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RESEARCH ARTICLE

No food for thought: an intermediate level of food deprivation enhances memory in *Lymnaea stagnalis*

Diana Kagan[1,*,‡], Veronica Rivi[2,*], Cristina Benatti[2,3], Fabio Tascedda[3,4,5], Joan M. C. Blom[2,3] and Ken Lukowiak[1]

ABSTRACT

Nutritional status plays an important role in cognitive functioning, but there is disagreement on the role that food deprivation plays in learning and memory. In this study, we investigated the behavioral and transcriptional effects induced by different lengths of food deprivation: 1 day, which is a short time period of food deprivation, and 3 days, which is an 'intermediate' level of food deprivation. Snails were subjected to different feeding regimens and then trained for operant conditioning of aerial respiration, where they received a single 0.5 h training session followed by a long-term memory (LTM) test 24 h later. Immediately after the memory test, snails were killed and the expression levels of key genes for neuroplasticity, energy balance and stress response were measured in the central ring ganglia. We found that 1 day of food deprivation was not sufficient to enhance snails' LTM formation and subsequently did not result in any significant transcriptional effects. However, 3 days of food deprivation resulted in enhanced LTM formation and caused the upregulation of neuroplasticity and stress-related genes and the downregulation of serotonin-related genes. These data provide further insight into how nutritional status and related molecular mechanisms impact cognitive function.

KEY WORDS: Operant conditioning, Nutritional status, Long-term memory, Invertebrates, Neuroplasticity

INTRODUCTION

Nutritional status can differently affect cognitive functioning in organisms (Beilharz et al., 2015; Leigh Gibson and Green, 2002). Although short-term fasting (12–24 h) has been shown to improve cognitive functioning in both invertebrates and vertebrates (Aonuma et al., 2016, 2018; Rivi et al., 2023; Totani et al., 2020), prolonged fasting (>24 h) can have severe consequences such as memory impairment (Papalini et al., 2017).

Among the challenges of studying the effects of different lengths of fasting on learning and memory, there is the difficulty of reproducing extended food deprivation without altering the organism's survival or inducing defensive responses and anxiety behaviors (Johnson et al., 2007). Moreover, because of the complexity of mammalian brains and behaviors, it is not too surprising to find disagreement in the literature on the role that food deprivation plays in learning and memory formation (Choi et al., 2016; Solianik and Sujeta, 2018).

In this context, the pond snail *Lymnaea stagnalis* represents an excellent model organism in which to study the behavioral and molecular mechanisms through which changes in nutritional status may affect learning and memory owing to its relatively simple nervous system and easily observable behaviors (Rivi et al., 2020, 2021b). Previous studies indicated that snails subjected to different lengths of food deprivation and then trained for the conditioning taste aversion (CTA) have different memory performances (Ito et al., 2015; Mita et al., 2014; Murakami et al., 2013; Sugai et al., 2007; Totani et al., 2019, 2020). In particular, snails that were food-deprived for 1 day before training were better learners than the *ad libitum*-fed ones, whereas 5 days of food deprivation was found to affect the expression of the formed memory (Ito et al., 2013, 2015).

We recently obtained similar results in snails trained for the Garcia effect, a unique form of CTA, the acquisition of which depends on animals experiencing a novel food substance and a visceral sickness (i.e. nausea) even hours after the taste of the novel food (Garcia et al., 1955; Rivi et al., 2021a, 2023). Although the Garcia effect was not apparent in 5-day food-deprived snails, snails food-deprived for 1 day before the training procedure were better memory performers than those fed *ad libitum* (Murakami et al., 2013; Rivi et al., 2023). Thus, the authors concluded that severely food-deprived snails (i.e. food-deprived for 5 days) participate in a 'conflict resolution process' when deciding either to eat or not (Ito et al., 2015) and, typically, hunger triumphed over memory (Ito et al., 2015).

Given the interconnectedness between food deprivation and long-term memory (LTM) formation for CTA and the Garcia effect in *L. stagnalis*, in this study, we investigated the effects of different lengths of food deprivation on LTM formation for a non-appetitive learning paradigm: the operant conditioning of aerial respiration. Thus, by utilizing operant conditioning of aerial respiration, which is a non-appetitive paradigm, we circumvented the problem of choosing eating over memory and prevented the behavioral phenotype of LTM from being occluded by a conflict resolution process.

During the operant conditioning procedure, snails are placed in an aquatic, hypoxic environment and receive a tactile stimulus (i.e. negative reinforcing stimulus) to the pneumostome area (i.e. respiratory orifice) every time they attempt to breathe (Lukowiak et al., 1996). Typically, following two 0.5 h training sessions separated by a 1-h interval, the lab-inbred strain of *L. stagnalis* utilized in Prof. Lukowiak's lab (hereinafter, the 'W strain') learns not to breathe and forms an LTM lasting for at least 24 h (Lukowiak et al., 1996). Thus, LTM is defined as a significant decrease in the number of attempted pneumostome openings between the first training session and the 24 h memory test (Lukowiak et al., 1996).

[1]Department of Physiology and Pharmacology, Hotchkiss Brain Institute, Cumming School of Medicine, University of Calgary, AB, Canada, T2N 1N4. [2]Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, 41125 Modena, Italy. [3]Centre of Neuroscience and Neurotechnology, University of Modena and Reggio Emilia, 41125 Modena, Italy. [4]Department of Life Sciences, University of Modena and Reggio Emilia, 41125 Modena, Italy. [5]CIB, Consorzio Interuniversitario Biotecnologie, 34148 Trieste, Italy.
*These authors contributed equally to this work

‡Author for correspondence (diana.kagan1@ucalgary.ca)

D.K., 0000-0001-7383-5509; C.B., 0000-0003-0236-9525; F.T., 0000-0002-3422-004X; J.M.C.B., 0000-0002-4974-1964; K.L., 0000-0001-9028-1931

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Importantly, environmentally relevant stressors have been shown to exert different effects (i.e. enhance, block or do not affect) on LTM formation (Hughes et al., 2017). For example, the scent of a crayfish predator enhances the snails' ability to form LTM in operant conditioning such that a single 0.5 h training session in crayfish effluent is sufficient to cause LTM formation (Lukowiak et al., 2008; Orr and Lukowiak, 2010). In contrast, an injection of *Escherichia coli* lipopolysaccharide before a single 0.5 h training session results in a sickness state that obstructs learning and memory formation (Rivi et al., 2022c).

In this study, we investigated the behavioral and transcriptional effects induced by different and still-unexplored lengths of food deprivation on the ability of W-strain snails to form LTM for the operant conditioning of aerial respiration following a single 0.5 h training session. To test this hypothesis, snails that had *ad libitum* access to food and those food-deprived for 1 or 3 days (i.e. which correspond to 'short-term' and 'intermediate-term' food deprivation, respectively) received a single 0.5 h training session for the operant conditioning of aerial respiration and LTM enhancement was tested 24 h later. Immediately after training, snails were killed and the expression levels of key genes for neuroplasticity, energy balance and stress response were measured in the central ring ganglia.

In particular, we first investigated whether short-term (i.e. 1 day) and intermediate-term (i.e. 3 days) food deprivation combined with the operant conditioning procedure of aerial respiration would differently alter the transcriptional regulation of the orthologues of the glutamate ionotropic receptor NMDA type subunit 1 (*Lym*GRIN1) and the transcription factor cAMP response element-binding protein 1 (*Lym*CREB1) (Iijima et al., 2009; Morris, 2013), which play key roles in learning and memory formation (Batabyal et al., 2021).

Further, as serotonin [5-hydroxytryptamine (5-HT)] regulates both feeding behavior and food satiety in *L. stagnalis* and mediates stress-induced arousal and vigilance behaviors (Il-Han et al., 2010; Totani et al., 2019; Yeoman et al., 2008), we focused our attention on two molecular targets of the serotoninergic system: tryptophan hydroxylase (*Lym*TPH), the rate-limiting enzyme for the synthesis of 5-HT, and *Lym*SERT, the transporter of 5-HT.

Another target we were interested in was the DOPA decarboxylase enzyme (*Lym*DDC), which is responsible for the synthesis of dopamine and 5-HT from L-DOPA and L-5-hydroxytryptophan, respectively (Aonuma et al., 2016). This multifunctional neurotransmitter is heavily involved in the networks that control feeding-related behaviors and mediates sensory stimuli with reference to the internal state (Miller, 2020). In addition, RPeD1, the neuron that was shown to be a necessary site of LTM formation of this behavior (Scheibenstock et al., 2002) is a dopaminergic neuron.

We also focused our attention on the orthologous of the molluscan insulin peptide II (*Lym*MIPII), which plays an important role in mediating CTA and the Garcia effect memory performances in *L. stagnalis* (Murakami et al., 2013). In particular, we found a significant upregulation of *Lym*MIPII (i.e. 'insulin spike) in 1-day food-deprived snails subjected to the conditioning procedure for the Garcia effect, which behaviorally led to better memory performances (Murakami et al., 2013; Rivi et al., 2023). Our working hypothesis was that 1 and 3 days of food deprivation coupled with activation of the insulin pathway would induce the synthesis of new proteins involved in the enhancing effects of short-term fasting on memory formation and memory recall.

The last target we investigated was Heat Shock Protein 70 (*Lym*HSP70), given its key role in mediating the stress response (Ambrose and Chapman, 2021). Indeed, we hypothesized that different lengths of food deprivation might be associated with different levels of stress perception and, therefore, might affect the mRNA levels of HSP70 (Ottaviani et al., 2013).

To summarize, the hypothesis underlying our study was that snails that are food deprived for 1 and 3 days will be 'smarter' (i.e. form memory following only a single training session) than the *ad libitum*-fed ones. To test our hypothesis, we performed behavioral tests and gene expression analyses to ask the following questions. (1) Is there a relationship between feeding regimens and the LTM formation for the operant conditioning of aerial respiration? And if so, (2) what are the transcriptional effects induced by the training procedure in *ad libitum*-fed snails and those food deprived for 1 or 3 days?

MATERIALS AND METHODS

Snails and animal maintenance

In this study, we used an inbred laboratory strain (W-strain) of *Lymnaea stagnalis* (Linnaeus 1758) maintained at the University of Calgary Biology Department. All snails were maintained at 20±1°C on a 16 h:8 h light:dark cycle and fed romaine lettuce *ad libitum*. The snails were housed in artificial pond water (0.25 g l^{-1} of Instant Ocean in deionized water, Spectrum Brands, Madison, WI, USA) supplemented with $CaCO_3$ to ensure calcium concentrations remained above 50 mg (Dalesman and Lukowiak, 2012).

Food-deprivation regimens

Snails were subjected to different lengths of food deprivation: (1) D1 snails were food deprived for 1 day; (2) D3 snails were food deprived for 3 days and (3) AL snails were fed *ad libitum* for the duration of the experiment. Snails that were food deprived were placed in a clean tank with no previous traces of lettuce and had no access to algal biofilms or conspecific carrion throughout the deprivation period. During the 24 h between the training session and the memory test, all snails had *ad libitum* access to lettuce. Mortality rates higher than what typically occurs owing to random chance (≤1 mortality per group) were not observed for any condition.

Operant conditioning of aerial respiratory behavior

Aerial respiration was defined as the spontaneous opening and closing of the pneumostome, the respiratory orifice (Lukowiak et al., 1996). A hypoxic environment was maintained to increase the frequency of aerial respiration (Moroz et al., 1993). This hypoxic environment was created by continuously bubbling 100% N_2 into the test beaker for 20 min before placing the snails in it and for the entire duration of the experiment. Tactile stimulation to the pneumostome area was used as the negative reinforcing stimulus. When a snail attempted to open its pneumostome, a weak tactile stimulus was applied to the pneumostome area via a thin wooden dowel. The stimulus intensity was sufficient to evoke pneumostome closure without eliciting a whole-animal withdrawal response. Snails underwent one 0.5 h training session and were subject to a 0.5 h memory test either 24 or 72 h later. Before beginning the training session, snails were removed from their home aquarium and placed into a 1 liter beaker filled with 500 ml of hypoxic pond water, and were given 10 min to acclimate. During the training session and memory test, snails received a tactile stimulus to the pneumostome area whenever they attempted to open their pneumostome. The number of 'pokes' applied to the pneumostome was recorded.

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

GenBank accession no.	Target	Product length (bp)	Type sequence
AB041522.1	*Lymnaea stagnalis* cAMP responsive element binding protein, *Lym*CREB1	180 (49–229)	FW: GTCAGCAGGGAATGGTCCTG RV: AACCGCAGCAACCCTAACAA
AY571900.1	*Lymnaea stagnalis* NMDA-type glutamate receptor, *Lym*GRIN1	140 (831–917)	FW: AGAGGATGCATCTACAATTT RV: CCATTTACTAGGTGAACTCC
AF129815.1	*Lymnaea stagnalis* tryptophan hydroxylase, *Lym*TPH	179 (238–417)	FW: AGGATACAGTCTACCGACAG RV: TGAGTTCACGGAAAACTATT
FX185022	*Lymnaea stagnalis* serotonin transporter, *Lym*SERT	177 (726–903)	FW: ATACCGTACCTTGTCATGTT RV: TGTTGTAGTACCAGGAGACA
FX186872	*Lymnaea stagnalis* DOPA decarboxylase, *Lym*DDC	99 (134–233)	FW: CACTGAGCTAGAAGTCTCCA RV: TATAACACCTCCACCTTTTC
X59302.1	*Lymnaea stagnalis* molluscan insulin-related peptide, *Lym*MIP II	186 (152–338)	FW: CCAATCATCTTGCAGTTTA RV: GTCGTCCAGATCTGTTTCT
DQ206432.1	*Lymnaea stagnalis* heat-shock protein 70, *Lym*HSP70	199 (134–333)	FW: AGGCAGAGATTGGCAGGAT RV: CCATTTCATTGTGTCGTTGC
DQ278441.1	*Lymnaea stagnalis* elongation factor 1-alpha, *Lym*EF1α	150 (7–157)	FW: GTGTAAGCAGCCCTCGAACT RV: TTCGCTCATCAATACCACCA

For each target, the accession number and the size (bp) of the PCR product obtained by amplification of the cDNA (mRNA) are given. The corresponding nucleotide position is shown in parentheses.

Total RNA extraction, reverse transcription and real-time polymerase chain reaction

A new cohort of snails underwent the same behavioral training and immediately after the memory test, AL, D1 and D3 snails were anesthetized on ice for 10 min, and the central ring ganglia (buccal ganglia were excluded) were dissected and stored at −80°C before analysis. Total RNA extraction and DNAse treatment were performed using GenElute™ Total RNA Miniprep Kit and DNASE70-On-Column DNAse I Digestion Set (Merck Millipore) as previously described (Batabyal et al., 2021; Benatti et al., 2017; Rigillo et al., 2018; Rivi et al., 2021a, 2022b). A single central ring ganglion was used for total RNA extraction. Six to seven samples were analyzed for each group. A 200-ng sample of total RNA was reverse transcribed with a High-Capacity cDNA Reverse Transcription Kit (ThermoFisher). Real-time quantitative PCR (qPCR) was carried out on 20 ng mRNA using a Bio-RadCFX Connect™480 Real-Time PCR Detection System with SYBR Green Master Mix (Bio-Rad).

The cycling parameters were 95°C for 2 min and 94°C for 10 s, 60°C for 30 s for 40 cycles, and a dissociation curve analysis followed the amplification. Cycle threshold (C_t) values were determined by CFX Maestro™ Software (Bio-Rad). Specific forward and reverse primers with a length of 19–23 nucleotides, a melting temperature between 58 and 62°C, a GC content between

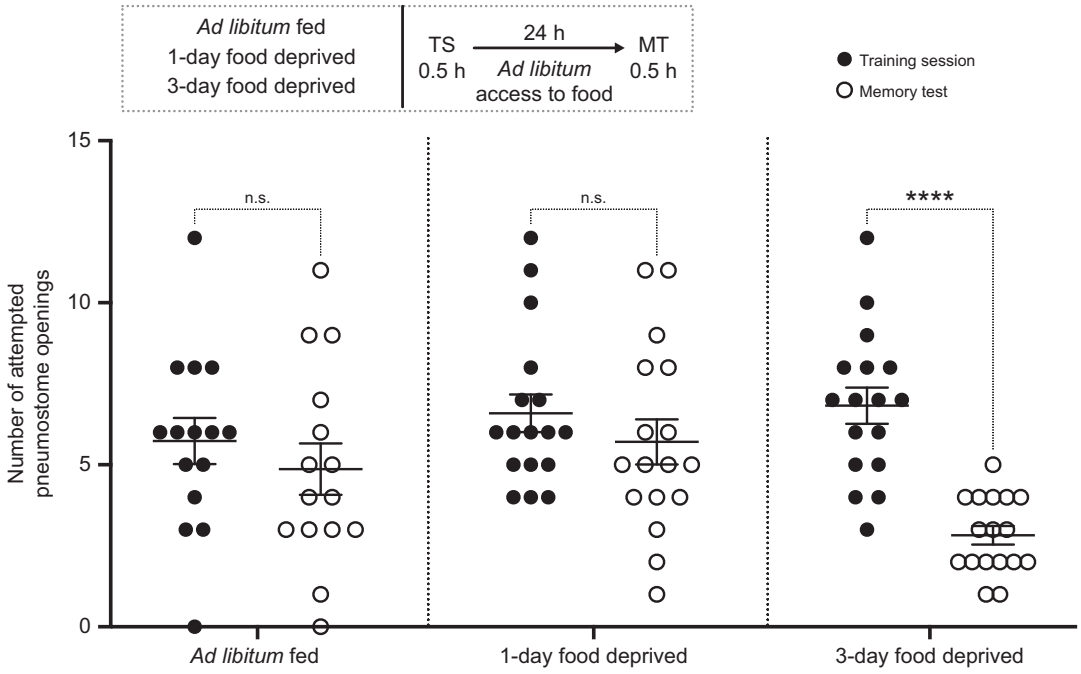

Fig. 1. Number of attempted pneumostome openings for each snail during the training session (TS) and the memory test (MT) of operant conditioning following different lengths of food deprivation. From left to right, snails were either fed *ad libitum* (n=15), food deprived for 1 day (n=17) or food deprived for 3 days (n=17). *Ad libitum*-fed and 1-day food-deprived snails demonstrated no statistically significant difference in pneumostome openings between TS and MT (P=0.3994 and P=0.3303), but 3-day food-deprived snails did (P<0.0001). Data were analyzed with a two-way repeated-measures ANOVA followed by a Šídák multiple comparisons test (****P<0.0001). Error bars represent ±1 s.e.m.

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40% and 60%, generating an amplicon between 100 and 200 bp, were used at the final concentration of 300 nmol l^{-1} (Table 1).

The mRNA levels of each target were normalized to a reference gene, elongation factor 1α (*Lym*EF1α; Young et al., 2019). It was demonstrated that the endogenous control mRNA levels were not affected by any procedure (one-way ANOVA) and the amplification efficiency of the target genes and endogenous control genes was approximately equal. For quantitative evaluation of changes, the comparative $2^{-\Delta\Delta C_t}$ method was performed using as a calibrator the average levels of expression of control animals (i.e. *ad libitum*-fed snails).

Statistical analyses

The behavioral data from the initial behavioral experiments and the behavioral experiments pre-RNA extraction were combined to increase the sample size and increase the power of statistical analyses. For behavioral experiments (Fig. 1), we performed a two-way repeated-measures ANOVA where the between-subjects factor was the level of food deprivation, and the within-subjects factor was the training/memory test. For *post hoc* analysis, we performed Šídák's multiple comparisons tests.

To assess 72 h LTM in 3-day food-deprived snails (Fig. 2), we performed a paired *t*-test to examine the differences in the number of attempted pneumostome openings between the training session and the memory test.

For gene expression analyses, first, we analyzed our data for the assumption of normality using the Kolmogorov–Smirnov one-sample test for normality (K–S distance and *P*), and a normal distribution was found for all targets investigated. One-way ANOVA was used to compare the expression levels of each target in AL, D1 and D3 snails trained for the operant conditioning procedure and killed immediately after the memory test. Significant changes were determined using Tukey's *post hoc* test.

Differences were considered significant if $P<0.05$. Data are presented as means±s.e.m. Molecular statistical analyses were performed using SPSS v. 26.0 (IBM Corp., Armonk, NY, USA), and behavioral data were analyzed using GraphPad Prism v. 9.00e for MAC® (GraphPad Software, Inc., La Jolla, CA, USA). GraphPad Prism v. 9.00e for MAC® was also used to generate graphs.

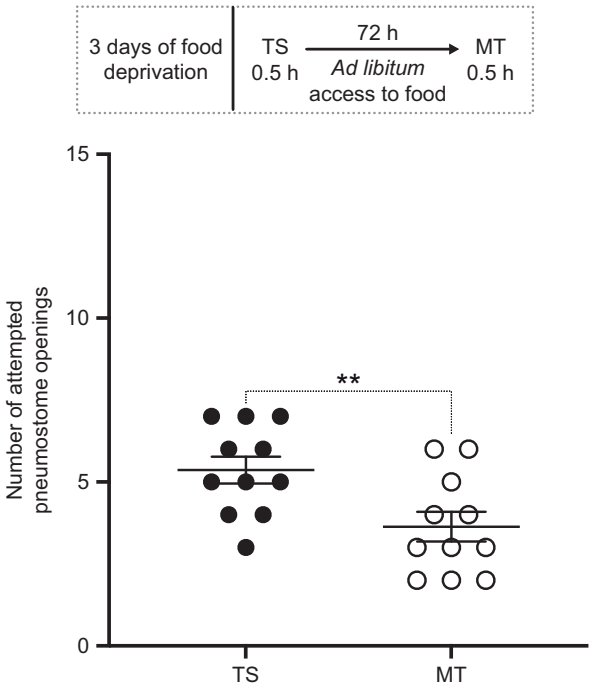

Fig. 2. Number of attempted pneumostome openings for each snail during the training session (TS) and the memory test (MT) of operant conditioning following 3 days of food deprivation. The MT was performed 72 h after the TS. Snails demonstrated a statistically significant decrease in pneumostome openings between the TS and the 72 h MT (paired *t*-test, $t=3.684$, d.f.=10, $P=0.0042$, $n=11$). **$P<0.01$. Error bars represent ±1 s.e.m.

RESULTS

Effects on memory formation following different lengths of food deprivation

A two-way ANOVA followed by a Šídák's multiple comparisons test revealed that there was a statistically significant interaction between the effects of food deprivation on the training/memory test ($F_{2,46}=10.00$, $P=0.0002$) (Fig. 1). Moreover, there was a main effect of the training/memory test on 3-day food-deprived snails ($F_{2,46}=10.00$, $P<0.0001$). In particular, 3-day food-deprived snails showed enhanced LTM formation following training. That is, the number of attempted pneumostome openings recorded during the 24 h memory test was significantly less than that during the training session ($P<0.0001$, $n=17$), indicating that LTM was formed. In contrast, neither *ad libitum*-fed snails nor 1-day food-deprived snails showed LTM formation following a single 0.5 h training session. That is, the number of attempted pneumostome openings recorded during the 24 h memory test and the 0.5 h training session did not differ significantly (*ad libitum*-fed: $P=0.3994$, $n=15$; 1-day food-deprived: $P=0.3303$, $n=17$).

We were interested in further exploring how robust the enhancement of LTM formation for 3-day food-deprived snails was. To do this, we repeated the 3-day food deprivation in a new naïve cohort of snails followed by one 0.5 h training session. We then tested for LTM 72 h later (instead of a regular 24 h LTM test). We found that 3 days of food deprivation caused a robust enhancement of LTM formation whereby one 0.5 h training session resulted in a LTM that persisted for at least 72 h (Fig. 2). That is, the number of attempted pneumostome openings recorded during the 72 h memory test was significantly less than during the training session (paired *t*-test, $t=3.684$, d.f.=10, $P=0.0042$, $n=11$).

Transcriptional effects induced by different lengths of food deprivation

Our behavioral data encouraged us to investigate the transcriptional effects induced by different lengths of food deprivation (Fig. 3). Thus, a naïve cohort of *ad libitum*-fed snails ($n=7$), a naïve cohort of 1-day food-deprived snails ($n=7$) and a naïve cohort of 3-day food-deprived snails ($n=6$) were first trained for the operant conditioning of aerial respiration 24 h after training LTM was tested.

We killed those animals immediately after the 24 h memory test and the mRNA expression levels of *Lym*GRIN1, *Lym*CREB1, *Lym*TPH, *Lym*SERT, *Lym*DDC, *Lym*MIPII and *Lym*HSP70 were measured in their central ring ganglia.

A one-way ANOVA followed by Tukey's *post hoc* test revealed a main effect of the behavioral procedure on the expression levels of *Lym*GRIN1 ($F_{2,17}=16.55$, $P=0.0001$; Fig. 3A) and *Lym*CREB1 ($F_{2,17}=8.4$, $P=0.0029$; Fig. 3B). In particular, we found an upregulation of these targets in 3-day food-deprived snails compared with the *ad libitum*-fed and day-1 food-deprived ones

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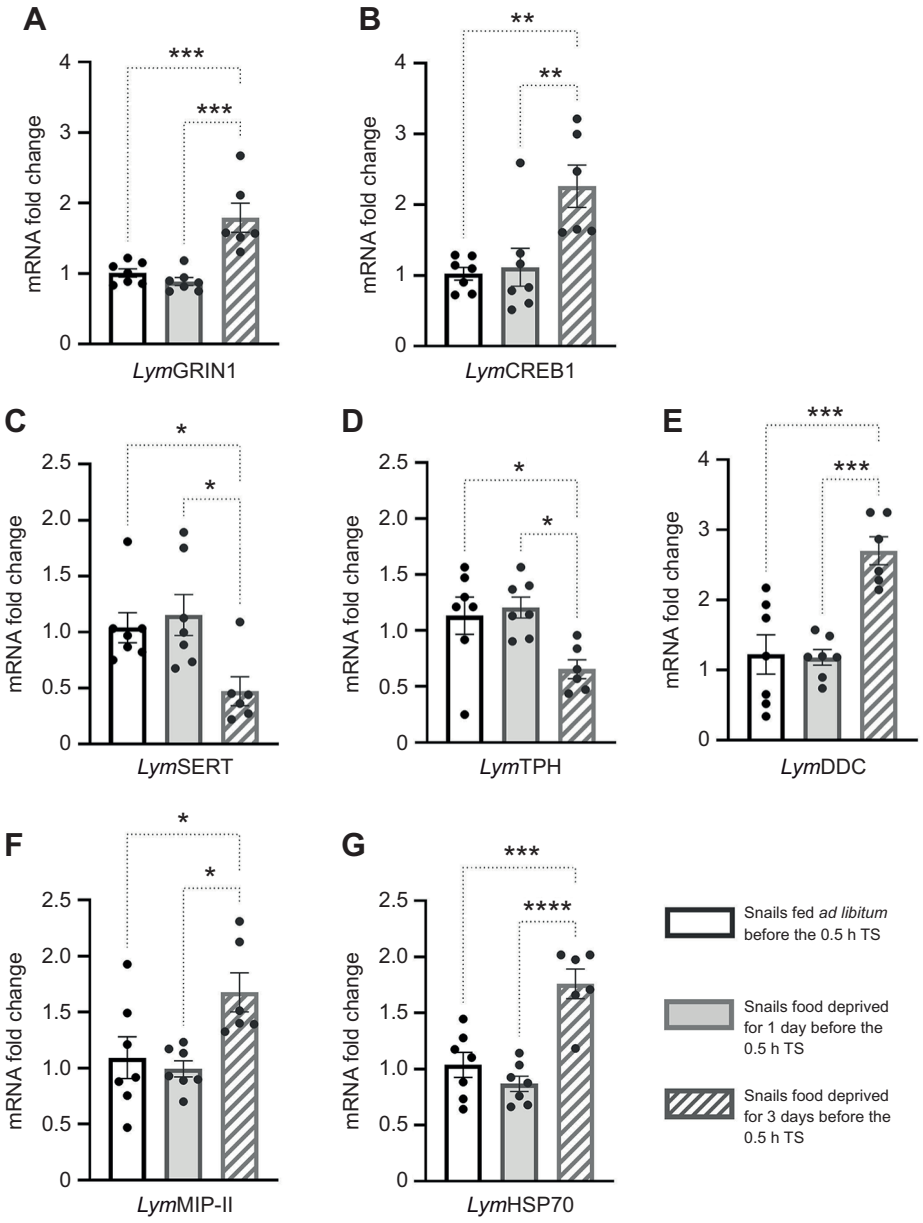

Fig. 3. Transcriptional effects induced by different lengths of food deprivation in the central ring ganglia of snails trained for the operant conditioning of aerial respiration paradigm. The expression levels of (A) *Lym*GRIN1, (B) *Lym*CREB1, (C) *Lym*SERT, (D) *Lym*TPH, (E) *Lym*DDC, (F) *Lym*MIPII and (G) *Lym*HSP70 were measured in the central ring ganglia of snails fed *ad libitum* (*n*=7, open bars), food deprived for 1 day (*n*=6, filled bars) and food deprived for 3 days (*n*=7, diagonal bars) before being trained for the operant conditioning of aerial respiration. Twenty-four hours after training, snails were killed, and the central ring ganglia were extracted. Data are represented as means±s.e.m. and were analyzed with a one-way ANOVA followed by Tukey *post hoc* analyses (**P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001).

(*Lym*GRIN1: *P*=0.007 and *P*=0.0001; *Lym*CREB1: *P*=0.0046 and *P*=0.0042, respectively). No differences were found in the expression levels of these targets between *ad libitum*-fed and day-1 food-deprived snails.

Moreover, one-way ANOVA revealed a main effect of the behavioral procedure on the expression levels of *Lym*SERT ($F_{2,17}$=5.35, *P*=0.016; Fig. 3C), *Lym*TPH ($F_{2,17}$=5.45, *P*=0.015; Fig. 3D) and *Lym*DDC ($F_{2,17}$=16.08, *P*=0.0001; Fig. 3E). In particular, *post hoc* analyses revealed that the expression levels of *Lym*SERT and *Lym*TPH were significantly reduced in 3-day food-deprived snails compared with the 1-day food-deprived and *ad libitum*-fed ones (*Lym*SERT: *P*=0.048 and *P*=0.018; *Lym*TPH: *P*=0.041 and *P*=0.018, respectively). In contrast, we found a significant upregulation of *Lym*DDC mRNA levels in 3-day food-deprived snails compared with the 1-day food-deprived and *ad libitum*-fed ones (*P*=0.0004 and *P*=0.0003, respectively).

Finally, for *Lym*MIPII (Fig. 3F) and *Lym*HSP70 (Fig. 3G) expression levels, we found a main effect of the operant conditioning paradigm ($F_{2,17}$=5.68, *P*=0.013 and $F_{2,17}$=19.44, *P*<0.0001, respectively). In particular, *post hoc* analyses revealed a significant upregulation in 3-day food-deprived snails compared with the *ad libitum* fed and day-1 food-deprived counterparts (*Lym*MIPII: *P*=0.004 and *P*=0.015; *Lym*HSP70: *P*=0.0005 and *P*<0.0001, respectively).

DISCUSSION

Here, we used the pond snail *L. stagnalis* and a well-known training procedure – operant conditioning of aerial respiration (Lukowiak et al., 1996) – to investigate the behavioral and transcriptional effects induced by different lengths of food deprivation. Contrary to what we hypothesized, 1 day of food deprivation was not sufficient to enhance snails' ability to form LTM. However, we found that 3-day food-deprived snails showed enhanced LTM formation, so a single 0.5 h training session was sufficient to cause an LTM that persisted for at least 72 h.

Our findings did not concur with the results obtained with the CTA and Garcia effect training procedures (Ito et al., 2017; Rivi et al., 2023; Totani et al., 2020). These studies, in fact,

demonstrated that 1-day food-deprived snails showed optimal LTM formation for CTA and the Garcia effect, whereas 5-day food-deprived snails did not. In contrast, the behavioral and molecular data obtained in snails trained for the operant conditioning of aerial respiration showed no differences between the *ad libitum* and the 1-day food-deprived snails. We hypothesize that the stress state resulting from 1 day of food deprivation, which did not cause enhancement of LTM formation, is not long enough to cause significant transcriptional changes that are necessary for LTM formation. The molecular data presented here are consistent with this hypothesis, as no significant differences were found in the expression levels of the selected targets between *ad libitum*-fed and 1-day food-deprived snails.

In contrast, in this study, we investigated for the first time in snails whether 3 days of food deprivation would affect (enhancing or blocking) memory formation for the operant conditioning of aerial respiration. Interestingly, we found that 3 days of food deprivation was sufficient to elicit a stress state that resulted in the enhancement of LTM formation.

The molecular data are correlated with enhanced LTM formation in the operant conditioning of aerial respiration. In particular, gene expression analyses revealed that 3 days of food deprivation combined with the operant conditioning procedure of aerial respiration resulted in a significant upregulation of the mRNA levels of *Lym*GRIN1 and *Lym*CREB1, which are key genes for learning and memory formation – compared with those of the *ad libitum*-fed and 1-day food-deprived snails, which did not form LTM. Thus, this upregulation may underlie the improvement of the learning abilities of these snails, as 3-day food-deprived snails showed enhanced LTM formation following a single 0.5 h training session.

Moreover, we observed a significant downregulation of *Lym*SERT, the 5-HT transporter, and *Lym*TPH, the rate-limiting enzyme for the synthesis of 5-HT, in 3-day food-deprived snails. A decrease in *Lym*TPH and *Lym*SERT mRNA levels is suggestive of a decrease in the serotoninergic tone in 3-day food-deprived snails. Thus, our data are consistent with and strengthen the findings from the traditional CTA studies performed by Ito and collaborators indicating that ‘smarter’ snails have a lower 5-HT content in their CNS (Aonuma et al., 2016, 2018; Rivi et al., 2023).

In addition, our data further strengthen the studies performed by Ito and collaborators (2017) indicating that snails showing better memory performances have a lower monoamine content (e.g. 5-HT, dopamine, noradrenaline and octopamine). 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.

Our results also show that in the 3-day food-deprived snails, compared with the *ad libitum*-fed and 1-day food-deprived snails, there was a significant upregulation of *Lym*DDC. These data suggest an increase in the dopaminergic levels in the central ring ganglia of 3-day food-deprived snails before the training session. In both vertebrates and invertebrates, dopamine plays a key role in reward systems and mediates signals related to salient but non-rewarding experiences such as aversive and alerting events (Bromberg-Martin et al., 2010; Baik, 2013; Ranaldi, 2014; Awata et al., 2015). In mollusks, this neurotransmitter has been shown to play an important role in reward classical and operant conditioning in *Aplysia* (Baxter and Bryne, 2006), and in the LTM consolidation of reward but not aversive conditioning in *Lymnaea* (Kemenes et al., 2011). Our data are consistent with the concept that there is a dopamine-dependent ‘wanting’ system that is activated by food deprivation. The idea of a wanting system was first proposed in mammalian systems and was thought to be the result of mesolimbic dopaminergic neural systems (Berridge and Robinson, 2016). Moreover, a wanting system has recently been shown to exist in honeybees, increasing their motivation to forage when they are hungry (Huang et al., 2022; Dong et al., 2023).

In the present study, we cannot claim that there is a causality between the LTM formation for the operant conditioning of aerial respiration and the dopamine contents in the central ring ganglia. However, memory has been found to be dependent on the level of stress the animal is encountering during training (Yerkes and Dodson, 1908; Hebb, 1955; Ito et al., 2015). Thus, our data suggest that 3-day food-deprived snails may be at optimal stress levels (as suggested by the upregulation of *Lym*HSP70), which may result in the upregulation of both the dopaminergic and glutamatergic systems (as suggested by the upregulation of *Lym*DDC and *Lym*GRIN1, respectively). It is possible then that this upregulation may modulate LTM consolidation in snails, as it does in mammals.

Of further interest was our finding that only in the 3-day food-deprivation group was there a significant upregulation of *Lym*MIPII, suggesting that 3 days of food deprivation and its combination with the operant conditioning procedure may result 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 (Mita et al., 2014; Hatakeyama et al., 2013; Murakami et al., 2013; Ito et al., 2012).

Finally, we also demonstrated that 3 days of food deprivation upregulated the expression levels of *Lym*HSP70 – which plays an important role in the stress response – in the snails’ central ring ganglia (Barco et al., 2003; Mayer and Bukau, 2005; Morris, 2013). These data are consistent with a previous study showing that HSP70 is enhanced in the hippocampus of mice after learning and during memory consolidation (Feder and Hofmann, 1999). Moreover, we know that in *L. stagnalis*, the upregulation of *Lym*HSP70elicited by heat shock is responsible for the enhancement of LTM formation (Teskey et al., 2012), whereas if the upregulation of *Lym*HSP70is blocked, LTM does not result (Rivi et al., 2021a, 2022a; Sunada et al., 2016). Thus, the results presented here should also be viewed in the context of the so-called Yerkes–Dodson/Hebb law, according to which ‘just the right amount of stress has a memory-enhancing effect’ (Yerkes & Dodson, 1908; Ito et al., 2015). In other words, stress (in this case provoked by 3 days of food deprivation) has a memory-enhancing effect as it corresponds to a level of stress that the individuals can cope with, acting as a motivator for learning and memory. Therefore, moderately fasting animals show higher memory performances when compared with those that have *ad libitum* access to food and those food deprived for 1 day. In contrast, short-term starvation may not be perceived as a stressor, resulting in no HSP upregulation.

Thus, the different memory phenotypes observed in 1-day and 3-day food-deprived snails and their *ad libitum*-fed controls may be due to the different lengths of food deprivation which, in turn, activate and modulate specific pathways. In particular, 3 days of food deprivation results in enhanced LTM memory formation for operant conditioning of aerial respiration and is associated with a significant upregulation of *Lym*MIPII and *Lym*DDC and the targets of the ‘neuroplastic’ pathway (i.e. *Lym*GRIN1 and *Lym*CREB1) and a significant downregulation of the serotoninergic targets. In comparison, 1 day of fasting results in no LTM formation and no differences in mRNA levels of key genes for neuroplasticity, stress

response and energy balance compared with the *ad libitum*-fed controls.

Although many previous studies included 5-day food-deprived snails, we did not include such a group because we saw enhanced LTM formation concomitant with significantly higher levels of gene activity correlated with learning and memory following 3 days of food deprivation. Thus, we did not find it necessary to kill more snails to test whether a 5-day food deprivation would show even further memory enhancement.

Conclusions

Together, our results suggested that 3 days of food deprivation created an optimal internal state in the central ring ganglia of *L. stagnalis*, allowing a spike in insulin and dopamine release and an upregulation of genes involved in neuroplasticity (i.e. *Lym*GRIN1 and *Lym*CREB1) when animals are trained for the operant conditioning of aerial respiration. Consequently, animals subjected to intermediate-term food deprivation show a robust enhancement of LTM that persists for 72 h. In contrast, low-intensity food deprivation (i.e. 1 day) did not affect memory performances at the behavioral or molecular levels. Thus, it is not enough to cause enhancement of LTM formation following operant conditioning of aerial respiration nor to induce transcriptional effects on the expression levels of the selected targets.

This study contributes to our understanding of the effects that nutritional status has on memory formation. To our knowledge, this is the first study investigating the effects of different lengths of starvation on the ability of an invertebrate model system to form LTM for the operant conditioning of aerial respiration. Although quite distant evolutionarily from humans, *L. stagnalis* shows molecular and behavioral properties that make it a versatile model to study the relationship between different lengths of food deprivation and memory performances for various (appetitive and non-appetitive) behavioral procedures. As such, results from *L. stagnalis* may pave the way for future studies in mammals and allow us to further explore how changes in nutritional status and related molecular mechanisms impact cognitive functioning.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: D.K., V.R., K.L.; Methodology: D.K., V.R., C.B., K.L.; Formal analysis: D.K., V.R.; Resources: F.T., J.M.B., K.L.; Writing - original draft: D.K., V.R.; Writing - review & editing: C.B., K.L.; Visualization: D.K., V.R.; Supervision: K.L.; Funding acquisition: F.T., J.M.B., K.L.

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Data availability

All relevant data can be found within the article and its supplementary information.

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