments. Veronica Rivi analyzed the molecular and behavioral data. Veronica Rivi and Pietro Actis drafted the article. Fabio Tascedda and Johanna Maria Catharina Blom provided resources. Fabio Tascedda, Cristina Benatti, and Johanna Maria Catharina Blom edited and provided feedback on the manuscript. All authors have reviewed the manuscript and approved the final version submitted for publication.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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