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Lymnaea stagnalis as model for translational neuroscience research: from pond to bench

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Introduction

Aims of the thesis

During my three years as PhD student in "Neuroscience", I focused my research on the characterization of a molluscan model, the pond snail *Lymnaea stagnalis* (*L. stagnalis*, Linnaeus, 1758), for addressing fundamental questions in Neuroscience.

The experiments described in this thesis were performed in the Laboratory of Neuropsychopharmacology at the University of Modena and Reggio Emilia (Modena, Italy) under the supervision of my thesis tutors, Prof. Fabio Tascedda and Dr. Cristina Benatti, and at the Hotchkiss Brain Institute (Cumming School of Medicine) under the supervision of Prof. Ken Lukowiak (Calgary, Canada).

By recurring to a multidisciplinary approach, which combines different methods and fields, including bioinformatics, genetics, molecular biology, and behaviour, the studies presented in this thesis illustrate how *L. stagnalis* can be used as a valid model system to open new frontiers towards Translational Neuroscience, the discipline that aims to improve human health by taking advantage of knowledge collected in non-human organisms.

The collected data provided important methodological information and original data, representing cutting-edge knowledge for the field, and providing a solid background for future experiments.

The goals I achieved in this project were four:

1. Development of an integrative approach to identify and characterize putative transcripts which codify for the enzymes of the kynurenine pathway (KP) in the central nervous system (CNS) of *L. stagnalis*.
2. Definition of a behavioural paradigm to study the Garcia effect in pond snails.
3. Description of the mechanisms through which nature and nurture modulate behaviours and cognitive functions in inbred and outbred populations of snails exposed to an environmental stressor.
4. Exploration of the memory-enhancing proprieties of a bioactive compound, the flavonoid quercetin.

Background

"Ought we, for instance (to give an illustration of what I mean), to begin by discussing each separate species-man, lion, ox, and the like-taking each kind in hand independently of the rest, or ought we rather deal first with the attributes which they have in common in virtue of some common elements of their nature, and proceed from this as a basis for the consideration of them separately?"
Aristotle, "On the Parts of Animals".

The human brain is the most complex entity known. Identifying conserved mechanisms that could elucidate its functions (and dysfunctions) is one of the main goals of neuroscience research.

However, the questions that can be answered by directly analyzing the human brain are limited by the difficulties in accessing samples. Since Ancient Greece, over 2500 years ago, it was known that animals, when appropriately used, could inform on the fundamental biology relevant to humans. To date, no animal model fully phenocopies the complexity of the human nervous system. However, as stated by a core principle of neuroethology termed the "Krogh's principle": "*for a large number of problems there will be some animal of choice or a few such animals on which it can be most conveniently studied*".

It is therefore fundamental to choose the most appropriate model system for answering specific questions. In that way, by navigating through the numerous data collected in different organisms, researchers are trying to decipher, piece by piece, the mechanisms underlying human neurophysiology and pathology.

Neuroscience research is currently dominated by studies on non-human primates and rodents, especially *Mus musculus* and *Rattus norvegicus*. These organisms provide ideal animal models for biomedical research because of the numerous similarities to human anatomy and physiology.

Although these systems are considered the gold standard for translational research and provide a wealth of knowledge, they are slow to grow, expensive to breed and keep, and sometimes difficult to work with for *in vivo* experiments.

Furthermore, in the last decades, the use of mammals for scientific purposes has fuelled numerous protests and triggered many scientific, economic, ethical, and social discussions (Alberts, 2010), which have led to profound changes in the laws associated with ethical restrictions in animal experiments. Because of that, the use of rodents in biomedical research has become increasingly expensive and restricted, generating a pressing demand for different models that are cheap, simple, and easy to handle but at the same time as effective as mammals. Many researchers have tried to solve the problem by recurring to a vast repertoire of *in vitro* cell lines. Compared to mammal models, cell systems require lower operating costs and ensure higher speed in collecting data. However, they are often limited in terms of maturation and complexity and resulted in inconclusive in elucidating the basis of complex physiological and pathological processes.

One avenue to solve the translational gap between rodents and cell cultures and to exceed the practical and conceptual limitations of experimentation on mammals, is the expansion of the portfolio of animal models, carefully considering their origin as well as the environmental conditions under which they are raised and tested.

In this complex scenario, invertebrates have been recognized as a flexible tool in which to study the basic and conserved mechanisms of CNS physiology and pathology (Corning et al., 1973; Kaang et al., 1993; Ottaviani et al., 2013).

Unlike mammals, invertebrates frequently have short generation times, numerous offspring, and can be more easily experimentally manipulated. Moreover, research using invertebrate models is associated with significant experimental efficiency due to the reduced time needed for experiments and the low costs required for their care.

Although invertebrates can never replace clinical research and mammal models in preclinical studies, these species are making a significant contribution to the understanding of basic Neuroscience that translates to the human situation. By virtue of the high level of conservation of fundamental signalling mechanisms which mediate synaptic plasticity, neurodevelopment, and learning and memory, the study of the nervous systems of invertebrates is elucidating the complexities of neural function mammals, including humans. Even though invertebrates are often identified as 'alternative models', the term 'complementary models' might be more appropriate, as they can and should be used in addition to, and not as an alternative, to classic mammalian models.

The most common and best characterized invertebrate models used in biomedical research are the fruit fly *Drosophila melanogaster* and the worm *Caenorhabditis elegans*.

Findings obtained in these models contributed to the understanding of the neural circuits underlying different behaviours and the effects of genetic mutations on neuronal activities. Moreover, they have become major experimental platforms for opening interesting perspectives concerning both the validation of the mechanism of action of existing pharmacological treatments and the preclinical studies of drugs in development. However, their very short life cycle (lasting approximately 2–3 months for *D. melanogaster*, whereas for *C. elegans* it is 2–3 weeks), limits their effectiveness in studies involving ageing and neurodegeneration or chronic diseases.

Molluscs as gold standard model organisms for Neuroscience

Molluscs played significant historical and practical roles in neuroscience research, providing important translational insight into basic neurobiological processes.

It was 1909 when Leonard W. Williams described for the first time the giant axons of the squid *Loligo forbesii*. This discovery remained unnoticed for almost 30 years when John Z. Young adopted this model (and its neurons) for investigating the mechanisms which underlie the generation of action potentials (Young, 1938). These studies enabled the ground-breaking voltage-clamp experiments performed in the 1940s by the American scientists Kenneth S. Cole, George Marmont, and Howard J. Curtis, and the European Alan L. Hodgkin, Andrew F. Huxley, and Bernard Katz.

Cole, the inventor of the Voltage-Clamp method performed his first experiments on the squid axons (Huxley, 1992). The strong collaboration between Hodgkin, Huxley, and Katz, led to the detailed description of the mechanisms of resting potential, action potential, and ionic nerve impulse transmission on the squid axon (Huxley, 1992; Hodgkin and Huxley, 1952; Hodgkin et al., 1952). The development of electrophysiological techniques allowed researchers to perform similar measurements on vertebrate neurons, making clear that all neurons (including human brain cells) maintain resting potential and create and mediate action potential, basically in the same way.

This discovery marked a turning point in Neuroscience.
Research utilizing molluscs, in fact, become a booming line not only for deciphering elementary neuronal phenomena but also to describe the molecular and cellular mechanisms underlying cognitive functions. For the latter purpose, the CNS of snails and sea slugs has proven to be particularly suitable because it consists of mostly large, easily accessible, and individually identifiable neurons for morphological, electrophysiological, and molecular analysis (Benjamin, 2008; Fodor et al., 2020a; Fodor et al., 2021; Kemenes and Benjamin, 2009; Moroz, 2011; Rivi et al., 2020).

It was the mid-1950s when Tauc introduced the sea slug *Aplysia californica* in neuroscience research. His studies inspired the investigation of several laboratories from the 1960s and 1970s which resulted in the identification of a core role for synaptic plasticity in learned behaviours (Castellucci et al., 1970; Sweatt, 2016). Because molluscan behaviours are regulated by a small number of large and accessible neurons, it has been possible to record directly from the circuits that underpin cognitive function (Kandel, 2016), revealing conserved cellular and molecular mechanisms that also occur in more complex organisms. In particular, these studies demonstrated that after training, short-term memory (STM) is consolidated into long-lasting memory: intermediate-term memory (ITM), which persists for 3-4h and is later transformed into long-term memory (LTM), lasting longer than 5h.

LTM differs from STM in requiring both *de novo* gene expression and new protein synthesis to be formed (Kandel et al., 2001). ITM, instead, is dependent on new protein synthesis but not altered gene activity (Emptage and Carew, 1993; Lukowiak et al., 2000; Braun and Lukowiak, 2011).
Since the initial studies on the identification of transcription factors required for LTM, it has emerged that the LTM formation resulting from exposure of *Aplysia* to repeated pulses of serotonin (5-HT) is activated by cAMP-dependent mechanisms and is mediated by the cAMP-response element-binding proteins (CREB) (Dash *et al.* 1990; Bourtchuladze *et al.* 1994; Yin *et al.* 1994; Bartsch *et al.* 1995; Silva *et al.* 1998; Scott *et al.* 2002; Yin and Tully 2006; Alberini 2009; Kandel 2012). Further studies from *A. californica* and *D. melanogaster*, indicated that the activation of the cascade cAMP-protein kinase A (PKA)-CREB is critical for plasticity and LTM formation (Yin *et al.* 1994; Kandel 2012; Perazzona 2004). Specifically, cAMP-PKA activation initiates short-term synaptic changes that subsequently link via nuclear translocation of PKA, MAPK (mitogen-activated protein kinases), and perhaps other kinases to the activation and recruitment of CREB proteins and gene transcription (Bacskai *et al.* 1993; Martin *et al.* 1997; Ch'ng *et al.* 2012). Most of these mechanisms have been conserved in the mammalian brain (Benito and Barco 2010; Barco and Marie 2011). This supports the notion that *L. stagnalis* has solid genetic and epigenetic background to investigate the cellular and molecular mechanisms of learning and memory.

Building on the findings obtained in *A. californica*, several laboratories have been set up in Europe using the pond snail *L. stagnalis* to further the knowledge on learning-related processes induced by different associative paradigms, establishing this species as a valid model for learning and memory research.

Rationale for the choice of the model system: the great potential of the pond snail Lymnaea stagnalis

Only a limited number of animal species meet the requirements to be model organisms in multiple biological disciplines. One of these is the pond snail *L. stagnalis* (**Figure 1**). Extensively used since the 1970s for studying fundamental mechanisms in neurobiology, the value of this pulmonated gastropod has been also recognized in ecotoxicology, host-parasite interactions, genome editing and 'omics', as well as human disease modelling (Rivi et al., 2020).

L. stagnalis is light to dark brown in colour and possesses a spiral shell long up to 55 mm (Benjamin, 2008). It lives in stagnant and shallow waters rich in vegetation (Lance et al., 2006) and is active all year round, even when there is a layer of ice on the water in wintertime. However, in the wild, the typical reproductive period goes from spring to late autumn (Nakadera et al., 2015). Snails develop in 2 weeks inside the eggs and, unlike the established models *D. melanogaster* and *C. elegans*, have a relatively long average life span. Under controlled laboratory conditions, *L. stagnalis'* life expectancy varies from one to two years depending on the environmental conditions (e.g., temperature, density, and salinity), whereas in wild conditions specimens can live for at least 2.5 years (Fodor et al., 2020).

The direct development and longevity of *L. stagnalis* allow the identification of age "stages" that could be connected to age-related modifications that, in turn, may involve genetic, molecular, and cellular mechanisms which usually take time to manifest their effects.

Figure 1. The pond snail, *L. stagnalis*.
Picture of an adult snail (8 months old). The shell is large with a massive body spiral and a small, relatively sharp spire. The surface is smooth, slightly shiny, and lacking the distinct spiral striae of related species. Despite its size, the shell is fragile. The colour pale is brown. The body length is between 3.5 and 4 cm.

The relatively simple CNS of *L. stagnalis* consists of approximately 25000 neurons (**Figure 2**), organized in a ring of eleven interconnected ganglia.

These neurons are large (diameter from 5 to 150 µm) and can be easily identified and analysed as part of specific circuits, allowing electrophysiological dissection of the networks involved in relatively simple rhythmic behaviours, such as feeding and aerial respiration (Ter Maat, 1992; Whelan and McCrohan, 1996; Jones, Kemenes and Benjamin, 2001; Jones et al., 2003; Feng et al., 2009).

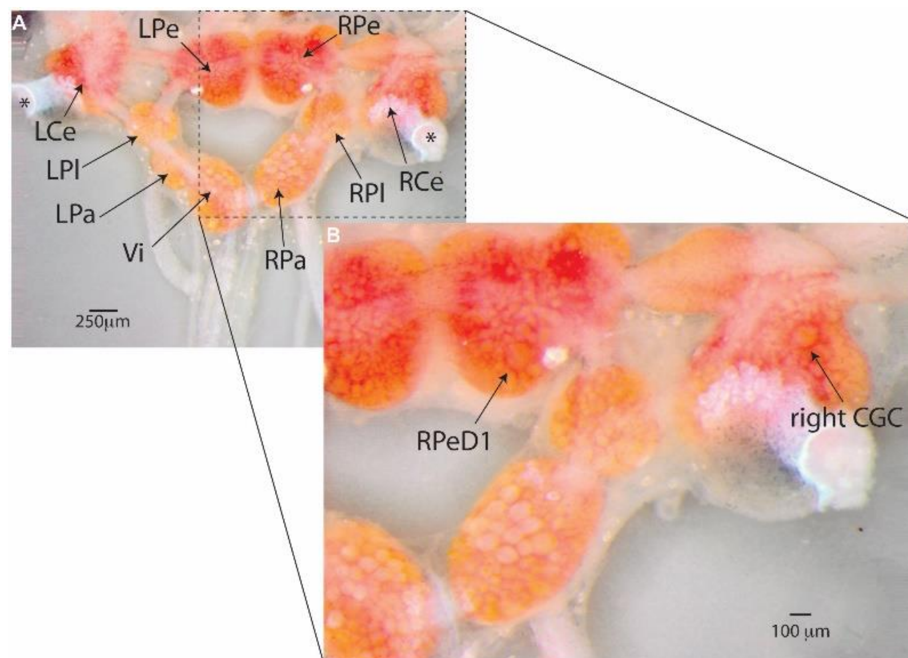


Figure 2. *L. stagnalis'* CNS and its identified neurons.

(**A**) *L. stagnalis'* CNS consists of nine ganglia plus two buccal ganglia that are not part of the inner ganglionic ring (LCD, left cerebral ganglion; RCe, right cerebral ganglion; LPe, left pedal ganglion; RPe, right pedal ganglion; LPl, left pleural ganglion; RPl, right pleural ganglion; LPa, left parietal ganglion; RPa, right parietal ganglion; Vi, visceral ganglion; the paired left and right buccal ganglia are not shown in this image).

(**B**) Enlargement showing the location of two identified neurons. Right pedal dorsal 1 (RPeD1) is a multi-modal neuron involved in the control of *L. stagnalis'* aerial respiration and an important locus of operant-conditioning induced plasticity of the animals' respiratory behaviour. The cerebral giant cell (CGC) is one of a bilaterally symmetrical pair of interneurons instrumental in the control of *L. stagnalis'* feeding behaviour. From Hermann et al., 2014

These rhythmic behaviours are induced by groups of central pattern-generating neurons (CPGs) (Katz, 2016), whose characterization is critical for understanding where and how the nervous system controls them and how the interplay between CPGs and external stimuli participates in the production of adaptive learned behaviours (Syed et al., 1990; Yeoman, Brierley and Benjamin, 1996; Spencer, Syed and Lukowiak, 1999; Spencer et al., 2002; Straub, 2004).

These CPG circuits, in fact, can be plastically reconfigured via environmental changes, experiences, and conditioning procedures to optimize the output to meet specific behavioural demands (Katz, 2016).

Moreover, the neuronal plasticity exhibited in the CPG circuits plays an important role in regulating the initiation and temporal output of behavioural rhythms in response to rewarding/aversive stimuli (as occurs in classical conditioning) and action-outcome contingencies (as occurs in operant conditioning) (Kojima et al., 1997). Using *in vitro* and semi-intact preparations (which allow monitoring the behaviour and neural activity simultaneously), the CPGs controlling feeding and aerial respiration have been well studied in *L. stagnalis* and the learning-induced changes have been elucidated with cellular precision (Lukowiak, 1991; Kemenes et al., 1997; Spencer et al., 1999, 2002; Lukowiak and Syed, 1999; McComb, 2005).

Studies such as these cannot easily be performed in most vertebrate preparations because their behaviours are more complex, and the underlying neuronal circuitries are more difficult to access for direct cellular and synaptic analyses (Kemenes, Staras and Benjamin, 1997).

Noteworthy, by using a combination of cell culture and *in vivo* transplantation techniques, Syed *et al.* (1990, 1992) demonstrated both the sufficiency and necessity of the three-neuron CPG to drive aerial respiratory behaviour. Few, if any, other neural circuits meet both the sufficiency and necessity criteria. This finding is one of the main reasons for moving from *A. californica* to *L. stagnalis* for studying learning and memory. In fact, because aerial respiratory behaviour can be operantly conditioned and consolidated into LTM, studies using *L. stagnalis* allow detecting the causal neuronal mechanisms of learning and memory directly.

An additional advantage offered by *L. stagnalis* is that its neurons are unipolar and the single process (i.e., the primary neurite) emerging from the soma is the site where most synaptic interactions and normal neuronal activities and behaviours are mediated (Syed et al., 1990, 1992; Scheibenstock et al., 2002). Fascinating is the finding that the primary neurite can not only survive after the surgical ablation of the soma but is also competent to synthesize new proteins without the soma being present (Spencer et al., 2000). This discovery allowed to distinguish between the sites (i.e., neurites and soma) in which memories are processed (Scheibenstock et al., 2002): following the removal of the soma, in fact, LTM (which is dependent on new protein synthesis and altered gene activity) cannot be formed, whereas ITM (which is dependent on new protein synthesis but not altered gene activity) can (Scheibenstock et al., 2002).

Thus, soma-less neurites can mediate associative learning and ITM, but not LTM. On the other hand, if the soma is ablated after LTM consolidation has occurred, LTM can still be accessed, suggesting that the soma is a site of LTM formation. These characteristics of *L. stagnalis*' neurons differ from most vertebrate and mammal preparations, where disruption of the neuronal soma usually causes the death of the entire cell (Saleuddin and Mukai, 2017).

Furthermore, the large neurons of *L. stagnalis* offer a great amount of biological material for molecular, morphological, and functional analyses, which can lead to the validation of the function of specific genes and the study of the molecules and metabolic pathways involved in neuronal regeneration (Hermann et al., 2000; Koert et al., 2001), synapse formation (Syed et al., 1992; Feng et al., 1997; Gardzinski et al., 2007), synaptic plasticity (Smit et al., 2001), neurodevelopment (Croll, 2000), ageing (Wildering et al., 1991; Klaassen et al., 1998; Patel et al., 2006), adaptive responses to stress (Hermann et al., 1998; Fei et al., 2007), and learning and memory (Benjamin et al., 2000; Lukowiak et al., 2003).

In particular, the study of the quantitative changes in gene expression induced by appetitive classical conditioning permitted to elucidate which molecules participate in the dialogue between the synapse and the nucleus and vice versa during memory and learning (Rivi et al., 2020). This created an integrated picture of the processes underlying learning and memory, such as consolidation, reconsolidation, extinction, and forgetting.

Memory formation after single-trial chemical appetitive classical conditioning involves N-methyl-D-aspartate (NMDA) receptors; the activation of PKA, mitogen-activated protein kinase (MAPK), Ca2+/calmodulin-dependent protein kinase II (CaMKII), and nitric oxide/cyclic GMP (NO/cGMP) signalling; the activation of CREB1 and parallel suppression of CREB2; regulation of gene expression by CREB1; and new protein synthesis (e.g., CCAAT enhancer-binding protein; IGF2) (Kemenes, 2008; Rosenegger, Wright and Lukowiak, 2010; Korneev et al., 2018; Rivi et al., 2020). The molecular machinery involved in LTM formation in *L. stagnalis* is summarized below (**Figure 3**).

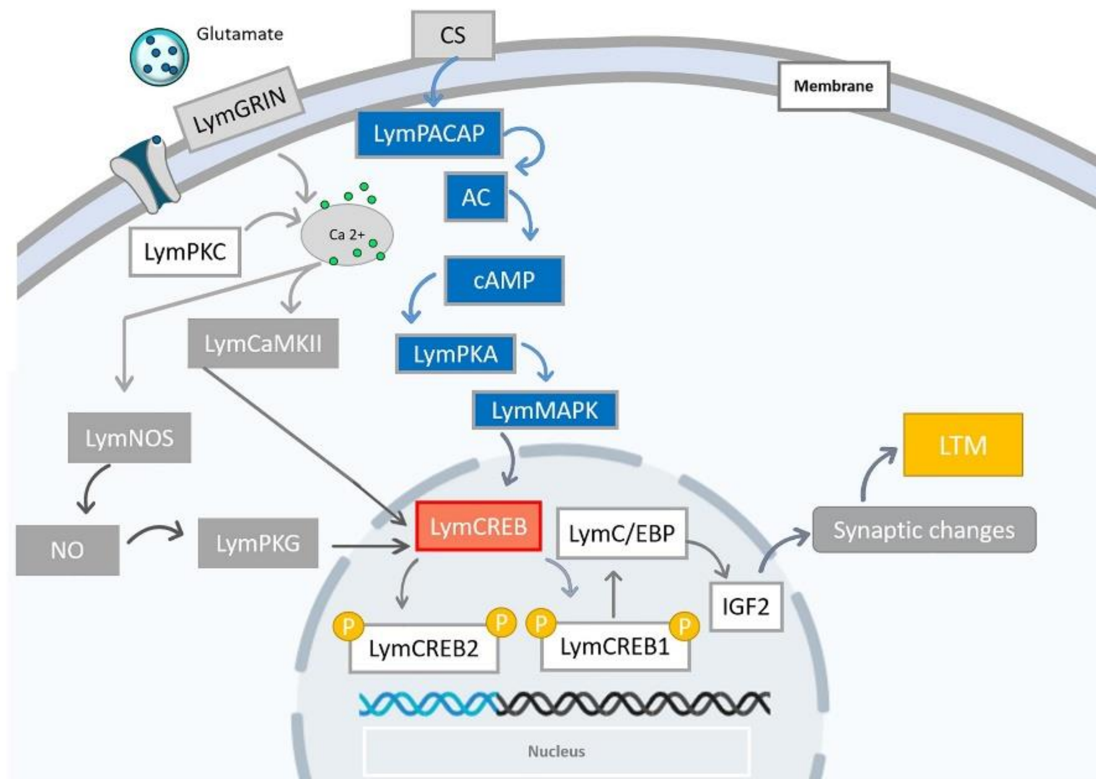


Figure 3. Schematic representation of the signalling molecules, receptors, and pathways involved in LTM formation after food-reward classical conditioning in *L. stagnalis*

Lym CREB-driven transcription results downstream of (1) the activation of cAMP which, in turn, mediates almost all its actions through the activation of *Lym* PKA and the subsequent activation of *Lym* MAPK; (2) the entrance of Ca2+ through NMDARs, that activates directly or indirectly numerous protein kinases, including PKC and *Lym* CaKMII, together with *Lym* NOS. *Lym* NOS, for its part, promotes the synthesis of NO, which regulates *Lym* CREB activation, acting via *Lym* PKG. After getting phosphorylated by PKA, *Lym* CREB1 initiates a cascade of altered gene activity and new protein synthesis (e.g., CCAAT enhancer-binding protein; IGF2), necessary for synaptic enhancement in memory consolidation. In contrast, *Lym* CREB2, which basically inhibits the function of *Lym* CREB1, is inactivated by *Lym* MAPK. In fact, the ratio of activator/repressor *Lym* CREBs has been proposed to act as a "molecular switch" in determining whether LTM is formed. Even if *Lym* PKA, *Lym* NMDA receptors, *Lym* CaMKII, *Lym* CREB, and *Lym* NOS/NO are selectively activated or upregulated, it seems likely that these and other signalling molecules are part of a synergistic effort and together contribute to the memory consolidation process, with none of them alone being sufficient for LTM. From Rivi et al., 2020.

These molecular pathways provided further evidence that the mechanisms of learning and memory are conserved across phylogenetic groups in a variety of learning paradigms, including non-associative or associative learning, and operant or classical conditioning (Benjamin and Kemenes, 2013; Fulton et al., 2005; Josselyn and Nguyen, 2005; Kemenes et al., 2002; Marra et al., 2013; Michel et al., 2008; Nikitin et al., 2008; Park et al., 1998; Pirger et al., 2010; Pirger et al., 2014; Ribeiro et al., 2003; Sadamoto et al., 1998; Sadamoto et al., 2010; Schacher et al., 1988; Vigil and Giese, 2018; Wan et al., 2010). Because the genome and transcriptome of *L. stagnalis* have been sequenced but the gene characterization has not yet been performed, the molecular information currently available has been obtained by cloning of partial cDNA sequences, or by recurring to *in situ* hybridization and immunohistochemistry.

As the lack of molecular information has so far delayed the progress of research in *L. stagnalis* (Rivi et al., 2020), **the first aim** of my project has been the development of a robust methodological approach for identifying and characterizing putative transcripts in *L. stagnalis* CNS. Instead of generating a new transcriptome, the unannotated draft genome combined with the transcriptome dataset has been used to screen for conserved sequences. This multidisciplinary approach based on bioinformatics and molecular studies led to the identification and characterization of new putative transcripts coding for the homologues of each enzyme of the KP in the ganglia of *L. stagnalis*.

What can we teach L. stagnalis?

Classical and operant conditioning paradigms of learning and memory

Learning about the predictive association between events and the consequences of specific behaviours is indispensable for animals to adapt and survive in complex and ever-changing environments. Remembering these associations, animals alter their behaviour appropriately and this alteration can be defined as learning (Dalesman and Lukowiak, 2012; Lapiedra et al., 2017).

While non-associative learning (i.e., habituation and sensitization) is the simplest and most primitive form of learning, associative learning is more complex and requires that stimuli occur in close temporal contiguity and fixed sequence (Byrne and Hawkins, 2015). Among the various categories of associative learning, classical and operant conditioning are the best known and well-studied. The core of classical conditioning (i.e., Pavlovian conditioning) lies in the temporal-contingent association between two stimuli: an initially neutral stimulus (i.e., the conditional stimulus, CS) and a biologically relevant stimulus (i.e., the unconditional stimulus, US). By the temporal and forward pairing of the CS with the US, the CS comes to evoke a response like that evoked by the US (Pavlov, 1927; Walters, Carew and Kandel, 1979). In operant conditioning, the frequency of a behaviour is increased or decreased (depending on the reinforcement used) by the consequences of the behaviour.

Reinforcement can either be negative, leading to a decrease in the frequency of the behaviour or positive, increasing the frequency (Brembs, 2003). Thus, operant conditioning is concerned with an association between the behaviour of an organism and its environment.

Although there are large phylogenetic differences and extensive variability in the neural organization in the animal kingdom, the cellular and molecular basis of learning and memory have been highly conserved (Byrne and Hawkins, 2015).

"What can we teach *L. stagnalis*?" This is the question answered by the researchers who developed the variety of sensitive, solid, easily reproducible, and simple behavioural procedures currently used to uncover *L. stagnalis*' memory abilities. These paradigms are summarized in the following sections.

Appetitive classical conditioning of the feeding behaviour

Feeding is a rhythmic behaviour that has proven to be remarkably useful to investigate both reward (Alexander et al., 1984; Kemenes and Benjamin 1989) and aversive classical conditioning (Kojima *et al.* 1996). *L. stagnalis*' feeding behaviour shares with vertebrates important aspects, such as a strong dependence on external and internal variables and stimulus generalization and discrimination (Kemenes and Benjamin 2009). In classical appetitive food-reward conditioning, there is a temporal-contingent repeated presentation of a neutral (i.e., which does not elicit feeding) CS with the US that elicits feeding (Kemenes and Benjamin 1989). Sucrose is typically used as the US, whereas tactile, chemical, or visual cues are all used as the CS.

In one of the first associative learning and LTM experiments using *L. stagnalis*, a single pairing of amyl acetate (i.e., the CS) with sucrose (i.e., the US) was sufficient to create an LTM trace that persisted for at least 15 days (Alexander, Audesirk, and Audesirk 1984).

Aversive classical conditioning of feeding (conditioned taste aversion)

A negative experience with food usually results in an aversive learned behaviour, called conditioned taste aversion (CTA), which results in a hedonic shift from positive to negative in the preference for that food (Chambers, 2018; Nakai et al., 2020). Standard CTA has been shown to occur in *L. stagnalis* and requires close temporal contiguity between the presentation of an appetitive food substance (i.e., the CS) and the application of a negative stimulus (i.e., the US) (reviewed Rivi et al., 2021). Following successful conditioning, the CS no longer acts as an appetitive stimulus, and this CTA persists as an LTM for more than a month. Whether CTA learning and subsequent memory formation are formed is dependent on the snail's internal state. The best CTA occurs when snails are trained after one day of food deprivation, suggesting that a modest level of food deprivation acts as a motivating factor for memory formation (Ito *et al.* 2015). On the other hand, if snails are severely food-deprived hunger triumphs over the memory and animals continue to respond to the CS (Ito *et al.* 2015). Further studies demonstrated that *L. stagnalis* can distinguish between appetitive stimuli during CTA and can even acquire second-order conditioning (Sugai et al., 2006), where the memory for one taste that initially elicited feeding response can be used following CTA, as a negative reinforcer for a second appetitive taste.

A 'special' example of CTA is the bait-shyness or Garcia effect.

While for the 'standard CTA' to occur there is a requirement for close temporal contiguity between the presentation of an appetitive food substance and the application of a stimulus that suppresses feeding, the acquisition of the Garcia effect depends on animals experiencing a visceral sickness (e.g., nausea). In both cases, the snails learn to avoid a taste that initially they were drawn to.

As no previous studies demonstrated the Garcia effect in any invertebrate model, **the second aim** of my thesis was to develop a behavioural paradigm to investigate whether *L. stagnalis* can form it.

Operant conditioning of the aerial respiratory behaviour

Compared with vertebrates, relatively few protocols for operant conditioning have been developed in invertebrates (Carew 1996; Lukowiak *et al.* 1996). The operant conditioning of the aerial respiratory behaviour in *L. stragnalis* is a fascinating exception and, since 1996 has provided insight into basic and advanced cognition (Dickinson, 1987). *L. stagnalis* breathes bimodally, using both cutaneous and aerial respiration, thus aerial respiration can be temporarily inhibited or suppressed without jeopardizing the viability of the animal. Furthermore, the behaviour is mediated by a three-neuron CPG (Syed et al., 1990, 1992). One of the CPG neurons, RPeD1, the neuron that initiates rhythmogenesis, is necessary for LTM formation, extinction, memory reconsolidation, and forgetting (Scheibenstock et al., 2002; Sangha et al., 2003a, c, 2005). Hence, *L. stagnalis* represents an excellent model system to deal with what Prof Lukowiak referred to as the "Holy Grail of memory formation": understanding where and how memory is formed (Linden, 2003). According to the highly standardized and solid behavioural procedure developed by Lukowiak *et al.* (1996), both the training and the memory phases are performed in hypoxic pond water (as hypoxia increases the aerial respiratory behaviour) and each time the animal attempts to open the pneumostome, a tactile stimulus (i.e., the negative reinforcement) is presented to the pneumostome area. Following a successful operant conditioning procedure, the number of attempted pneumostome openings recorded during the memory test is significantly reduced compared to the training session(s). Associative learning, ITM, and LTM can be differentially produced by altering the duration, the number, and the interval between the training sessions (Lukowiak *et al.* 2000). This behavioural paradigm of learning and memory is extremely useful for understanding how behaviourally relevant stressors and bioactive compounds alter LTM formation and/or its strength/persistence. Typically, inbred laboratory-reared snails require two 0.5h training sessions separated by a 1h interval is to form an LTM that persists for at least 24h (Lukowiak et al., 2000). However, the ability to form and recall LTM in *L. stagnalis* can be enhanced by several different stressors (e.g., heat shock and predator scent) as well as various biologically active substances (e.g., cocaine, ketamine, methamphetamine, and epicatechin). Each enhancer causes LTM formation after only a single 0.5h training session.

Configural learning

L. stagnalis is also competent to exhibit configural learning, a higher-order form of learning and memory consisting in the ability to assign importance to different stimuli (Swinton *et al.,* 2019). In particular, following the simultaneous exposition to an appetitive taste with the predator effluent. Thus, the appetitive food acquires a new motivational state (i.e., elicited anti-predator behaviours) as opposed to its intrinsic motivational state (i.e., enhanced rasping). This higher-order conditioning is more 'cognitive', as the animal's behaviour depends on its ability to make meaningful comparisons between current sensory stimuli and its representation of previous sensory experiences (Sahley *et al.*, 1981; Hawkins *et al.,* 1998; Giurfa, 2003, 2007; Devaud *et al*., 2015; Onuma and Sakai, 2016).

What can L. stagnalis teach us?

New frontiers in neuroscience research

The highly reproducible and solid behavioural tests raised the question: 'What can *L. stagnalis* teach us?' In other words, how *L. stagnalis* and its nervous system, can help to understand the flow of neural transformation from behavioural output to sensory coding in more complex systems like the mammalian brain?

In snails, as in mammals, the success of classical conditioning training depends on both internal (e.g., food deprivation/satiety and ageing) and external variables (e.g., water conditions) (Kemenes and Benjamin, 1994; Murakami et al., 2013). Hermann and colleagues (2007) demonstrated memory retention and consolidation are progressively impaired with advancing age (Herman et al., 2007). Interestingly, the memory decline occurring in aged *L. stagnalis* is consistent with age-dependent impairment in learning and memory functions observed in many animal species, including humans (Hermann et al., 2007). In this context, *Lymnaea* represents a versatile model of ageing (Fodor et al., 2021) and provides a great opportunity to investigate the footprint of ageing at all levels of biological organization (Janssens and Houtkooper, 2020; Partridge et al., 2020). Two recent studies have identified several evolutionarily conserved sequences in *L. stagnalis*, such as klotho, huntingtin, presenilin, DNMT1, and RbAp48/RBBP4, that are associated with normal and pathological ageing and age-related memory impairment in vertebrates, including humans (Fodor et al., 2020b; Fodor et al., 2021).

L. stagnalis is also 'teaching us' the role of context in memory formation and consolidation. Typically, snails trained in one context only showed memory if memory testing was performed in the same context. Similar results have been obtained in other invertebrate models such as *A. californica, C. elegans,* honeybees, *Drosophila*, and ants (Ardiel and Rankin, 2010; Boset al., 2010; Kahsai and Zars, 2011; Fujinaka et al., 2016; Panoz-Brown et al., 2016; Giurfa, 2017). However, snails trained on consecutive days in two different contexts have the ability to remember in both contexts (Haney and Lukowiak, 2001).

Moreover, *L. stagnalis* has been used to demonstrate for the first time the concept of an unfaithful memory (i.e., memory infidelity) in a molluscan model system and for a non-declarative memory in any model system. These studies are consistent with those from mammals, showing that activated memory can be updated and reconsolidated.

Depending on the context in which the reconsolidation occurs, there can be consequences on the accuracy of memory, resulting in what is called 'memory infidelity' (Lukowiak et al., 2007). These findings on memory reconsolidation have triggered great interest among mental health professionals who treat disorders based on pathological memories (Alberini and Ledoux, 2013).

In recent years, memory reconsolidation has been adopted in therapeutic settings to make learning and memory creation more efficient and adaptive, to prevent or rescue memory impairments, and to ameliorate maladaptive memories linked to psychopathologies, such as those associated with post-traumatic stress disorders and addiction (Bisaz et al., 2014). Although many such studies have focused on humans and rodents, a translational approach based on *L. stagnalis* may be a rapid and cost-effective option for elucidating the causal, neuronal and molecular changes underlying memory at the level of a single cell. *L. stagnalis* is also 'teaching us' how memory formation is affected by environmental stress. Stress is defined here as a state that requires a physiological and/or behavioural readjustment or modification to maintain the well-being of the organism. Since Bacon (Bacon, 1620) it has been known that stress affects learning and memory. As exemplified in the 'Yerkes–Dodson Law' (Brown, 2020), too much or too little stress impedes LTM formation, while 'just the right amount' of stress enhances LTM. As LTM formation requires 'neuronal cost' (in terms of altered gene activity and new protein synthesis), organisms invest energy only for 'relevant' events. This relevancy is in part determined by the level of stress perceived at the time of learning. However, the same stimulus may be perceived as a stressor for one organism but not for another, or only at certain times and not at others in the same organism. Because of the complexities of the mammalian brain and the multitude of behaviours tested, in addition to the different ways in which stressors act, is not surprising that there is disagreement in the literature on the role that stress plays in learning and memory formation. In this contest, studies using *L. stagnalis* may help to decipher how stress affects memory at both the behavioural and neuronal levels.

Strain-specific differences of the effects of stress on memory in L. stagnalis

Populations within *L. stagnalis* species may vary greatly in their responses to environmental stress, reflecting adaptation to local conditions. Because stress affects cognition, this variation in response between geographically distinct strains may result in differential learning abilities to form memory in response to the same stressor (Hughes et al., 2017; Shymansky et al., 2017; Rothwell et al., 2019). To date, most of the knowledge about memory formation and factors that affect it in *L. stagnalis* has been carried out using an inbred strain that has been reared in laboratory standardized conditions over many generations. This laboratory-reared strain (herein named 'W strain') was originally collected from a polder near Utrecht, the Netherlands, in the 1950s, and has been laboratory-reared since then.

Most of the wild strains of *L. stagnalis* sampled exhibit learning and memory-forming capabilities identical to those of the W strain. However, some freshly collected populations have higher LTM-forming capabilities. Furthermore, 'average' W snails require exposure to stress for optimal memory formation, whereas in 'smart' snails the same outcome occurs under control conditions without any external stress applied (Orr et al., 2009). Using relevant stressors that snails are likely to encounter in their natural environment, it is possible to study how stress can alter LTM formation and/or its persistence in both 'smart' and 'average' snails.

Effect of heat stress on behavioural and cognitive functions: predicting the impact of the current climate change

Among the various stressors, attention should be paid to heat. Global warming in recent decades has turned the spotlight on the impact of thermal variations on the aquatic environment. As poikilothermic ectotherms, molluscs are dependent on the thermal conditions of their environment. Perturbations of water temperatures are perceived as stressful events, requiring behavioural and physiological adjustments to maintain homeostasis. The influence of thermal perturbations depends on the extent of the variation and its duration. However, across animal *taxa*, the increase in environmental temperature affects physiological, psychological, behavioural, and cognitive functions (Selye, 1973; Salo et al., 2017; Abram et al., 2016; Zhang et al., 2018). In this complex picture, investigations into the thermal tolerance limits of molluscs are necessary for a holistic understanding of the ecology of a species or population and to predict and infer the impact of the current climate change.

A common limitation of many thermal studies is their emphasis on organismal survival, whereas the additive and interactive effects of temperature on respiration, foraging/feeding, locomotion, predator-prey relationships, and cognitive abilities are poorly understood, limiting the ability to predict the effects of the global change. Moreover, the upper and lower heat thresholds governing these functions are dependent on both the thermal/physiological history of the organism being studied, as well as the conditions in which it has been raised and maintained. In this context, *L. stagnalis* serves as an optimal model system to answer some of the questions about thermal tolerance and the effects of heat stress on cognitive functions (Lukowiak et al., 2010; Hughes et al., 2017; Shymansky et al., 2017; Fodor et al., 2020; Rivi et al., 2020; Rivi et al., 2021a; Rivi et al., 2021b).

In the laboratory, the maximum growth rate of *L. stagnalis* is achieved between 11 and 28°C (Vaughn, 1953) and the lowest mortality occurs between 15.7 and 20.1°C (McDonald, 1969). On the other hand, wild snails typically face broad temperature fluctuations depending on prevailing weather conditions over both daily and seasonal time courses (Brown, 1979).

Thus, the different thermal environmental conditions of the laboratory and wild may induce different behavioural responses to the same heat stressor. In this scenario, the highly reproducible and solid learning paradigms available in *L. stagnalis* can provide a strong measure of the effect of the current rise in temperatures on animals' cognitive functions and behaviours.

Thus, the **third aim** of my thesis has been the study of the effects of different thermal treatments and durations (which mimic the thermal fluctuations occurring in wild environments) on both laboratory-reared and field-collected *L. stagnalis* to elucidate how different are inbred strains from the natural populations as a result of different selection pressures influencing their sensitivity to thermal fluctuations. Moreover, investigating whether the memory abilities of wild snails and the responses to stressors had been conserved in successive generations reared in the laboratory, may open new scenarios on the genetic or epigenetic basis to these abilities and responses (Orr et al., 2009; Dalesman et al., 2011).

Effects of bioactive compounds on cognitive functions

Memory formation, retention, and recall are dynamic processes and can be modified not only by environmental stressors but also by lifestyle choices. One key lifestyle choice that may impact memory formation and retention is diet. In this context, *L. stagnalis* has been recognized as a useful organism to examine the effects of bioactive compounds, like flavonoids, on learning and memory.

Flavonoids are a group of phytochemicals found in plants that have been associated with a cognitive enhancement in a wide variety of species including invertebrates (Swinton et al., 2018).

Studies from *L. stagnalis* indicated that the flavonoid (-)-Epicatechin, can enhance LTM formation and reverse memory obstruction caused by stress (Swinton et al., 2018). The positive cognitive effects of flavonoids in mammals have been attributed to the protection of neural functioning, stimulation of neuronal regeneration, and increased blood flow to the brain.

Another important flavonoid is quercetin (Q), whose promising bioactive effects include antidiabetic, anti-inflammatory, antioxidant, antimicrobial, anti-Alzheimer's, antiarthritic, cardiovascular, and wound-healing effects. Moreover, growing evidence underlies that the dietary consumption of flavonoids may improve memory and neurocognitive performances. However, the actions that these bioactive compounds have in improving cognitive functions are still unclear.

That raises the question of whether quercetin would exert similar learning and memory-enhancing effects in *L. stagnalis*. The ability of quercetin to enhance operant conditioning LTM has been the **fourth aim** of my thesis.

Bridging the gap between in vitro studies and research on mammals

The findings presented in this thesis illustrate how a reductionist, but not simplistic, approach based on the use of a simple model system such as *L. stagnalis*, might be useful to address fundamental questions in Neuroscience. Although the use of a reductionist approach in the 21st-century may be an arduous undertaking for some researchers, it is important to remember that if neurological functions are common to all animals with a complex nervous system, conserved processes must exist in the underlying molecular mechanisms that can be studied more effectively in simple invertebrate animals. The ultimate and future goal of this project is to "*translate*" data obtained in *L. stagnalis* with that of more complex organisms until arriving at human beings.

AIM 1

A bioinformatics and molecular approach for the identification of the kynurenine pathway in the CNS of L. stagnalis

Background

The kynurenine pathway (KP) has been studied in detail since the beginning of the 20th century, when it was primarily viewed as the synthetic route to nicotinamide and hence nicotinamide adenine dinucleotide (NAD+), which is a key co-factor for numerous enzymes (Muneer 2020). As many of the major enzymes in the KP are vitamin B6-dependent, the molecular cascade was also studied for elucidating the link between the dietary availability of vitamin B6 and the tryptophan metabolism (Badawy *et al.* 2016). No specific neurobiological activities were demonstrated for the KP until 1978 when it was shown for the first time that several metabolites of the pathway cause convulsions when injected directly into the brain (Lapin *et al.* 1996). At that time, it was already known that NMDA receptors can induce an excitotoxic neuronal loss in the CNS (Schwarcz and Stone 2017), suggesting that an overexcitation of these receptors plays a role in the etiology of neurodegenerative disorders (Dong and Dong 2008). It was during a screening of putative NMDA agonists that quinolinic acid, a major component of the KP, was identified as an endogenous, selective agonist of NMDA receptors (Lapin *et al.* 1996). Consistently with this agonistic activity, quinolinic acid was then found to cause excitotoxic neuronal damage and degeneration (Guillemin 2012; Baranyi *et al.* 2015; Lovelace *et al.* 2017). By testing other compounds of the KP, it was found that kynurenic acid acts as a glutamate antagonist, blocking the actions of NMDA receptors, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid, and kainic glutamate receptors as well (Guillemin 2012; Albuquerque and Schwarcz 2013). In line with the demonstration that NMDA receptor antagonists prevent excitotoxicity (Schwarcz *et al.* 1983; Schwarcz and Stone 2017), kynurenic acid was then shown to possess marked neuroprotective effects (Wirthgen *et al.* 2018).

In those same years, attention was also focused on the biological functions of one of the rate-limiting enzymes of the KP, IDO, which converts tryptophan to L-kynurenine.

As Pfefferkorn observed in 1984, IDO can be activated by interferon-ɣ during the immune response to an infection and it inhibits the detrimental effects of the infectious agent (Pfefferkorn 1984).

This discovery was followed by numerous studies which showed that an interaction exists between inflammation and the KP in neurological and psychiatric disorders (Fujigaki *et al.* 2019).

Research on the physiology and pharmacology of brain kynurenines has made great progress since these initial studies (Schwarcz and Stone 2017). Today it is known that kynurenic acid is also an inhibitor of the α7 nicotinic acetylcholine receptor, which, in turn, results in the reduced release of neurotransmitters such as glutamate, acetylcholine, and dopamine, each of which has a critical role in cognitive processes (Albuquerque and Schwarcz 2013). On the other hand, excesses of quinolinic acid are also responsible for energy deficit, oxidative stress, and cellular death. Other metabolites, including 3-hydroxy kynurenine, 3-hydroxy anthralin acid, and anthranilic acid, instead, participate in complex pro-oxidative and anti-oxidative processes but have no direct effect on neuronal activity (Darlington *et al.* 2010). However, to date, the exact role of kynurenines on brain function is still not fully understood.

The kynurenine pathway (KP)

The KP is a complex multi-step cascade formed by the main branch and different lateral arms. It is responsible for the tryptophan degradation - more than 95% of dietary tryptophan is catabolized through the KP - into several neuroactive metabolites, known as kynurenines (**Figure 4**) (Savitz 2020). The initial KP metabolic step is the oxidative cleavage of tryptophan into N-formylkynurenine by IDO or tryptophan 2,3-dioxygenase (IDO). Next, N-formylkynurenine is metabolized by kynurenine formamidase to the pivotal KP intermediate L-kynurenine. Then, the KP metabolism segregates along several branches that give rise to the production of physiologically and neurochemically active kynurenine metabolites. The main route of the KP leads to the conversion of L-kynurenine into 3-hydroxykynurenine by the enzyme kynurenine 3-monooxygenase (KMO).

An additional lateral branch of the pathway leads to the formation of kynurenic acid - which is usually considered to be neuroprotective - by kynurenine aminotransferase isoenzymes (Allison and Ditor 2014). Continuing along the KMO-branch, 3-hydroxykynurenine is metabolized into 3-hydroxykynurenine.

The following reaction is catalyzed by the enzyme hydroxyanthranilate 3,4-dioxygenase and generates a highly unstable intermediate product, 2-amino-3-carboxymuconic-6-semialdehyde, which can spontaneously rearrange to form quinolinic acid or can be converted by 2-amino 3-carboxymuconate 6-semialdehyde decarboxylase (ACMSD) to picolinic acid (Foster 1983).

Quinolinic acid is normally presented in nanomolar concentrations in the human brain and cerebrospinal fluid, and it is further metabolized into nicotinic acid dinucleotide, which is further converted into NAD+.

Increased levels of quinolinic acid (0.3–10 mM), instead, can lead to excitotoxicity. Thus, ACMSD is a key enzyme responsible for maintaining the balance between quinolinic acid and picolinic acid, which has been suggested to exert a neuroprotective role (Thirtamara-Rajamani *et al.* 2013). Under normal conditions, the KMO branch seems to be the major metabolic route of the KP

because of its high affinity for L-kynurenine, whereas the metabolism via the KYNU branch only occurs when L-kynurenine is produced at elevated concentrations (Savitz 2020). Under inflammatory insults, instead, pro-inflammatory mediators like LPS may upregulate the activity of IDO, inducing a severe increase of the level of the kynurenine in brain tissues (Wang *et al.* 2010).

Thus, the kynurenine pathway can orchestrate the complex interactions between the central nervous- and immune systems (Golia *et al.* 2019).

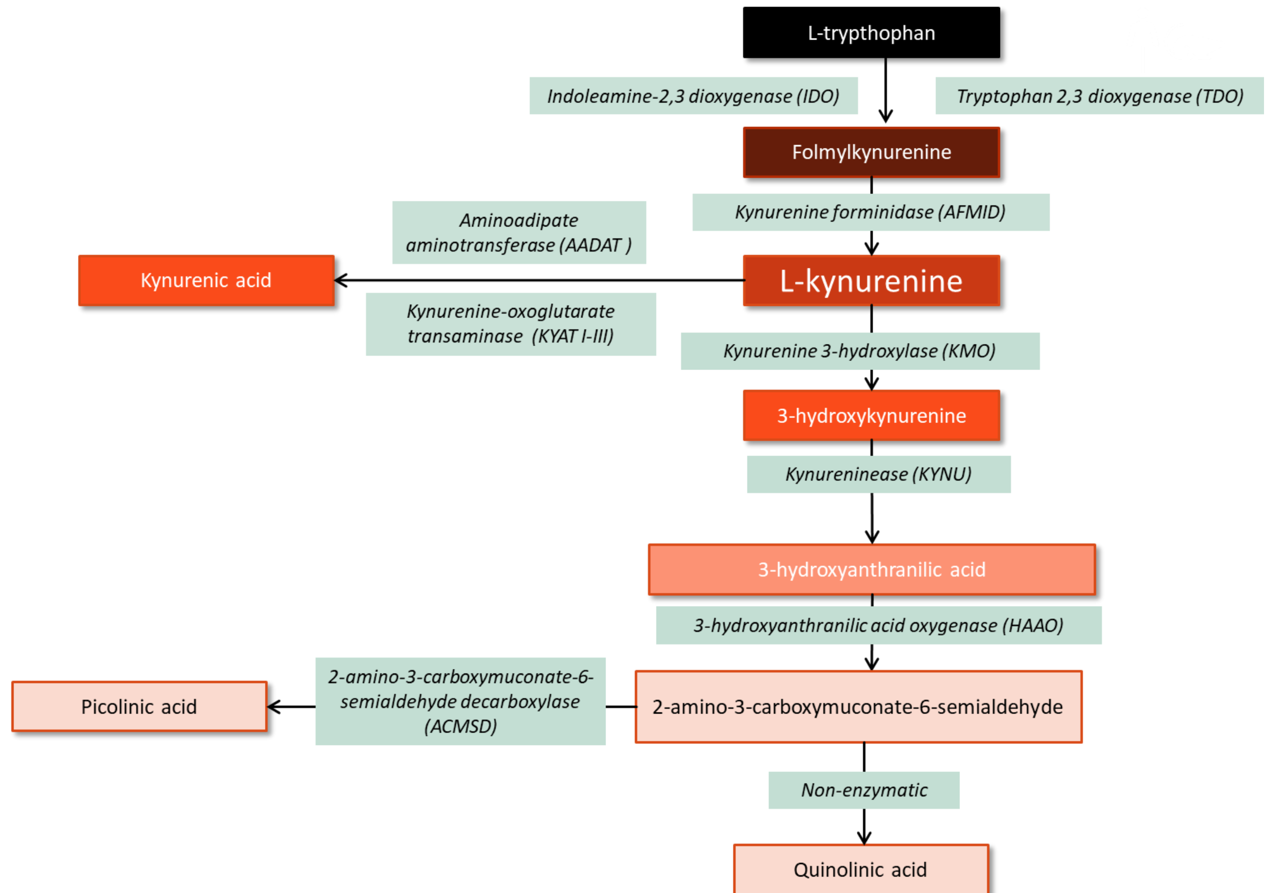


Figure 4. Simplified illustration of the kynurenine pathway (KP)

L-Tryptophan is predominantly converted into formylkynurenine by indoleamine 2,3-dioxygenase (IDO) and tryptophan dioxygenase (TDO). Then kynurenine formamidase (AFMID) hydrolyzes formylkynurenine into L-kynurenine, which, in turn, can be transamined into kynurenic acid by kynurenine aminotransferase isoenzymes (kynurenine-oxoglutarate transaminase (KYAT I-III) and aminoadipate aminotransferase – (AADAT)), or can be metabolized by hydroxylation into 3-hydroxykynurenine by kynurenine 3-monooxygenase (KMO). 3-hydroxykynurenine is then hydrolyzed into 3-hydroxyanthranilic acid by kynureninase (KYNU). Then the enzyme hydroxyanthranilate 3,4-dioxygenase (HAAO) converts 3-hydroxyanthranilic acid into a highly unstable intermediate product, 2-amino-3-carboxymuconate-6-semialdehyde. The pathway favors its nonenzymic cyclization to quinolinic acid. However, the enzyme 2-aminomuconic acid semialdehyde dehydrogenase (ACMSD) competes with this cyclization, catalyzing the formation of picolinic acid.

To date, there are many unresolved issues that pertain to the role of the KP in inflammatory processes. For example, are the kynurenines the drivers of the functional changes occurring during inflammation, or are they merely biologically inert mediators? Are the changes in the levels of the metabolites a direct consequence of immune regulations of individual KP enzymes or the result of secondary reactions to different stimuli? What are the effects induced by chronic inflammation on the KP?

Why characterize the kynurenine pathway in the CNS of L. stagnalis?

Based on the previous premises, there are several reasons to characterize the KP in *L. stagnalis*.

First, kynurenine metabolism is highly conserved throughout the Eukaryotic lineage from yeast to humans (Schwarcz and Stone 2017). Second, the pathway is the major route for tryptophan catabolism, and many of the intermediates and products of this pathway can either block or activate neurotransmitter receptors in the CNS (Schwarcz and Stone 2017). Third, the KP is considered to link the immune and neurotransmitter systems as the breakdown of tryptophan via the KP is activated by proinflammatory cytokines (Savitz 2020).

While considerable evidence suggests a role for inflammation in the pathophysiology of many neurodegenerative diseases and chronic psychiatric disorders, the mechanism by which neuroinflammation influences neurotransmitter systems is still unknown (Dantzer 2016).

Considering the importance of the KP in different fields from neuroscience to immunology, and pharmacology, it seemed noteworthy to study this pathway in the CNS of *L. stagnalis*. In fact, the characterization of this highly conserved pathway in a simple model system could give important translational contributions for elucidating the effects of inflammation on the CNS.

A collaborative effort with the bioinformatics group of the University of Modena and Reggio Emilia (Prof. Silvio Bicciato-UNIMORE) enabled to overcome the lack of an annotated genome and led to the characterization of putative transcripts coding for the homologue enzymes of the KP in the CNS of *L. stagnalis*. The quick and efficient multidisciplinary approach developed in this study, which combines bioinformatics and molecular analyses, provides the first insights into the conserved molecular cascade and function of the KP in an invertebrate model system.

Material and methods

Part 1

Bioinformatics analysis

BLAST annotation

The sequenced genome of *L. stagnalis* is deposited into the genome sequence database NCBI (http://www.ncbi.nlm.nih.gov/genome). DNA fragments are assembled at the contig level (i.e., by overlapping the sequences' ends).

L. stagnalis genome assembled at the contig level (328,378 contigs) was downloaded from the NCBI genome database. Gaps between contigs were filled post-assembly with Gap-closer, leading to a more complete genome that benefits downstream genome annotation and characterization. The conserved genes in the *L. stagnalis* genome were identified using Blast-X from the 2.2.28+ release of NCBI-Blast (http://www.ncbi.nlm.nih.gov/BLAST/).

Then, protein sequences from three different species were downloaded from the NCBI ftp site (https://ftp.ncbi.nih.gov/genomes):

(1) *Biomphalaria glabrata* (i.e., one of the most phylogenetically related organisms *to L. stagnalis* https://ftp.ncbi.nih.gov/genomes/Biomphalaria_glabrata/protein/);

(2) *Mus musculus* (https://ftp.ncbi.nih.gov/genomes/M_musculus/protein/);

(3) *Homo sapiens* (https://ftp.ncbi.nih.gov/genomes/Homo_sapiens/protein/).

These protein sequences were annotated with RefSeq IDs and were aligned with *L. stagnalis* contigs. 81,845 contigs having a hit in *B. glabrata*, 30,478 having a hit *in M. musculus*, and 30,001 having a hit in *H. sapiens*, were found. The queryMany function in the R package "mygene" was used to provide for each RefSeq IDs the Gene Ontology (GO - http://geneontology.org/) terms and the Kegg pathways (https://www.genome.jp/kegg/pathway.html). The association of each protein RefSeq IDs with GO terms and Kegg pathways provided a framework to elucidate the biological role(s) of each gene by semantic analysis and the relative pathways. GO contains terms in a structured format within three domains: biological processes, molecular functions, and cellular components. Moreover, protein sequences were mapped to HUGO gene symbols (https://www.genenames.org/) to assign standardised nomenclature to the genes.

Transcriptome assembly and transcript abundance estimation

RNA-seq reads from wild-type *L. stagnalis* CNS were downloaded from DDBJ (i.e., the DNA Data Bank of Japan, http://www.ddbj.nig.ac.jp) (Sadamoto *et al.* 2012) and the *L. stagnalis* transcriptome shotgun assembly (TSA) was assembled by aligning RNA-seq reads on *L. stagnalis* genome using TopHat v2.1.0 and Cufflinks v2.0.2.

Then, 81,851,004 RNA-seq reads were trimmed and qualitatively filtered using Trimmomatic (GPL V3 – Bolger et al., 2014), a high-performance tool that handles paired-end data.

In line with previous studies, the RNA-seq reads were trimmed when base quality was above 20, and a head crop of 9 nucleotides was performed (Bolger et al., 2014).

Thus, 3,605,974 reads were dropped, whereas the remaining 78,245,030 reads were re-aligned with Top-Hat.

Then, non-multimappers reads per contig were counted and converted in transcripts per kilobase million (TPM) using the formula:

$$\boldsymbol{TPM_i = \frac{X_i}{l_i} \cdot \left(\frac{1}{\sum_j \left(\frac{X_j}{l_j} \right)} \right) \cdot 10^6}$$

where X_i is the reads count from a contig *i* and l_i is the length of the contig. This normalization method allowed us to estimate the RNA sequencing abundance.

RNA-Seq track visualization

For visualizing the RNA-seq reads from the *L. stagnalis* CNS and extracting the exonic sequences from the intronic ones, the Integrative Genomic Browser (IGV, http://www.broadinstitute.org/igv/) has been used (Thorvaldsdóttir et al., 2013).

Sequence analysis

The deduced amino acid sequences were determined using the NCBI open reading frame (ORF) finder website (https://www.ncbi.nlm.nih.gov/orffinder/). Alignments of nucleotide and translated protein sequences were performed with http://nadv.herokuapp.com/. ORF sequences were fed into the protein family database, PFAM (https://pfam.xfam.org/), to identify the major conserved characteristic domains belonging to each previously organized sequence group (El-Gebali *et al.* 2019).

Part 2

Molecular analysis

Animals

Laboratory-reared *L. stagnalis* (Linnaeus 1758), originally derived from a stock generously donated by the Vrije University in Amsterdam, were used in this study. Adult (8-9 animals) snails having shell lengths of 20-25 mm were used in these experiments. Animals were maintained in 12 L aquaria at the University of Modena and Reggio Emilia (Italy) at 21-23°C in well-aerated dechlorinated tap water on a 12/12 h light/dark cycle. Snails were fed pesticide-free lettuce and goldfish pellets three times a week. The aquaria were cleaned on alternate days. Every effort was made to minimize the number of animals used and their suffering

Phylogenetic analysis

To determine the phylogenetic organization and distribution of the sequences found and to provide further evidence of the presence of probable proteins found in the snail, phylogenetic analysis was performed using Molecular Evolutionary Genetics Analysis (MEGA)-X using the UPGMA method (Kumar *et al.* 2018). For the analysis, amino acid sequences of deuterostomes and protostomes organisms such as *Homo sapiens, Mus musculus, Rattus norvegicus, C. elegans, A. californica* and *B. glabrata*, obtained from NCBI were used. The phylogenetic distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The deduced amino acid sequences of the *L. stagnalis*-like KP enzymes were aligned with those of *H. sapiens, M. musculus, R. norvegicus, C. elegans, A. californica,* and *B. glabrata* using the T-Coffee tool and visualized with BoxShade.

RNA extraction and retrotranscription

Total RNA was extracted from *L. stagnalis* CNS and 8-10 replicates were analysed for each group. Total RNA extraction was performed using TRIzol® reagent and GenElute™ Total RNA Miniprep Kit (Sigma–Aldrich) and a DNAse treatment (DNASE70 – On-Column DNase I Digestion Set, Sigma–Aldrich) was performed to remove genomic contamination. 500 ng of total RNA were reverse transcribed with a High-Capacity cDNA Reverse Transcription Kit (Life Technologies Corporation) in 20 µl of the reaction mix.

Qualitative PCR analysis and sequencing

Qualitative PCR was performed using Dream Taq DNA polymerase® (Thermo Scientific, Waltham, Massachusetts, United States) under the general 3-step amplification of 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s; 72°C for 30 s, and final extension of 72°C for 7 min.

Primer sequences were designed by NCBI Primer-BLAST software and were synthesized by Sigma-Genosys Ltd. (Cambridgeshire, UK) (**Table 3**). PCR products were electrophoresed on agarose gel (2%) and DNA fragments were visualized by UV illumination to confirm the correct amplicon size. PCR products (600-800 bp) were purified using a High Pure PCR Product Purification Kit (Roche Diagnostics Corporation, USA) following the manufacturer's instructions, and were directly sequenced using the Sanger sequencing method. Sequencing was performed on the Ion Torrent™ Personal Genome Machine System (Life Technologies Ltd., Paisley, UK) using an Ion 314™ Chip v2 following the Ion PGM™ Sequencing 200 Kit v2 manual. The sequence analysis of the PCR fragments was performed using Sequence Scanner Software 2.0 (Applied Biosystems®, California, USA) and sequences were compared with the contigs of *L. stagnalis* in the Transcriptome Shotgun Assembly (TSA) Sequence Database using BLAST_X (http://www.ncbi.nlm.nih.gov).

Design, validation, and optimization of primers for quantitative PCR analysis

Candidate primers intended for quantitative Real-Time PCR (qPCR) were designed with NCBI Primer-BLAST software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and were synthesized by Sigma-Genosys Ltd. Primers were designed to have a length of 19–23 nucleotides, a melting temperature between 58 and 62 °C, a GC content between 40% and 60% and generating an amplicon between 100 and 200 bp. Primer specificity was assessed by qualitative PCR as previously described. Each experiment contained two biological replicates of cDNA from central ring ganglia of *L. stagnalis* and *Pomacea canaliculata* (kindly provided by Professor Davide Malagoli - UNIMORE), and controls were minus reverse transcription (RT) controls to assess the genomic DNA and non-template controls. PCR products were run as previously described. Primer efficiency was evaluated in Bio-Rad CFX Connect. Curves were generated from four-fold serial dilutions of cDNA run in triplicate. Amplification of all genes was detected with SyBR Green dye which generates fluorescence based on the synthesis of double-stranded DNA. The reactions contained 5 µL of cDNA with 10 µL of Bio-Rad SsoAdvanced Universal SyBR Mix, 300 nM forward and reverse primer concentration, and topped to 20 µL with H2O. Each point was run in triplicate. The qPCR reactions took place in a Bio-Rad CFX Connect thermocycler running a custom program. The custom qPCR program consisted of 95 °C for 30s; 40 cycles of 95 °C for 15 s, 60°C for 30 s. The plate was read by the machine to measure fluorescence at the end of each cycle. All gene-specific primers used for the mRNA expression analysis are listed in **Table 5.** Expression levels of Elongation factor-1a (Lym EF1a , 5'-GTGTAAGCAGCCCTCGAACT-3' and 3'- TTCGCTCATCAATACCACCA-5') and b-tubulin (Lym bTUB 5'- GAAATAGCACCGCCATCC-3' and 3'- CGCCTCTGTGAACTCCATCT-5') were used as internal controls.

LPS treatment

To confirm and compare the tissue expression of putative enzymes of the kynurenine pathway identified from the transcriptome, mRNAs were quantified by Real-Time quantitative polymerase chain reaction in the following experimental paradigm. Sixty adult snails were divided in two groups receiving either: freshwater snail solution (41.15 mM NaCl; 0.54 mM KCl; 3.55 mM CaCl2; 2.61 mM MgCl2; 5 mM Tris; pH 7.5 Ottaviani, 1983); and 25 mg (estimated concentration 6.25 µg/mL) of Escherichia coli-derived LPS serotype O127:B8 (L3129) dissolved in freshwater snail saline (Sigma-Aldrich, USA). The calculated volume of hemolymph in a snail with a 20-mm shell length was 400 µl (Murakami *et al.* 2013), a single injection of 40 mL was performed intramuscularly in the foot of the snail using a 31G syringe. Thirty LPS-injected snails and their relative sham-injected controls were sacrificed 2 h after the injection, thirty animals 6 h after injection, while the remaining snails were sacrificed after 24h. In all groups, snails were anesthetized by placing them on ice for 10 min. Treatments and sacrifices were performed between 9 a.m. and 3 p.m. local time.

Statistical analysis

First, NormFinder (http://moma.dk/normfinder-software) has been used for calculating the stability values for each candidate housekeeping gene (i.e., elongation factor 1-a, *Lym* EF1α, and b-tubulin, *Lym* TUB), considering intra and intergroup variation. As a calibrator, the geometric mean of Cqs of the reference genes was used. For an appropriate application of the comparative ΔΔCt method, it was demonstrated that amplification efficiency of the target genes and endogenous control genes were approximately equal (data not shown).

For quantitative evaluation of changes, the comparative ΔΔCt method was performed, using as calibrator the average levels of expression of saline-receiving animals sacrificed 2 hours after injection.

Statistical analysis of relative gene expression data was performed using a two-way ANOVA to assess the main effect of time after injection, treatment, and interaction between the two terms in all the experimental groups, followed by Bonferroni *post-hoc* test to identify significant differences (with $p<0.05$ significance level). Extreme outliers were excluded before statistical analysis using the boxplot tool in SPSS (more than 3x the interquartile range outside of the end of the interquartile box). Analyses were conducted using SPSS for Windows® v.26 (SPSS Inc., Chicago, USA).

Results

Part 1

Bioinformatics analysis

Contigs' table for identifying conserved sequences

A contigs' table indicating for each *L. stagnalis* contig, the ID, the length, the RNA seq TPM value, the accession number of each non-redundant sequences of the *L. stagnalis* TSA (FX_), and the homology association generated using Blast-X with *B. glabrata, M. musculus,* and *H. sapiens* protein sequences has been generated. An extrapolation of this table is attached below (**Table 1**).

Table 1. Contigs' table

Lymnaea stagnalis			Homology association by BlastX						Functional annotation			
CONTIGS			*Biomphalaria glabrata*		*Mus musculus*		*Homo sapiens*					
ID	Length (bp)	RNAseq TPM value	RefSeq protein ID	Gene symbol	RefSeq protein ID	Gene symbol	RefSeq protein ID	Gene symbol	GO Cellular component	GO Biological process	GO Molecular function	Kegg pathway
FCFB01000001.1	6275	0.061	XP_013071679	LOC106058735	NA	NA	NA	NA	NA	NA	NA	NA
FCFB01000003.1	29634	4.026	XP_013079973, XP_013079974	LOC106065648	XP_006514269, XP_006514270, NP_001074525, XP_006514267, XP_006514271	Xpot	XP_016874237, NP_009166	XPOT	nuclear pore, nucleoplasm, nucleoplasm, cytoplasm, cytosol, cytosol, nuclear matrix	tRNA export from nucleus, tRNA export from nucleus, tRNA re-export from nucleus	tRNA binding, Ran GTPase binding	RNA transport - Homo sapiens (human)
FCFB01000008.1	18455	0.844	XP_013071679	LOC106058735	NA	NA	NA	NA	NA	NA	NA	NA
FCFB01000010.1	7107	0.009	XP_013092901, XP_013092900	LOC106076624	XP_017174855	Gm46731	NA	NA	NA	NA	NA	NA
FCFB01000012.1	12121	0.011	XP_013079458	LOC106065228	NA	NA	NA	NA	NA	NA	NA	NA
FCFB01000013.1	13712	0.093	XP_013079830, XP_013079829	LOC106065540	XP_006503420	Zmym1	NP_001276020, XP_016857850	ZMYM1	Nucleoplasm, cytoplasm	Regulation of transcription from RNA polymerase II promoter	RNA polymerase II transcription factor activity, sequence-specific DNA binding, DNA binding, zinc ion binding, protein dimerization activity	
FCFB01000014.1	8727	0.147	XP_013079458	LOC106065228	NA	NA	NP_659802, XP_011514215, XP_011514213	FAM200A	Nucleoplasm, cytoplasm, integral component of membrane	regulation of transcription from RNA polymerase II promoter	RNA polymerase II transcription factor activity, sequence-specific DNA binding, DNA binding, protein binding	

Identification and characterization of putative transcripts of the kynurenine pathway in L. stagnalis: alignment and phylogenetic analysis

The contigs' table was then used for identifying in *L. stagnalis*' genome putative genes coding for the KP enzymes. To do that, specific genes matching with the annotated genome of *B. glabrata* were searched. All the putative enzymes (whose transcript was associated with an FX_) were identified univocally (**Figure 5**).

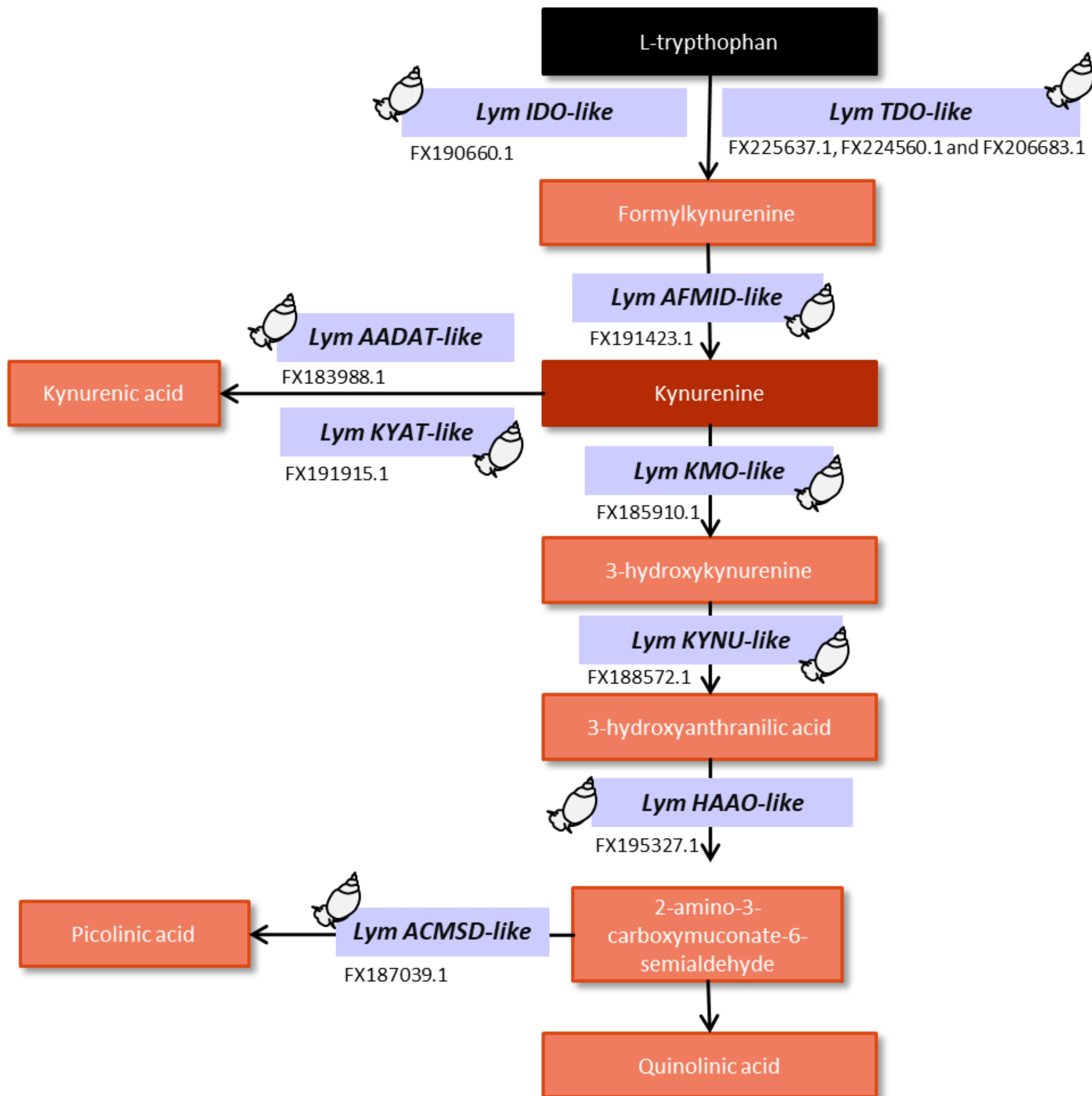


Figure 5. Putative enzymes of the kynurenine pathway (KP) identified in the CNS of *L. stagnalis*.
Putative enzymes (purple boxes) and metabolites (red boxes) of the kynurenine pathway (KP) identified in the CNS of *L. stagnalis* are reported. *Lym* IDO-like (putative indoleamine 2,3-dioxygenase); Lym TDO-like (putative tryptophan dioxygenase); Lym AFMID-like (putative kynurenine formamidase); Lym KYAT-like (putative kynurenine oxoglutarate transaminase); Lym AADAT-like (putative aminoadipate aminotransferase); Lym KMO-like (putative kynurenine 3-hydroxylase); Lym KYNU-like (putative); Lym HAAO-like (putative 3-hydroxyanthranilic acid oxygenase), and Lym ACMSD-like (putative 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase). For each enzyme, the corresponding FX_is reported.

The IDO-like orthologue was named 'myoglobin-like' instead of IDO (LOC106056257) as it was first identified in mollusks' buccal mass where it acts as myoglobin (**Table 2**). Moreover, two putative isoforms of aminotransferases were included in the analysis: KYAT I/III and AADAT (KYAT II) as in human and rodents' genomes KYAT I and KYAT III share the highest sequence identity and are phylogenetically distant from AADAT. Several *L. stagnalis* contigs matching the KP enzymes were isolated and, using IGV. Then, the exonic sequences were extracted and blasted against the transcriptome shotgun assembly (TSA) of *L. stagnalis*. This step allowed the identification of a specific transcript for each enzyme. All the transcripts had a TMP>0, with the sole exception of TDO for which 3 small contigs were identified (FX225637.1, FX224560.1 and FX206683.1).

Table 2 – Putative transcripts codifying for the enzymes of the KP

Lymnaea stagnalis				Homology association by BlastX						
Contigs			Transcriptome	*Biomphalaria glabrata*			*Mus musculus*		*Homo sapiens*	
Contig ID	Contig length (bp)	RNAseq TPM value	FX_TSA	RefSeq protein ID	Gene symbol	GENE definition	RefSeq protein ID	Gene symbol	RefSeq protein ID	Gene symbol
FCFB01037212.1	19708	0.211	FX190660.1	XP_013068349, XP_013068353, XP_013068352, XP_013068350, XP_013068354, XP_013068351, XP_013068348	LOC106056257	**myoglobin-like**	NA	NA	NA	NA
FCFB01037213.1	10282	2.481		XP_013068350, XP_013068348, XP_013068351, XP_013068349, XP_013068354, XP_013068353, XP_013068352			NA	NA	NA	NA
FCFB01037214.1	5124	4.716		XP_013068348, XP_013068349, XP_013068351, XP_013068353, XP_013068352, XP_013068350			NA	NA	NA	NA
FCFB01109750.1	7853	0.726	FX206683.1	XP_013068953, XP_013068952, XP_013068951, XP_013068954	LOC106056678	**tryptophan 2,3-dioxygenase-like**	NA	NA	NA	NA
FCFB01109751.1	5146	0.324	FX225637.1	XP_013068954, XP_013068952, XP_013068951, XP_013068953			NA	NA	NA	NA
FCFB01255639.1	922	1.112	FX224560.1	XP_013068951, XP_013068954, XP_013068952, XP_013068953			NA	NA	NA	NA
FCFB01077058.1	4544	0.621	FX191423	XP_013073423	LOC106060173	**kynurenine formamidase-like**	NA	NA	NA	NA
FCFB01161380.1	3637	1.745		XP_013073423			NA	NA	NA	NA
FCFB01232167.1	1538	1.084		XP_013073423			NA	NA	NA	NA
FCFB01020652.1	28977	15.087	FX185910.1	XP_013064773	LOC106053720	**kynurenine 3-monooxygenase-like**	NP_598570	Kmo	NP_003670, XP_016858139, XP_005273394, XP_016858138, XP_005273395	KMO
FCFB01033100.1	41166	4.438	FX191915.1	XP_013067258	LOC106055518	**kynurenine--oxoglutarate transaminase 1/3-like**	NP_001280489, XP_006501494, XP_011238416, NP_776124	Kyat3	NP_001008662	KYAT3
FCFB01016053.1	18986	8.370	FX183988.1	XP_013087071	LOC106071491	**2-aminoadipate transaminase-like**	NA	NA	NA	NA
FCFB01070793.1	13125	2.085	FX188572.1	XP_013068429	LOC106056296	**kynureninase-like**	XP_011237480, NP_001276522, XP_011237481	Kynu	NP_003928, XP_011510404, NP_001186170	KYNU
FCFB01141163.1	10604	2.545	FX195327.1	XP_013070925	LOC106058097	**3-hydroxyanthranilate 3,4-dioxygenase-like**	NA	NA	NA	NA
FCFB01269750.1	775	0.331		XP_013070925			NA	NA	NA	NA
FCFB01196791.1	649	0.593	FX187039.1	XP_013081879, XP_013081878	LOC106067265	**2-amino-3-carboxymuconate-6-semialdehyde decarboxylase-like**	NP_001028213	Acmsd	NP_612199	ACMSD
FCFB01209656.1	3764	1.976		XP_013081879, XP_013081878			NP_001028213	Acmsd	XP_005263647	ACMSD
FCFB01252153.1	971	16.042		XP_013081878, XP_013081879			NP_001028213	Acmsd	NP_001294912, XP_016858814, XP_016858815, XP_005263646, XP_005263645, XP_011508894	ACMSD
FCFB01272611.1	752	4.688		XP_013081879, XP_013081878			NP_001028213	Acmsd	XP_005263646, NP_001294912, XP_011508894, XP_016858815, XP_016858814, XP_005263647, XP_005263645	ACMSD
FCFB01319919.1	524	6.973		XP_013081879, XP_013081878			NP_001028213	Acmsd	XP_005263646, NP_001294912, XP_005263647, XP_005263645, XP_011508894, XP_016858815, XP_016858814	ACMSD

To assess whether these transcripts were expressed in the *L. stagnalis* CNS, primers were designed spanning the matching region (**Table 3**). For *Lym* TDO-like, a combination of primers on the different contigs was used and a single PCR product resulting from the combination of the three contigs FX225637.1, FX224560.1 and FX206683.1 with a 15bp gap (TATTCCTCTATAAGG) between the first two was amplified and sequenced.

Table 3 – Primers used for sequencing each putative enzyme of the KP.

For each putative enzyme of the KP, are reported the relative transcript FX_, and the position of the forward (FW) and reverse (R) primers.

MATCH ON	PREDICTED ENZYME		POSITION ON TRANSCRIPT	SEQUENCE
FX190660.1	**Lym IDO-like** Predicted *Lymnaea stagnalis* indoleamine 2,3-dioxygenase (IDO) 1 288 1619 1725bp << ORF 443aa << 60 1719	FW	60	CGGTTCTAACATAATTCCTC
		RV	1719	GCGATACAACAAAAATACAA
FX225637.1, FX225637.1, FX206683.1	**Lym TDO-like** Predicted *Lymnaea stagnalis* tryptophan 2,3 dioxygenase (TDO) 1 38 1294 1451bp >> ORF 418aa >> 247 1117	FW	247	TGTCAGAGATATGTTCATGC
		RV	1117	CAGTGGTGGTATGTAGTCAC
FX191423.1	**Lym AFMID-like** Predicted *Lymnaea stagnalis* kynurenine formamidase(AFMID) 1 791 1609 1630bp << ORF 272aa << 828 1517	FW	828	ACAAAATTAGGTTCCGTAAG
		RV	1517	AAGTTACGGAGACTCAGAGA
FX185910.1	**Lym KMO-like** Predicted *Lymnaea stagnalis* kynurenine 3-monooxygenase (KMO) 1 896 2335 2560bp << ORF 479aa << 954 2230	FW	954	GGAGTTTCTAGATTCCAATG
		RV	2230	TTCAAGGTTGATTTGTATGA
FX183988.1	**Lym AADAT-like** Predicted *Lymnaea stagnalis* kynurenine/alpha-aminoadipate aminotransferase II (AADAT) 1 3 1353 3127bp >> ORF 450aa >> 106 1214	FW	106	ACTTGCTTATGAAATGGAGT
		RV	1214	GTAAACACCTGGAAGAAAAC
FX191915.1	**Lym KYAT I-III-like** Predicted *Lymnaea stagnalis* kynurenine oxoglutarate transaminase 1-3 (KYAT I-III) 1 84 1472 1574bp << ORF 462aa << 205 1256	FW	208	GGAGGTATCACTGTCAATTT
		RV	1256	TGATATATCCAAACTTGCAG
FX188572.1	**Lym KYNU-like** Predicted *Lymnaea stagnalis* kynureninase (KYNU) 1 279 1736 2024bp >> ORF 485aa >> 318 1666	FW	318	ACTACACCTTTGCAAGAACT
		RV	1666	ATGAATCTGTGAACATCCTC
FX195327.1	**Lym HAAO-like** Predicted *Lymnaea stagnalis* 3-hydroxyanthranilate 3,4-dioxygenase (HAAO) 1 67 422 447 949 1263bp >> ORF 293aa >> 99 819	FW	99	TGACAGTTGGATAGATGAAA
		RV	819	TGAGTAACAACTGACTCACC
FX187039.1	**Lym ACMDS-like** Predicted *Lymnaea stagnalis* 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase (ACMSD) 1 1153 2220 2300bp << ORF 355aa << 1280 2052	FW	1280	TCAGGACTAAATTCTTCCAT
		RV	2052	GAAGCACGAATTAAAGAAAT

Qualitative PCR was performed for each target and the PCR products were purified, sequenced, and matched with the deposited sequence for FX190660.1 (*Lym* IDO-like), FX191423.1 (*Lym* AFMID-like), FX185910.1 (*Lym* KMO-like), FX183988.1 (*Lym* AADAT-like), FX191915.1 (*Lym* KYAT I/III-like), FX188572.1 (*Lym* KYNU-like), FX187039.1 (*Lym* ACMSD-like). The amplified sequence of *Lym* HAAO-like with respect to the original deposited sequence of FX195327.1 missed an A in position 422 and presented a C in position 447 instead of a T. All the identified transcripts contained an ORF (i.e., a portion of a DNA molecule that, when translated into amino acids, contains no stop codons) (**Table 4** and **Figure 6**).

Table 4 – Contigs, transcripts, and ORFs for each sequenced putative enzyme of the KP

ENZYME	*Lymnaea stagnalis*				
	Contig	Transcript	ORF	Predicted size (aa)	Position
IDO1 **indoleamine 2,3-dioxygenase 1**	FCFB01037212.1 FCFB01037213.1 FCFB01037214.1	FX190660.1	**MRQSFDLSKYNVSYDTGLMLKSPLKTLPSYFNPWNELAETLPKLVKEKKVREAVRRLPLLDH RQLVGHRQLRLAHLQLSFITAGYVWQDGDKGVPQVLPKCLAFPFYNISKELGIQPILGHVD LALANYHLINPSGPYEFENLQCLYHIPGGLAGDWFCIVTFMVEFSFARCLKHLVRLLDLLEV AHVQMRADEKGRKDTEREMAERLDDITLAVQTMEASLARMHDNLDAKMFFNVIRPFLG GWGGEGNPLPDGLIYEGISDYPIKMPGGSAAQSTTLQILDALLGVEHSEDKRAFLVHMRSF MPPDHCRLIVDLEQRPHKLRDLVESACSRELCDAYNNCVSALVHLRNYHIQIVTKYVIQAS QTVNEGNYESLDKKGTGGTSLIPFLKDIRSDTESKIMPIPHTQSKKSQGKLMSWQKVAVVV AITASMAVGTIKLLKYK**	**443**	288-1619bp
TDO **tryptophan 2,3-dioxygenase**	FCFB01109750.1 FCFB01109751.1 FCFB01255639.1 FCFB01297038.1	FX224560.1 FX206683.1 FX225637.1	**MAECPMNNGVCKEDGNDLNYHSYLMLDNVLTSQVMQSAVKNKVVHDEHLIITHQAYEL WFKQILYEVDSVRDMFMLPVMDETKTILILNRLSRVTLILKLMVDQIDILETMTPLDFLEFRL QLSSASGFQSLQFRLLENKMGVLETYRVKYNQQHYTKVFSKPDQVKMLQDSIEQPSLFNLL EKWLERTPGLTVKSFHFWDLYKRSVERWLQDLLYIPAQKETDEKIKQSLIEEYKKQKESFDSIL DEDKYNASVARGDRRLSHQAFKGALLISLYHDQPRFNQPSKLLSLLMDIDSLLTKWRYNHV MMVQRMIGSKVGTGGSSGYQYLRSTVSDRYKVFLDLFNMSTYLIPRDYIPPLSRSLKRQLSI LIHEYMPRDPEDSDDYDLDDGTDDNSPEGKNVDSCPNEETRPKVKFEI**	**418**	38-1294bp
AFMID **kynurenine formamidase** **AFMID arylformamidase**	FCFB01077058.1 FCFB01161380.1 FCFB01232167.1	FX191423.1	**MAPEAVTVAHLEALVQGSRNSLWTLDCETEISYGDSERQKFDIFHKKNASKNGAPMFAYIH GGYWQVKEVSREISSFMAVPLCNAGASVISIGYDVAPDVSLNTIVLQIKRALCLIIKIAKERQ SSGIYLSGHCVGAHLAAMMLMADLDGFDRDLIKGAVLISGVYDLRPLVKTSINEPLGLTEV DAWRLSPHQFVQEIAYQSQDRHIVVAVAEYDPPEFRRQSGEMEMALRKFGVKTSYLDVP DTDHFNLVDKLTEPNFVLTKECIRLMGL**	**272**	791-1609bp
KMO **kynurenine 3-monooxygenase**	FCFB01020652.1	FX185910.1	**MTPKRESQSQRKKHVAVCGGGLVGALNACYMAKRGFKVDLYEMRPDIRTLEVVKGRSIN LALSCRGREALRRVGLEDTITKNGIPMHARMIHDLNGKRRPLLYGNEDQYIMSIDRRLLNEV MLTEVEKFPDVNIHFSHKLVSCDFNSGEAIFENEKGECLTKQFDLIIGCDGAYSSVRKQLMKL SRFDYQQEYIPHGYMELNIPPNKKNESAMEINYLHIWPRNDYMMIALPNLDKSFTTTLFMP FEIFESITSEEKLIQFFKDEFPDALPLLGEAELKQSFFSCKALPLVSIKCHPYHVNDKAVIMGDA AHAMVPFYGQGMNCGMEDCIVFDELLDKYNNNFAKVLPAYTKIRNPDAKAICDLAMYN YTEMRSKVNSRLFLVRKKLDNMLFKLFPNTWVPLYTMVSFTRIRYHECVQRREWQDKILTR SLILSAVCILLGFTALIYNANKFGDHWNLETPQSLLTGSFDKFQKWISRW**	**479**	896-2335bp
AADAT **aminoadipate aminotransferase**	FCFB01016053.1	FX183988.1	**MIFRILGFKYYNSADNIVNYNSMTEEDESSDKEMNLLMKWSNGISFVAGSPGVELMKRCSE MLLTASKSVLGSSTIDIGNLFRYGAEQGDADFREQLSCFLSHEYRDKVKSANLMVTAGATQ GLHAILSLLCDKASPIFVEDPSYFFAVRMIRDDLQMNLVPVPCDECGINPELLDQFLIAHKRE FYKGNNSNINKRYTRFWAAAYLIPVFNNPTGQTYSETRCKELIQLARKHNILLIAEDIYNLIHF DESGLAPQRLLHYDNTSDPDYGGGHVLSNGTFSKIFAPSIRLGWIEGPEQLLFKMYNSNLT WSGGSFNHYMAKLMANILASGILYDHLKFTRSAYKERMFALCAALDELCPDGWSYPRPQ GGYFLWITFPQNIDARKFELFARDNYGVGFLPGVYCSPTNSFINCARLSISSLQVDTIIEGAKK LFSAYQGYVIHIKPITD**	**450**	3-1353bp
KYAT I-III **kynurenine-oxoglutarate transaminase 1/3-like**	FCFB01033100.1	FX191915.1	**MHKELKSCTRFLLSCVQQHRNRKRVLKSPFSTCLTCFSTSSVNNKMTSKKFELAKRVQGTEK NVWVDISKLAVEQKSINLGQGFPDFMAPQDIVNCLKDTVNSDSPAVHQYARSFGHPRLV KALAELYEPFLGRQIDPMTEILISVGAYGALFCVIQGLVNPGDEVIIIEPFFDCYQPMVKAAG GTPVFVPLRPTKDGSVTSSADWKLDEGELSSKFNSKTKLIIVNTPNNPLGKVFTREELLVIANL CKKHDVVCVADEVYEWMTYNGNQHIKIATLPDMWERTVTIGSAGKTFSVTGWKIGWTV GPQHLVHCAQIMHQNCTYNCPTPIQEAVARGLELEKSRLGSPGSYLISLAASLKPKRDKLAS VLEEVGMKPVIPEGGYFMMADYTSMEADVPESDEAKDFRFSKWMTINKKLTVIPPSAFYC KEHEHMAENYVRFCFIKEDSTLEKAANVLRHWKN**	**462**	84-1472bp
KYNU **kynureninase-like**	FCFB01070793.1	FX188572.1	**MGDVNNATANSHSTTPLQELEKIANENNLKLGDVEFARHMDKRDPLKYLRDEFHYPKMG DVLNTDPSIVDLSEDCVYFCGNSLGLCPKKTKEYMNIQIDKWAKLGVQGHTNGELPWAW CDELLEEDMAKIVGCKREEVSLMNGLTVNLHLLLISFYRPTKDRYKILCESKAFPSDHYTFESQ SRLHGFDPKDAMICVEPREGEFTLRTEDILDVIEKQGDKIAVVCFSGVQYYTGQLFDIPKITQ AGKAKGCHVGWDLAHAVGNVPLKIHDWGVDFACWCTYKYLNASAGGLAGLFIHENFQT NDFPKLLGWWGHDMKTRFQMNNQMDLIPGARGYRISNTPGFLCVPLKASLEIFKKTSVEE LRKKSLVLTAYLEHLIQKSYQRPAGKAPEDDCDAVYIDIFTPSDPKQRGAQLSLAFNVCIEQ VFKELEKRGVVCDKRFPRVIRITPVPMYCSFEDVHRFMGYLKDALVAAKGSLRHVDTHKV**	**485**	279-1736bp
ACMSD **2-amino-3-carboxymuconate-6-semialdehyde decarboxylase**	FCFB01196791.1 FCFB01209656.1 FCFB01252153.1 FCFB01272611.1 FCFB01319919.1	FX187039.1	**MKIDLHTHILPENWPDLKERYGYGGWVQLHHHCAGRAKMMKDGKLFREIEENCWSPEA RIKEMDAHGVDIQVLSTVPVMFGYWAEPKDTLDLCEMLNNHLAGIVKSCPSRFVGLGTVP MQAPELAVKELIRCKKELGFPGIQIGSHINNWNLDAPELQQVFAAAEEHNCVIFVHPWD MEQGGRMQKYWLPWLVGMPTETTVAICSMIFGGVLERFPKLKVCFAHGGGAFPFTIGRIE HGFNSRPDLCAVENNVNPRKYLGQIYTDSLVHDTEALKLLVNVIGKNRIVLGSDYPFPLGEQ HPGKLVESMEEFSPELKHQILAGNAMEFLGLDPSLYKPCIGEDTQMDSTNTHLPCP**	**355**	1153-2220bp
HAAO **3-hydroxyanthranilate 3,4-dioxygenase-like**	FCFB01141163.1	FX195327.1	**MSVSGPVVYNIDSWIDENKQYFLPPVCNKMMHNDGQMKVMYVGGPNQRKDYHIEEG EELFYMLKGDMCLKVVEHGQHRDVVINEGEIFLLPGKIAHSPQRQGDTIGLVIERERDNSEL DGLRYYQEVDGVPTAESLYEEWFHCTDLGTQLGPVIKRYFGSEQHKTGKPVPGTIPESPPIIL DSEISMQDPFNLHVWIEQNRKELDSKGFVQLFGDNFQFSVKLYGKGENTGLCDNAETWIW QMEGESVVTQNDKEFKLQKNDSLLIRVGERYKAARPEGSIALICYQDPSRKK**	**293**	67-949bp

Thus, for each putative enzyme of the KP, it has been predicted the aminoacidic sequences (**Figure 6**).

A Lym IDO-like (FX190660.1)

B Lym TDO-like (FX225637.1, FX224560.1, FX206683.1)

C Lym AFMID-like (FX191423.1)

D Lym KMO-like (FX185910.1)

E Lym AADAT-like (FX183988.1)

F Lym KYAT I/III-like (FX191915.1)

G Lym KYNU-like (FX188572.1)

H Lym HAAO-like (FX195327.1)

I Lym ACMSD-like (FX187039.1)

Figure 6. Nucleotide sequences and deduced aminoacidic sequences of each enzyme of the KP in *L. stagnalis* CNS.
(**A**) *Lym* IDO-like (**B**) *Lym* TDO-like (**C**) *Lym* AFMID-like (**D**) *Lym* KMO-like (**E**) *Lym* AADAT-like (**F**) *Lym* KYAT I/III-like (**G**) *Lym* KYNU-like (**H**) *Lym* HAAO-like (**I**) *Lym* ACMSD-like. The nucleotide sequences are black, whereas the deduced aminoacidic sequences are red.

Next, the ORF of each putative KP enzyme in *L. stagnalis* was aligned with the amino acid sequences of the corresponding enzyme in *H. sapiens*, *M. musculus*, *R. norvegicus, A. californica*, *B. glabrata*, *P. canaliculata*, and *C. elegans*, as putative kynurenine pathway-related genes have been identified in this nematode (Jaronen and Quintana 2014) (**Table 5**). 6/9 orthologs from *C. elegans* were identified and had a mean homology with the corresponding sequence of *L. stagnalis* around 48%. A similar degree of homology was found with mammals (around 48%). For mollusks a higher degree of homology was observed: 63% for *P. canaliculata*, 64% for *A. californica*, and 70% for *B. glabrata*. AADAT was the less conserved gene (mean homology 30% from 23% in *A. californica* to 49% in *B. glabrata*) while ACMSD (mean homology 71% from 70% in *H. sapiens*, *M. musculus*, *R. norvegicus* to 86% in *B. glabrata*) was the more conserved. This trend has been confirmed also with a multiple sequence alignment with T-Coffee between each putative enzyme identified in *L. stagnalis* with homologues from other species (**Figure 7**).

Table 5. Homology between the ORF of the KP enzymes in *H. sapiens*, *M. musculus*, and *R. norvegicus*

ENZYME	*Lymnaea stagnalis*			
	Contig	Transcript	ORF	Predicted size (aa)
Lym IDO-like	FCFB01037212.1 FCFB01037213.1 FCFB01037214.1	FX190660.1	MRQSFDLSKYNVSYDTGLMLKSPLKTLPSYFNPWNELAETLPKLVKEKKVREAVRRLPLLDHRQLVGHRQLRLAHLQLSFITAGYVWQDGDKGVPQVLPKCLAFPFYNISKELGIQPILGHVDLALANYHLINPSGPYEFENLQCLYHIPGGLAGDWFCIVTFMVEFSFARCLKHLVRLLDLLEVAHVQMRADEKGRKDTEREMAERLDDITLAVQTMEASLARMHDNLDAKMFFNVIRPFLGGWGGEGNPLPDGLIYEGISDYPIKMPGGSAAQSTTLQILDALLGVEHSEDKRAFLVHMRSFMPPDHCRLIVDLEQRPHKLRDLVESACSRELCDAYNNCVSALVHLRNYHIQIVTKYVIQASQTVNEGNYESLDKKGTGGTSLIPFLKDIRSDTESKIMPIPHTQSKKSQGKLMSWQKVAVVVAITASMAVGTIKLLKYK	**443**
Lym TDO-like	FCFB01109750.1 FCFB01109751.1 FCFB01255639.1 FCFB01297038.1	FX224560.1 FX206683.1 FX225637.1	MAECPMNNGVCKEDGNDLNYHSYLMLDNVLTSQVMQSAVKNKVVHDEHLFIITHQAYELWFKQILYEVDSVRDMFMLPVMDETKTILILNRLSRVTLILKLMVDQIDILETMTPLDFLEFRLQLSSASGFQSLQFRLLENKMGVLETYRVKYNQQHYTKVFSKPDQVKMLQDSIEQPSLFNLLEKWLERTPGLTVKSFHFWDLYKRSVERWLQDLLYIPAQKETDEKIKQSLIEEYKKQKESFDSILDEDKYNASVARGDRRLSHQAFKGALLISLYHDQPRFNQPSKLLSLLMDIDSLLTKWRYNHVMMVQRMIGSKVGTGGSSGYQYLRSTVSDRYKVFLDLFNMSTYLIPRDYIPPLSRSLKRQLSILIHEYMPRDPEDSDDYDLDDGTDDNSPEGKNVDSCPNEETRPKVKFEI	**418**
Lym AFMID-like	FCFB01077058.1 FCFB01161380.1 FCFB01232167.1	FX191423.1	MAPEAVTVAHLEALVQGSRNSLWTLDCETEISYGDSERQKFDIFHKKNASKNGAPMFAYIHGGYWQVKEVSREISSFMAVPLCNAGASVISIGYDVAPDVSLNTIVLQIKRALCLIIKIAKERQSSGIYLSGHCVGAHLAAMMLMADLDGFDRDLIKGAVLISGVYDLRPLVKTSINEPLGLTEVDAWRLSPHQFVQEIAYQSQDRHIVVAVAEYDPPEFRRQSGEMEMALRKFGVKTSYLDVPDTDHFNLVDKLTEPNFVLTKECIRLMGL	**272**
Lym KMO-like	FCFB01020652.1	FX185910.1	MTPKRESQSQRKKHVAVCGGGLVGALNACYMAKRGFKVDLYEMRPDIRTLEVVKGRSINLALSCRGREALRRVGLEDTITKNGIPMHARMIHDLNGKRRPLLYGNEDQYIMSIDRRLLNEVMLTEVEKFPDVNIHFSHKLVSCDFNSGEAIFENEKGECLTKQFDLIIGCDGAYSSVRKQLMKLSRFDYQQEYIPHGYMELNIPPNKKNESAMEINYLHIWPRNDYMMIALPNLDKSFTTTLFMPFEIFESITSEEKLIQFFKDEFPDALPLLGEAELKQSFFSCKALPLVSIKCHPYHVNDKAVIMGDAAHAMVPFYGQGMNCGMEDCIVFDELLDKYNNNFAKVLPAYTKIRNPDAKAICDLAMYNYTEMRSKVNSRLFLVRKKLDNMLFKLFPNTWVPLYTMVSFTRIRYHECVQRREWQDKILTRSLILSAVCILLGFTALIYNANKFGDHWNLETPQSLLTGSFDKFQKWISRW	**479**
Lym AADAT-like	FCFB01016053.1	FX183988.1	MIFRILGFKYYNSADNIVNYNSMTEEDESSDKEMNLLMKWSNGISFVAGSPGVELMKRCSEMLLTASKSVLGSSTIDIGNLFRYGAEQGDADFREQLSCFLSHEYRDKVKSANLMVTAGATQGLHAILSLLCDKASPIFVEDPSYFFAVRMIRDDLQMNLVPVPCDECGINPELLDQFLIAHKREFYKGNNSNINKRYTRFWAAAYLIPVFNNPTGQTYSETRCKELIQLARKHNILLIAEDIYNLIHFDESGLAPQRLLHYDNTSDPDYGGGHVLSNGTFSKIFAPSIRLGWIEGPEQLLFKMYNSNLTWSGGSFNHYMAKLMANILASGILYDHLKFTRSAYKERMFALCAALDELCPDGWSYPRPQGGYFLWITFPQNIDARKFELFARDNYGVGFLPGVYCSPTNSFINCARLSISSLQVDTIIEGAKKLFSAYQGYVIHIKPITD	**450**
Lym KYAT-like	FCFB01033100.1	FX191915.1	MHKELKSCTRFLLSCVQQHRNRKRVLKSPFSTCLTCFSTSSVNNKMTSKKFELAKRVQGTEKNVWVDISKLAVEQKSINLGQGFPDFMAPQDIVNCLKDTVNSDSPAVHQYARSFGHPRLVKALAELYEPFLGRQIDPMTEILISVGAYGALFCVIQGLVNPGDEVIIIEPFFDCYQPMVKAAGGTPVFVPLRPTKDGSVTSSADWKLDEGELSSKFNSKTKLIIVNTPNNPLGKVFTREELLVIANLCKKHDVVCVADEVYEWMTYNGNQHIKIATLPDMWERTVTIGSAGKTFSVTGWKIGWTVGPQHLVHCAQIMHQNCTYNCPTPIQEAVARGLELEKSRLGSPGSYLISLAASLKPKRDKLASVLEEVGMKPVIPEGGYFMMADYTSMEADVPESDEAKDFRFSKWMTINKKLTVIPPSAFYCKEHEHMAENYVRFCFIKEDSTLEKAANVLRHWKN	**462**
Lym KYNU-like	FCFB01070793.1	FX188572.1	MGDVNNATANSHSTTPLQELEKIANENNLKLGDVEFARHMDKRDPLKYLRDEFHYPKMGDVLNTDPSIVDLSEDCVYFCGNSLGLCPKKTKEYMNIQIDKWAKLGVQGHTNGELPWAWCDELLEEDMAKIVGCKREEVSLMNGLTVNLHLLLISFYRPTKDRYKILCESKAFPSDHYTFESQSRLHGFDPKDAMICVEPREGEFTLRTEDILDVIEKQGDKIAVVCFSGVQYYTGQLFDIPKITQAGKAKGCHVGWDLAHAVGNVPLKIHDWGVDFACWCTYKYLNASAGGLAGLFIHENFQTNDFPKLLGWWGHDMKTRFQMNNQMDLIPGARGYRISNTPGFLCVPLKASLEIFKKTSVEELRKKSLVLTAYLEHLIQKSYQRPAGKAPEDDCDAVYIDIFTPSDPKQRGAQLSLAFNVCIEQVFKELEKRGVVCDKRFPRVIRITPVPMYCSFEDVHRFMGYLKDALVAAKGSLRHVDTHKV	**485**
Lym ACMSD-like	FCFB01196791.1 FCFB01209656.1 FCFB01252153.1 FCFB01272611.1 FCFB01319919.1	FX187039.1	MKIDLHTHILPENWPDLKERYGYGGWVQLHHHCAGRAKMMKDGKLFREIEENCWSPEARIKEMDAHGVDIQVLSTVPVMFGYWAEPKDTLDLCEMLNNHLAGIVKSCPSRFVGLGTVPMQAPELAVKELIRCKKELGFPGIQIGSHINNWNLDAPELQQVFAAAEEHNCVIFVHPWDMEQGGRMQKYWLPWLVGMPTETTVAICSMIFGGVLERFPKLKVCFAHGGGAFPFTIGRIEHGFNSRPDLCAVENNVNPRKYLGQIYTDSLVHDTEALKLLVNVIGKNRIVLGSDYPFPLGEQHPGKLVESMEEFSPELKHQILAGNAMEFLGLDPSLYKPCIGEDTQMDSTNTHLPCP	**355**
Lym HAAO-like	FCFB01141163.1	FX195327.1	MSVSGPVVYNIDSWIDENKQYFLPPVCNKMMHNDGQMKVMYVGGPNQRKDYHIEEGEELFYMLKGDMCLKVVEHGQHRDVVINEGEIFLLPGKIAHSPQRQGDTIGLVIERERDNSELDGLRYYQEVDGVPTAESLYEEWFHCTDLGTQLGPVIKRYFGSEQHKTGKPVPGTIPESPPIILDSEISMQDPFNLHVWIEQNRKELDSKGFVQLFGDNFQFSVKLYGKGENTGLCDNAETWIWQMEGESVVTQNDKEFKLQKNDSLLIRVGERYKAARPEGSIALICYQDPSRKK	**293**

ENZYME	*Homo sapiens*			*Mus musculus*			*Rattus norvegicus*			*Aplysia californica*			*Biomphalaria glabrata*			*Pomacea canaliculata*			*Caenorhabditis elegans*		
	Ortholog	Identities	E-value Blastp	Ortholog	Identities	E-value Blastp	Ortholog	Identities	E-value Blastp	Ortholog	Identities	E-value Blastp	Ortholog	Identities	E-value Blastp	Ortholog	Identities	E-value Blastp	Ortholog	Identities	E-value Blastp
Lym IDO-like	NP_002155.1	152/412 (37%)	1.0E-84	NP_032350.1	147/407 (36%)	1.0E-84	NP_076463.1	154/408 (38%)	8.0E-90	XP_005113130.1	74/211 (35%)	1.0E-31	XP_013068348.1	262/443 (59%)	0.0E+00	XP_025080939.1	191/404 (47%)	5E-140	/	/	/
Lym TDO-like	NP_005642.1	198/380 (52%)	4.0E-133	NP_064295.2	185/354 (52%)	4.0E-127	NP_071798.1	189/355 (53%)	5.0E-130	XP_012941285.1	117/170 (69%)	7.0E-74	XP_013068951.1	339/419 (81%)	0.0E+00	XP_025088197.1	264/398 (66%)	0.0E+00	NP_498284.1	200/370 (54%)	9.0E-135
Lym AFMID-like	NP_001010982.2	95/242 (39%)	6.0E-53	NP_082103.1	98/270 (36%)	1.0E-49	NP_001104836.1	99/274 (36%)	2.0E-48	XP_005112504.2	178/275 (65%)	6.0E-138	XP_013073423	115/155 (74%)	1.0E-80	XP_025114029.1	160/277 (58%)	2.0E-119	/	/	/
Lym KMO-like	NP_003670.2	238/437 (54%)	0.0E+00	NP_598570.1	249/440 (57%)	0.0E+00	NP_067604.1	235/417 (56%)	5.0E-175	XP_012945866.1	324/464 (70%)	0.0E+00	XP_013064773.1	59/138 (43%)	2.0E-35	XP_025105148.1	280/437 (64%)	0.0E+00	NP_506025.1	225/460 (49%)	2.0E-156
Lym AADAT-like	NP_001273611.1	84/329 (26%)	9.0E-28	NP_035964.1	98/405 (24%)	3.0E-19	NP_058889.1	88/334 (26%)	1.0E-22	XP_012941811.1	78/334 (23%)	4.0E-14	XP_013087071.1	138/279 (49%)	1.0E-87	/	/	/	/	/	/
Lym KYAT-like	NP_001008661.1	234/442 (53%)	1.0E-175	NP_001280489.1	236/436 (54%)	2.0E-168	NP_001015037.1	231/444 (52%)	2.0E-167	XP_012935490.1	323/425 (76%)	0.0E+00	XP_013067258.1	144/184 (78%)	8.0E-109	XP_025114352.1	280/451 (62%)	0.0E+00	NP_001024823.1	187/412 (45%)	2.0E-133
Lym KYNU-like	NP_001186170.1	247/461 (54%)	5.0E-177	NP_081828.1	243/463 (52%)	4E-172	NP_446354.1	247/455 (54%)	4.0E-171	XP_005108249.1	379/470 (81%)	0.0E+00	XP_013068429.1	107/132 (81%)	9.0E-76	XP_025090853.1	311/482 (65%)	0.0E+00	NP_509023.1	243/454 (54%)	7.0E-175
Lym ACMSD-like	NP_612199.2	232/331 (70%)	0.0E+00	NP_001028213.1	236/336 (70%)	0.0E+00	NP_599199.1	235/336 (70%)	0.0E+00	XP_005102237.1	282/333 (85%)	0.0E+00	XP_013081878.1	301/352 (86%)	0.0E+00	XP_025094288.1	249/349 (71%)	0.0E+00	NP_001022935.1	167/383 (44%)	2.0E-106
Lym HAAO-like	NP_036337.2	143/283 (51%)	3.0E-111	NP_079601.1	143/284 (50%)	2.0E-100	NP_064461.1	143/284 (50%)	3.0E-102	XP_005102960.1	210/289 (73%)	5.0E-170	XP_013070925.1	167/221 (76%)	4.0E-130	XP_025104598.1	201/291 (69%)	1.0E-157	NP_505450.1	111/270 (41%)	3.0E-62

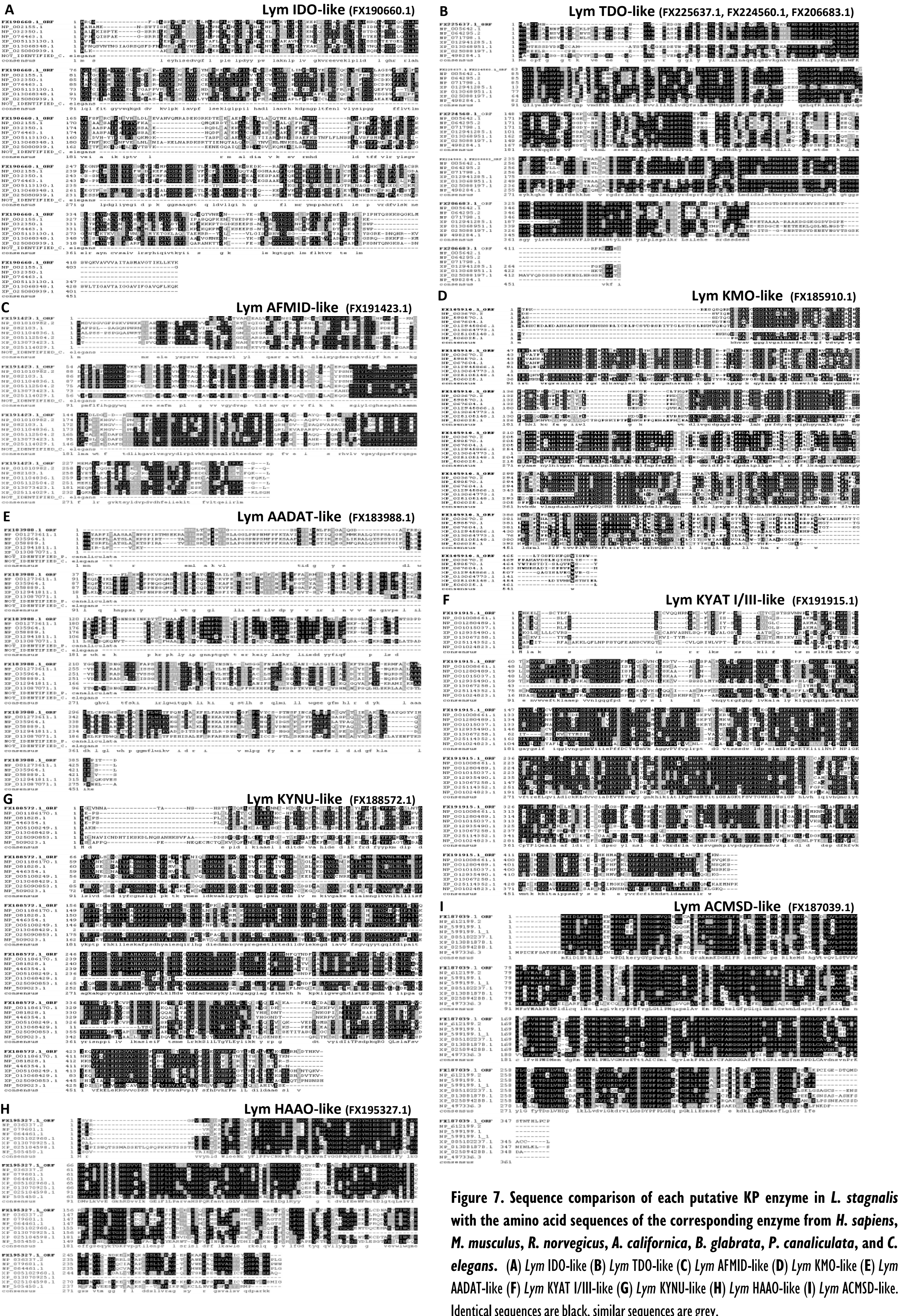


Figure 7. Sequence comparison of each putative KP enzyme in *L. stagnalis* with the amino acid sequences of the corresponding enzyme from *H. sapiens*, *M. musculus*, *R. norvegicus*, *A. californica*, *B. glabrata*, *P. canaliculata*, and *C. elegans*. (**A**) *Lym* IDO-like (**B**) *Lym* TDO-like (**C**) *Lym* AFMID-like (**D**) *Lym* KMO-like (**E**) *Lym* AADAT-like (**F**) *Lym* KYAT I/III-like (**G**) *Lym* KYNU-like (**H**) *Lym* HAAO-like (**I**) *Lym* ACMSD-like. Identical sequences are black, similar sequences are grey.

The phylogenetic analysis revealed a predictable pattern in the relatedness of *L. stagnalis* sequences to those of other *Mollusca* (**Figure 8**).

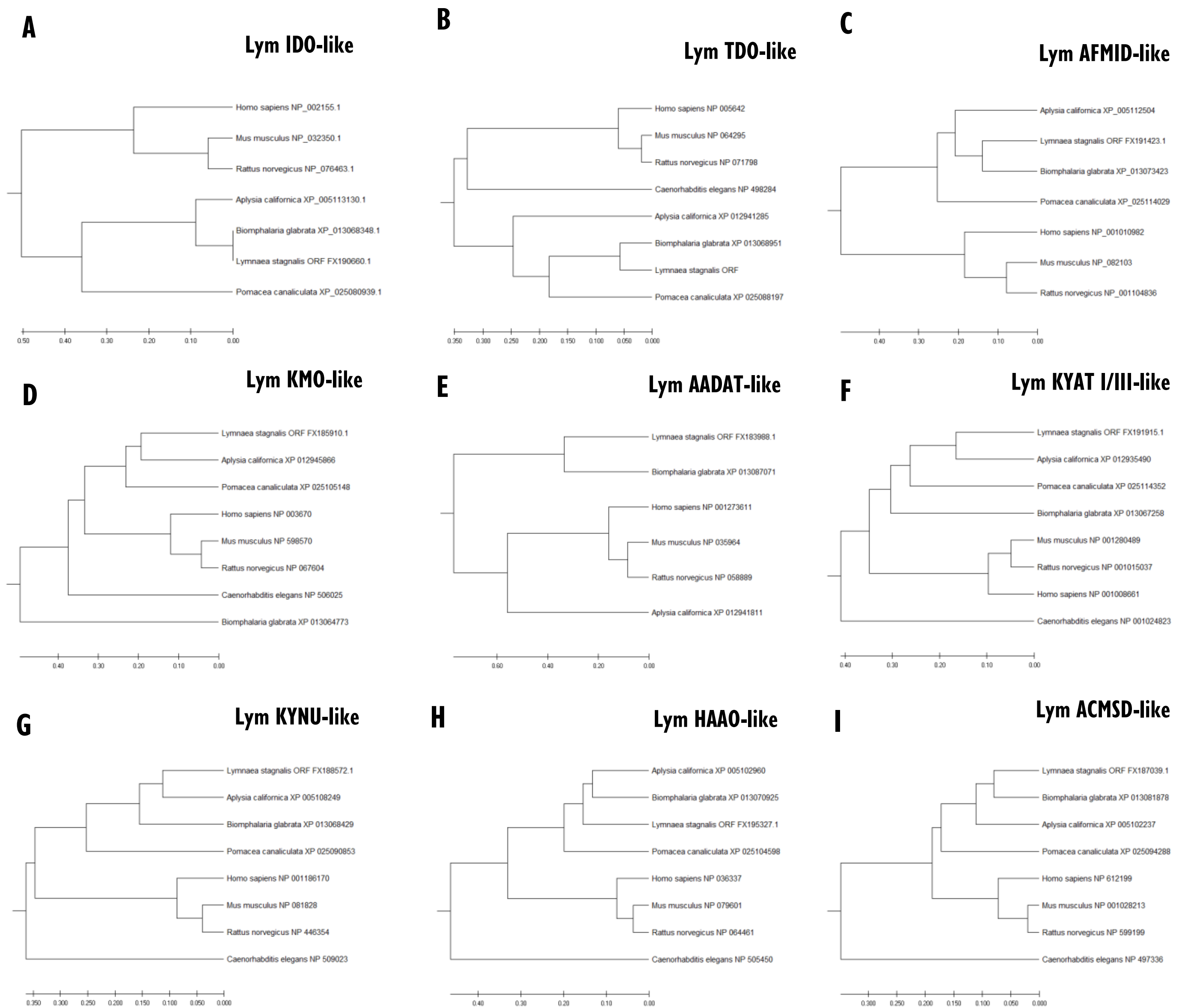


Figure 8. Molecular phylogenetic tree of *L. stagnalis stagnalis* KP-like enzymes

Lym IDO-like (**B**) *Lym* TDO-like (**C**) *Lym* AFMID-like (**D**) *Lym* KMO-like (**E**) *Lym* AADAT-like (**F**) *Lym* KYAT I/III-like (**G**) *Lym* KYNU-like (**H**) *Lym* HAAO-like (**I**) *Lym* ACMSD-like. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. This analysis involved 7-8 amino acid sequences. Analyses were conducted using MEGA X.

The analysis performed by PFAM showed that all the KP-predicted enzymes have the expected domains (**Figure 9**).

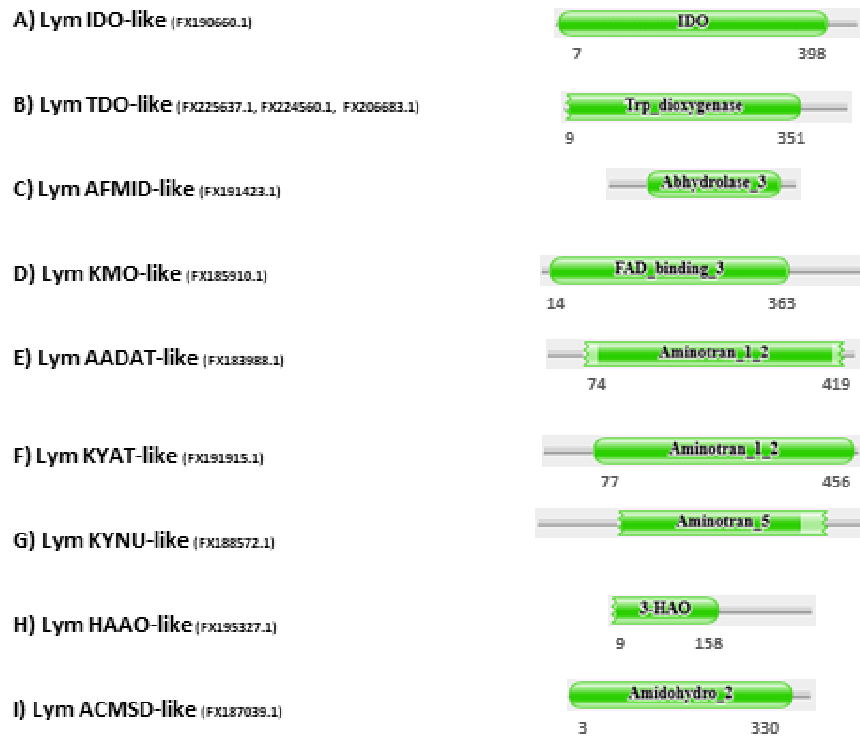


Figure 9. Identification and location of the conserved domain for each putative enzyme of the KP in *L. stagnalis stagnalis*
(**A**) *Lym* IDO-like (**B**) *Lym* TDO-like (**C**) *Lym* AFMID-like (**D**) *Lym* KMO-like (**E**) *Lym* AADAT-like (**F**) *Lym* KYAT I/III-like (**G**) *Lym* KYNU-like (**H**) *Lym* HAAO-like (**I**) *Lym* ACMSD-like. Analyses were performed using PFMA.

Part 2

Molecular analysis

Primer specificity, efficiency, and validation

qRT-PCR was used to study the changes in mRNA levels of the putative *L. stagnalis* KP-enzymes.

The mean Cq of all the putative KP-enzymes ranged from 25.41 to 30.02 for 20 ng of cDNA with moderately abundant mRNA levels in the ganglia of *L. stagnalis* (**Table 6**).

Table 6. Validated primers for gene expression analysis

TARGET	Transcript	PRIMER FW SEQUENCE	PRIMER RV SEQUENCE	SIZE (bp)	EFFICIENCY	R2	Ct value 20 ng
Lym IDO-like	FX190660.1	ACTTAGGAAGAGTTTCAGCA	TTAAACCTAATCCCACAGAC	179 bp	115.66%	0.981	25.41
Lym TDO-like	FX225637.1	CTCTAGAATGTCGATTTGGT	TGTCAGAGATATGTTCATGC	120 bp	119.89%	0.994	30.02
Lym-AFMID-like	FX191423.1	ACAAAATTAGGTTCCGTAAG	ATGATCCTCCAGAGTTTAGA	138 bp	114.07%	0.990	26.86
Lym-KMO-like	FX185910	TTTTGTGATGGTGTCTTCTA	TTCAAGGTTGATTTGTATGA	137 bp	94.82%	0.996	25.84
Lym-AADAT-like	FX183988.1	GTCCAACTCTTTGGAGATAA	TGAGTAACAACTGACTCACC	189 bp	119.37%	0.995	28.33
Lym-KYAT I/III-like	FX191915.1	GGAGGTATCACTGTCAATTT	TACTTCATGATGGCTGATTA	118 bp	125.30%	0.995	27.41
Lym-KYNU-like	FX188572.1	TTCCTTCAGATCATTACACA	CTGTACACCAGAAAAACAAA	178 bp	114.89%	0.991	27.34
Lym-HAAO-like	FX195327.1	CTCTAGAATGTCGATTTGGT	TGTCAGAGATATGTTCATGC	124 bp	117.46%	0.988	28.53
Lym-ACMSD-like	FX187039.1	TTTAGGAAATCTCTCAAGGA	AAGAGCACAACTGTGTGATA	138 bp	119.56%	0.996	26.82

Primers for all putative *L. stagnalis* KP-enzymes were evaluated to ensure that they could produce consistent results and not amplify off-target products or generate primer dimers. Following amplification, each primer pair produced amplicons that yielded single bands at the correct size after electrophoresis in 2% agarose gels (**Figure 10**). Additionally, no amplification was observed in cDNA from another pulmonated gastropod *P. canaliculata* nor in controls that lacked reverse transcriptase in the RT-PCR or cDNA template.

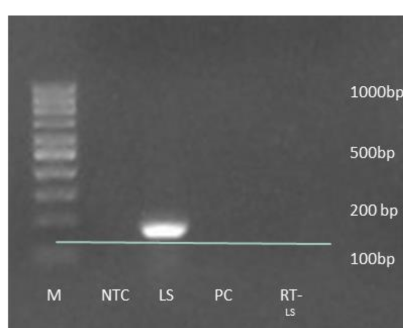


Figure 10. Example figure illustrating the species-specificity of one of the putative enzymes of the KP (*Lym* IDO-like).

The same results have been obtained for all the putative enzymes of the KP. Amplification is observed only in the cDNA from *L. stagnalis* (LS) but not in the cDNA of another pulmonated gastropod, *P. canaliculate* (PC) nor in the negative control (NCT), nor in the control lacking the reverse transcriptase in the RT-PCR (RT- LS). The product size is between 100 and 200 bp, as expected. Agarose gel 2%.

Primer specificity was checked also by melt curve analysis. A single sharp peak with no primer-dimer was observed in all primers (**Figure 11**). The PCR efficiency was comprised between 94.82% to 125.30% and the R^2 of primers was greater than 0.98.

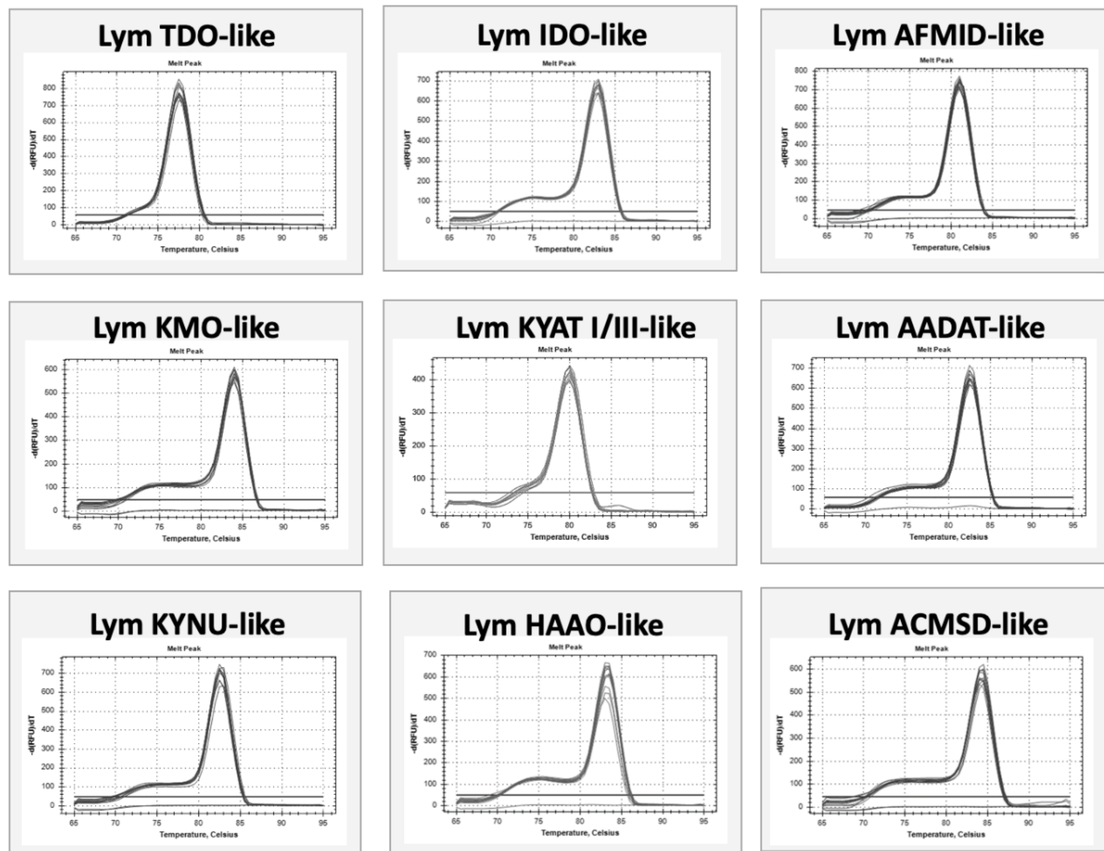


Figure 11. **Melting curves analysis**.

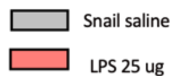
For each primer, a single sharp peak with no primer-dimer was observed.

Gene expression analysis

The expression levels of the KP enzymes were analyzed following treatment with 25 µg of LPS. No significant differences were revealed between saline exposed animals sacrificed at different times after injection. Results revealed a main effect of the treatment [F (1,44=15.62) = 1.45, P=0.0003], no main effect of time, and an interaction between the two terms [F (2,44) = 4.17, P=0.022] on *Lym* TDO-like mRNA levels. Bonferroni's multiple comparisons test showed a significant upregulation in animals injected with LPS at both 2 and 6 hours (p=0.025 and p=0.003, respectively) compared to their saline-injected counterparts (**Figure 12A**). Similarly, a main effect of the treatment [F (1,45) = 16.46, P=0.0002] and the interaction time x treatment [F (2,45) = 4.25, P=0.02] on the expression levels of *Lym* IDO-like was found. *Post-hoc* analyses showed a significant upregulation at 2 hours (p=0.025) and 6 hours (p=0.002) post LPS injection (**Figure 12B**). *Lym* AFMID-like mRNA levels, instead, were significantly affected only by the time after injection [F (1.6,36.13) = 5.66, P<0.0001] and no difference was found between treated and control animals at 2h, 6h or 24h (**Figure 12C**). In these experimental conditions, the expression levels of *Lym* KMO-like and *Lym* KYAT-like were not affected either by the treatment or the time after injection (**Figure 12D** and **Figure 12E**). On the other hand, a main effect of the treatment [F (1,43) = 18.17, P<0.0001] and interaction between the two terms [F (2,43) = 7.4, P=0.002] was revealed for *Lym* AADAT-like mRNA expression levels. *Post-hoc* analysis showed significant downregulation in animals injected with LPS and sacrificed 6 hours (p=0.003) or 24 hours later (p=0.03) in respect to their saline-injected counterparts (**Figure 12F**). Two-way ANOVA for *Lym* KYNU-like revealed a main effect of the time of the treatment [F (1.77,38.13) = 7.25, P=0.003] and the interaction between treatment and time [F (2,43) = 8.18, P=0.001]. *Post-hoc* analysis showed that 6h after the LPS injection, *Lym* KYNU-like expression levels were significantly decreased with respect to snails receiving saline (p=0.003). No effects were reported in the cohorts of animals sacrificed 2 or 24h after injection (**Figure 12G**). For *Lym* HAAO-like expression levels, the two-way ANOVA analysis revealed only a main effect of treatment [F (1,46) = 4.78, P=0.03], subsequent Bonferroni's multiple comparisons test failed to reveal a main effect in any of the cohorts sacrificed together (**Figure 12H**). Finally, the expression levels of *Lym* ACMSD-like were found to be affected by the treatment [F (1,16) = 148.5, P<0.0001], the time after injection [F (1.4,18.6) = 41.76, P<0.0001], and the interaction between them [F (2,27) = 46.77, P<0.0001].

Post-hoc analysis showed a 7-fold increase in mRNA levels of *Lym* ACMSD in animals receiving LPS at 2 hours post-injection with respect to the saline counterpart (p<0.0001).

Six hours after treatment *Lym* ACMSD-like mRNA was still upregulated in animals receiving LPS with respect to saline-exposed animals (p<0.0001) (**Figure 12I**).



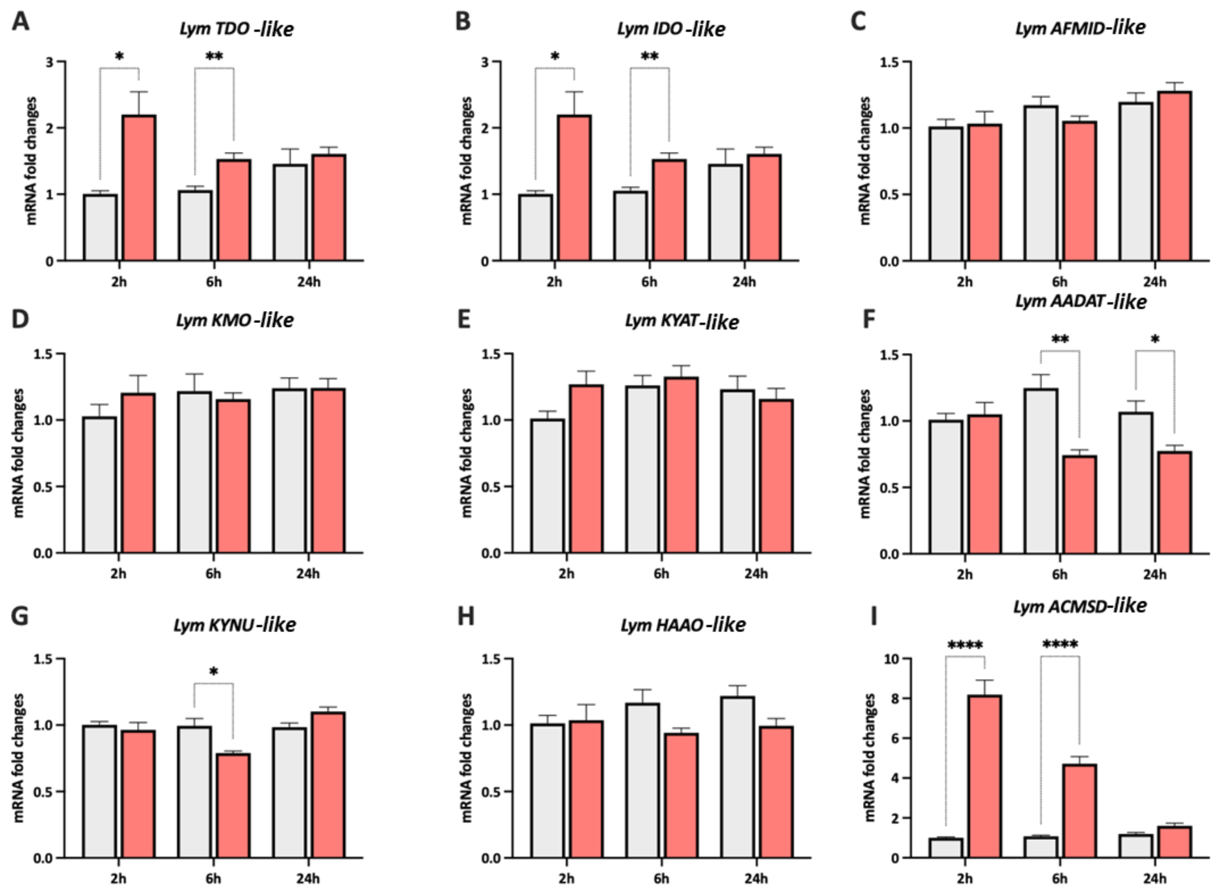


Figure 12. Effect of an immune challenge on expression levels of putative KP enzyme in the CNS of *L. stagnalis stagnalis*.

Adult snails were injected with either snail saline (grey bars) or 25 µg of LPS (red bars) and sacrificed 2, 6 or 24hlater. (**A**) *Lym* IDO-like (**B**) *Lym* TDO-like (**C**) *Lym* AFMID-like (**D**) *Lym* KMO-like (**E**) *Lym* AADAT-like (**F**) *Lym* KYAT I/III-like (**G**) *Lym* KYNU-like (**H**) *Lym* HAAO-like (**I**) *Lym* ACMSD-like. mRNA expression in the ganglia, with *Lym* TUB as endogenous control, were measured by Real-time PCR. N=8-10. Data are represented as means ± S.E.M. and were analysed with Two-way ANOVA Mixed effects followed by Bonferroni *post-hoc* analysis. **** $p < 0.0001$, ** $p < 0.01$, and * $p < 0.05$.

Summing up the results presented:

1. the combined approach of bioinformatics and molecular biology developed in this study represents a quick, solid, and well-reproducible strategy for the characterization of key pathways involved in neurophysiology and pathology, like the KP.
2. The enzymes involved in tryptophan catabolism via kynurenine formation are highly conserved in invertebrate and vertebrate model systems.
3. The highly conserved enzymes of the KP are expressed in *L. stagnalis*' CNS and are differentially regulated by an immune challenge (i.e., LPS).

AIM 2

Defining and characterizing a behavioural protocol for inducing a Garcia effect-like phenotype in L. stagnalis

Background

In what is considered a pioneering paper, Garcia and colleagues (Garcia *et al.* 1955) demonstrated a long-lasting (~30 days) gustatory aversion to a specific taste (i.e., saccharin-water) following its pairing with a dose of low-intensity gamma radiation, which caused nausea in the rats some hours later. It was later shown (Garcia *et al.* 1966) that a single paired presentation of the novel food and the negative reinforcing stimulus (even with a long interval between the stimuli) is sufficient for a long-lasting taste aversion to be formed (Garcia *et al.* 1967). These findings added to the concept of 'bait shyness' (Rzóska 1953). In humans this is known as the 'Sauce Bearnaise-effect' after a researcher became nauseous some hours after eating Bearnaise sauce (Seligman 1970). However, it is unclear if the Bearnaise sauce was the causative agent in the development of nausea, as nausea may have been caused by the stomach flu. Anyways, the researcher avoided eating Bearnaise sauce in the future as it was this taste that was associated with the later developed nausea (Stensmyr and Caron 2020).

In this study, a new behavioural paradigm has been developed to induce the formation of a Garcia-like effect in *L. stagnalis*. To this purpose, a slurry of carrot (C) was used as the novel, appetitive taste, and a heat shock stressor (HS, 30 °C for 1 h) was adopted as the aversive nausea-inducing stimulus.

This learning paradigm allowed me to demonstrate that snails respond to nausea with a dietary strategy in its choice of future decisions about which foods to eat.

Materials and methods

Animals

The populations of *L. stagnalis* (Linnaeus 1758) used in the current study were established at the University of Calgary (Canada) and the University of Modena and Reggio Emilia (Italy) and both derived from the same Vrije Universiteit Amsterdam colony (the standard laboratory strain used worldwide for *L. stagnalis* studies). Adult animals having shell lengths of 20 to 25 mm were used in these experiments. For snails' maintenance information see Aim 1.

Carrot slurry (C)

Snails were exposed to a new chemosensory stimulus: a carrot slurry (C), which is an appetitive food stimulus that elicits a robust feeding behaviour (Sugai 2006; Swinton *et al.* 2020). This was done by preparing a C slurry by putting two fresh medium carrots in a blender along with 500 mL of pond-water (PW). Following blending and straining of the mixture, a carrot–PW slurry without any observable pieces of carrot (i.e., C) was obtained. The snails used in these experiments had not previously been exposed to C; except for one specific cohort (N=12) that was only given slices of carrot to eat for one week in their home aquarium before the Garcia-effect experiments.

Sucrose solution

In some of the behavioural experiments, it was used a sucrose solution to determine whether there was an overall suppression of feeding behaviour, or it was only specific to C that was used as a CS for pairing with heat shock. Sucrose solution was prepared by dissolving 1.71g of sucrose in 1L of PW (5mM). In naïve W snails (N=15) the average number of rasps per minute in sucrose is mean±se: 16.6 ±1.39.

Heat shock stressor

In humans, a prolonged exposure to or physical exertion in high temperatures results in nausea and vomiting (Becker and Stewart 2011) Moreover, studies from rodents demonstrated that the paired presentation of an appetitive taste with a thermal shock can induce a Garcia-like effect. In this study, it has been assumed that a heat shock exposure may result in some sort of visceral illness (reaction/sickness) in snails. As for laboratory reared snails, a prolonged (> 3h) exposure to temperatures of 30°C or above is lethal, the heat procedure adopted in this study was 30 °C for 1h.

Snails were placed in a 1-litre beaker filled with 500 mL of PW which was previously heated to 30°C. The beaker containing *L. stagnalis* was then maintained in a water bath at 30°C for 1h. The heat shock has been chosen as the aversive stimulus because studies from

Quercetin administration

Quercetin (3,3′,4′,5,7-pentahydroxyflavone, Q - MW: 302 g/mol) was obtained from Sigma-Aldrich (St Louis, MO, USA - CAS number 117-39-5). A stock solution was prepared by dissolving quercetin in 1 mL of 100% dimethyl sulfoxide (DMSO) at a concentration of 1M. A quercetin final solution (Q) was prepared by dissolving 50µl of stock solution (1M) in 500mL of pond water (PW). This concentration of quercetin (100 mmol L-1) has been successfully used previously in *L. stagnalis* (Sunada *et al.* 2016). Snails were removed from their home aquarium and placed in 500 mL of 100 µmol/L quercetin PW for 1h. After that, animals were returned to their home aquarium, where they were fed *ad libitum*.

Behavioural procedure

Feeding behaviour or rasping in *L. stagnalis* is a rhythmic motor behaviour in which repeated movements of the radulae scrape the surface of a substrate, leading to the ingestion of food(Benjamin and Kemenes 2013). In this experiment, snails were placed into a 14 cm diameter Petri dish with enough PW or C for the snails to be partially submerged. To observe the feeding behaviour, 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 where the number of rasps was counted for 2 min in PW. Previous studies demonstrated that some snails open their mouths by chance in the absence of any delivered stimulus and such spontaneous openings occur at a rate of about one per minute (Kojima *et al.* 1996). One hour after the PW stimulus, snails were exposed to C and were similarly monitored for 2 min while recording rasping behaviour, following an acclimation. Animals were then returned to their home aquaria (e.g., 1 or 24h) until they were exposed to the HS for 1h. The rasping behaviour was again determined for 2 min in C at different time intervals following the HS.

In all experiments, after the 2-min C re-exposure following HS, snails were allowed *ad libitum* access to food (i.e., lettuce). The behavioural experiments were performed in the morning because it has been shown that the learning scores are better in the morning than at other times (Wagatsuma *et al.* 2005).

Snail marks-scheme

Snails were given marks or grades on an individual basis to show how well (or how poorly) they learned. The following grading scheme was used to assess learning: a mark of 'A' was assigned if the feeding behaviour in C at 24h post-HS had a greater than 50% reduction with respect to the first C exposure (C pre); a mark of 'B' was assigned if there was a 35–49.99% reduction in the number of rasps in C-post 24h compared to the initial C exposure; a mark of 'C' was assigned when there was a 20–34.99% decrease, and an 'F' was assigned if the decrease was less than 20%. This marking scheme has been successfully used before (e.g., Aonuma *et al.* 2016).

Total RNA extraction, reverse transcription, and Real-Time polymerase chain reaction

For more detail, See the Material and Methods of Aim 1. In this experiment, total RNA was extracted from the central ring ganglion of *L. stagnalis* and 6 samples were collected for each group. Specific forward and reverse primers were used at the final concentration of 300 nM. Two different reference genes were used: elongation factor-1 and b-tubulin. The nucleotide sequences of the primer used for the target investigated were as follows: Heat shock protein 70 (*Lym* HSP70): 5'- AGGCAGAGATTGGCAGGAT-3' and 3'-CCATTTCATTGTGTCGTTGC-5' (product length: 199 bp); Heat shock protein 40 (*Lym* HSP40) 5'- GTGTTTGGTCACCTTCTTT -3' and 3'-AAGGTCTTGAATCCTGATG -5' (product length: 120 bp).

Statistical analysis

All behavioural data were first analysed for normality assumption using Kolmogorov–Smirnov one-sample test for normality (K–S distance and P). Parametric data were then further analysed using a repeated-measures analysis of variance (RM ANOVA) followed by a Tukey's *post-hoc* test. For non-normal data, a Friedman's test was conducted, followed by a Dunn's multiple comparison *post-hoc* test. A two-way repeated-measures ANOVA having one between subjects' factor, where independent groups of snails went through different interstimulus intervals (ISI: 4h, 24h, and 48h) and one within-subjects factor (i.e., feeding response at different time points) was used to determine whether memory was present (i.e., whether there was a significant difference in the number of rasps compared to the C pre-HS response and whether that was different across the groups). For gene expression analyses, mRNA levels of each target were normalized to two reference genes, elongation factor 1α (Lym EF1α) and β-tubulin (Lym β TUB).

The stability of mRNA expression of these endogenous controls was assessed using Normfinder®, considering intra and intergroup variations. 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 ΔΔCt method was performed using as calibrator the average levels of expression of control animals. Statistical analyses were performed using One-way ANOVA. Significant changes were determined by Tukey's *post-hoc* test. Significant differences between two groups were examined by Student's t-test. All tests were defined as significant at $p < 0.05$. R squared (R^2) values have been reported for all our ANOVA models and effect sizes by providing z or q scores wherever applicable. All statistical analyses were performed using GraphPad Prism v. 9.00e for MAC® (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Heat-induced suppression feeding behaviour to C

First, it was investigated whether the exposure for 1h to the heat shock stressor (HS; 30°C for 1h) altered the snails' rasping response to C, which was a novel food taste for these snails. Initially, it was demonstrated that C has a positive hedonic value. The feeding response to C has been examined in a naïve cohort of snails (N=12) 1h before and 3h after the exposure to the HS. An RM One-way ANOVA [F (1.290, 14.19) = 71.27, P<0.0001, R2=0.86, **Figure 13A**] followed by Tukey's *post-hoc* test revealed that the rate of rasping in C was significantly increased with respect to PW (q=13.32, p<0.0001). However, 3h after the HS presentation the feeding behaviour elicited by C was significantly decreased with respect to the first exposure (q=10.35, p<0.0001), suggesting that, following HS, C no longer acted as a strong appetitive stimulus. Then it was investigated whether this significant suppression in rasping behaviour was due to the repeated stimulation of the snails by C. A first control cohort of naïve snails (N=10; **Figure 13B**) did not experience the HS but was exposed to C two times with an interval of 5h (i.e., the same interval between two subsequent C exposures in the previous experiment) and it was found that the increased feeding behaviour induced by C with respect to PW [RM One-way ANOVA, F (1.718, 15.4) = 38.8, P<0.0001, R2=0.13] was not reduced following two repeated presentations of C (PW vs C: q=13.95; p<0.0001; PW vs C 5h: q=9.94, p=0.002; C vs C 5h: q=0.04, p=0.999, *post-hoc* Tukey's test), indicating that in the absence of the HS there was no shift in the hedonic value of C due to the second presentation of C. A second control cohort of snails (N=10; **Figure 13C**) was exposed to C only after the HS. Snails did not experience C before the HS. As the HS procedure did not suppress the feeding response to C, which was significantly increased with respect to the response in PW [RM One-way ANOVA, F (1.207, 10.87) = 136.5, P<0.0001, R2=0.93], it was concluded that the presentation of C after the HS did not alter the hedonic value of C.

Together these data led to the conclusion that snails were forming an association between C and the HS when C preceded the HS. This association persisted for at least 3h; even though there was an interval of 1h between the presentation of C and HS. Once the association is formed, C is no longer perceived by the snails as an appetitive stimulus, suggesting that *L. stagnalis* exhibited a 'Garcia-effect' and became 'bait shy to C.

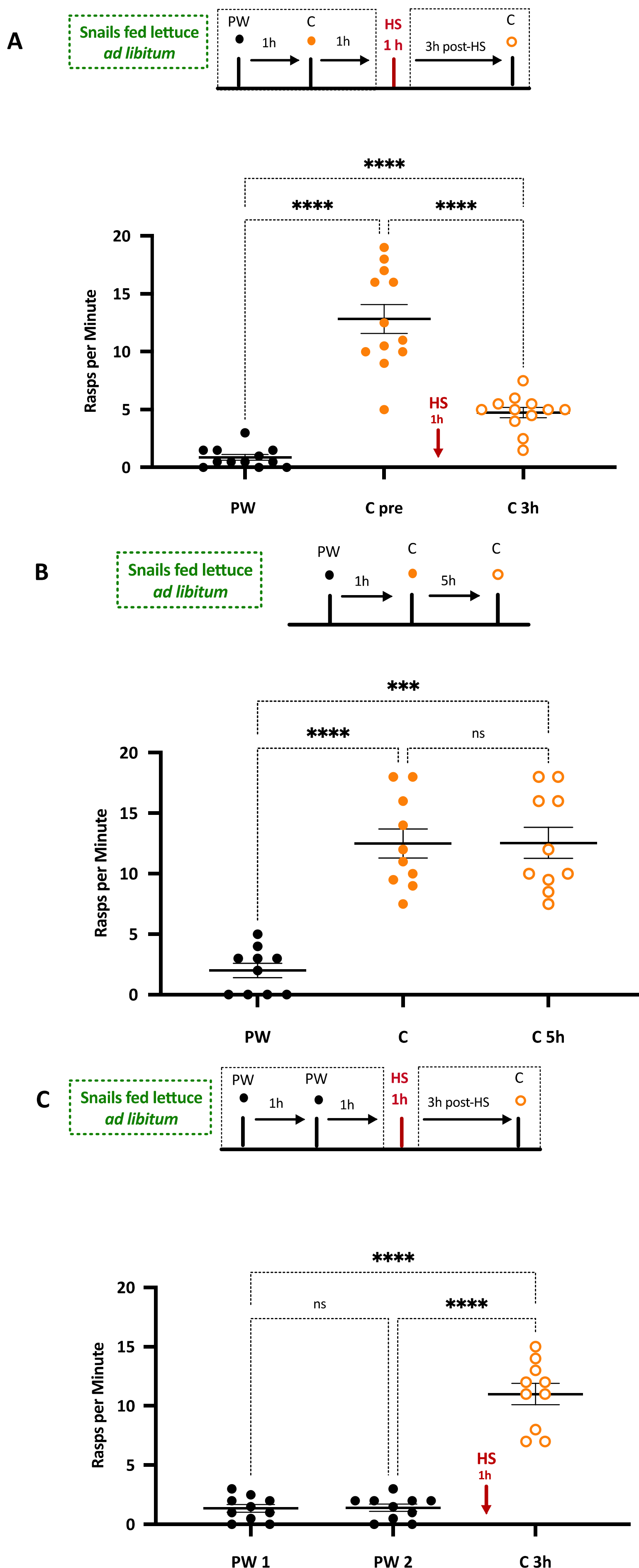


Figure 13. Heat-induced suppression feeding behaviour to C.

The timeline of each experiment is presented above the data. The snails used in this experiment were fed lettuce *ad libitum* and never experienced C before.

(A) In a cohort of snails (N=12) spontaneous rasping was first counted in PW for 2 min (PW) followed by C (C pre) for 2 min, before experiencing the HS (30°C for 1h). At 3h post-HS, the number of rasps in carrot (C 3h) was significantly suppressed with respect to C pre-HS.

(B) In the second cohort of snails (N=10) rasping was first counted in PW for 2 min (PW) and in C (C) for 2 min. There was no reduction in the number of rasps in subsequent exposure to C (C 5h).

(C) In the third cohort of snails (N=10) rasping was first counted in PW for 2 min (PW1) followed by another PW (PW2). Snails next received the HS and 3 hours later their response to C was observed (C 3h). There was a significant increase in the number of rasps compared to what was observed in PW.

The solid line is the mean, and the error bars are the s.e.m. Comparisons were made by RM One-way ANOVA, followed by *post-hoc* Tuckey's test. **** $p<0.0001$; *** $p<0.001$; ns = not significant as $p>0.05$.

The persistence of memory following the one-trial conditioning C-HS procedure

Next, it was examined how long the negative hedonic shift to C persisted. A new naïve cohort of snails (N=10; **Figure 14A**) was given the same C-HS training procedure (i.e., C preceded HS by 1h) and then C was presented 3h, 24h, and 48h after the HS. It was found an overall significant effect of the treatments [RM One-way ANOVA, F (2.306, 20.75) = 31.03, P<0.0001, R2 =0.77], such that when snails were exposed to C 3h and 24h after the HS their feeding response was significantly decreased compared to the feeding response 1h prior to the HS (q=6.987, p=0.005 and q=11.1, p=0.0002, respectively, Tukey's *post-hoc* analysis). In addition, no differences were observed in the feeding response elicited by C at 3h and 24h after the HS exposure (q=1.40, p>0.05). Moreover, the number of rasps elicited by C 48h after HS was not significantly reduced compared to the response before the HS (q=0.05, p=0.99). Finally, the feeding response to C 48h after HS was significantly greater than the response to C at 3h and 24h (q=9.37, p=0.0007, and q=9.25, p=0.0008, respectively). Thus, after a single C-HS exposure, the negative hedonic shift in *L. stagnalis'* response to C (i.e., a suppression of feeding elicited by C) persisted for at least 24h.

To control for the possibility that the repeated presentation of C 3h and 24h following HS, resulted in a reduced response to C at 24h, a new cohort of snails (N=10) was trained as in Figure 14A but only tested at 24h and 48h after HS (**Figure 14B**). The results were like those presented in Figure 14A [RM One-way ANOVA, F (1.774, 15.97) = 66.95, P<0.001, R2 =0.88]. The feeding response elicited by C at 24h post-HS was significantly suppressed with respect to C pre-HS (q=11.2, p=0.0001, *post-hoc* Tukey's test). In addition, no significant differences were found between the initial C response and the rasping behaviour at 48h following the HS (q=0.53, p=0.98). Thus, after a single C-HS pairing, there was a negative hedonic shift in the snails' response to C that persisted for at least 24h but less than 48h.

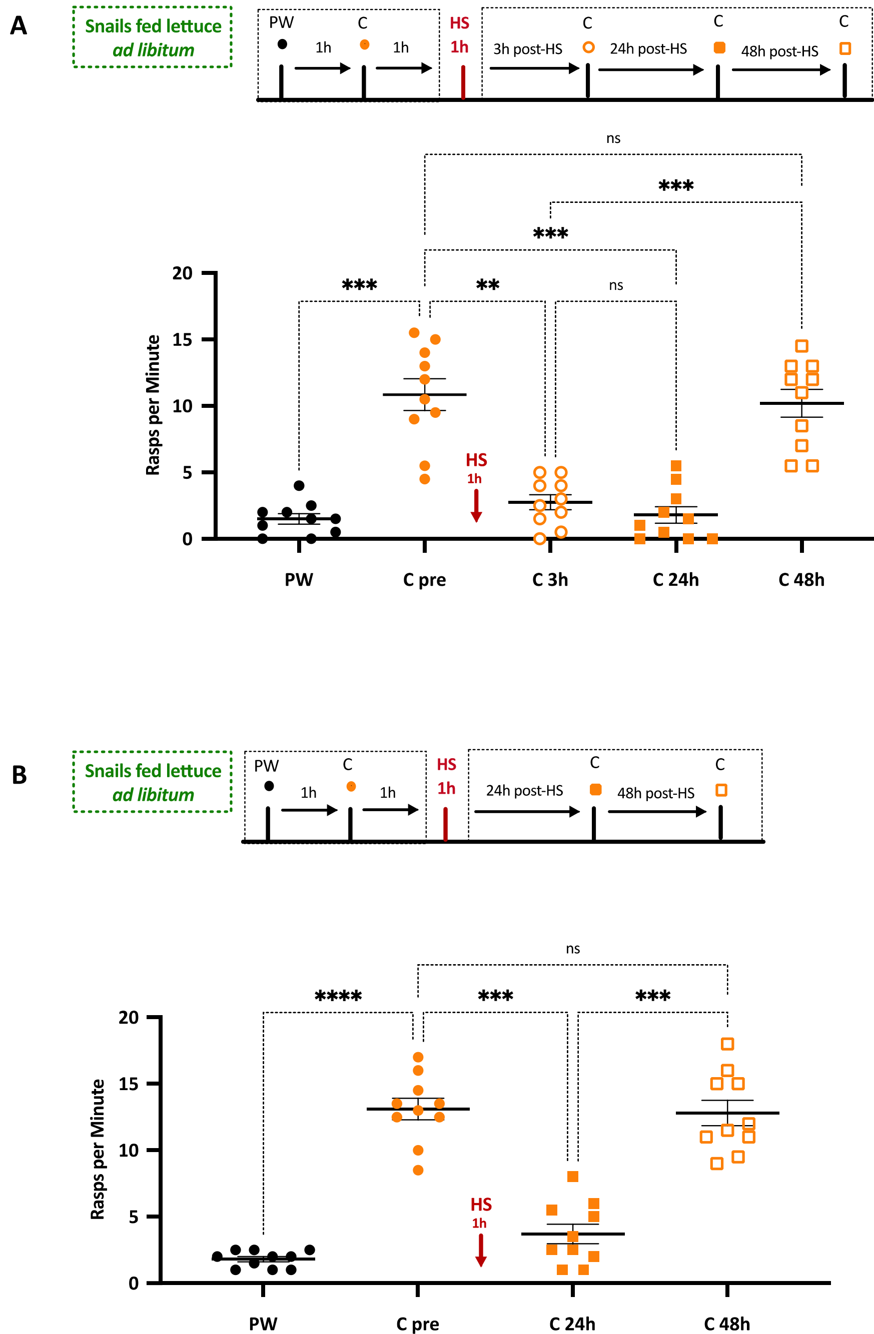


Figure 14. The persistence of memory following the one-trial conditioning C-HS procedure. The timeline of each experiment is presented above the data.

(**A**) In a cohort of snails (N=10) spontaneous rasping was first counted for 2 min in PW (PW) and, 1h later, in C (C pre), which was a novel taste. One hour later snails were exposed to the HS for 1h. The response to C was then recorded for 2h, 3h (C 3h), 24h (C 24h), and 48h (C 48h) after the HS. The number of rasps in C was significantly suppressed for 24h but not 48h with respect to C pre.

(**B**) In a cohort of snails (N=10) spontaneous rasping was counted for 2 min in PW and, 1h later, in C, which was a novel appetitive taste. Following this, snails experienced the HS for 1h. At 24h post-HS exposure, C (C 24h) elicited significantly lower rasps with respect to the pre-heat session. C 48h, instead, was not different from C pre. The solid line is the mean, and the error bars are the s.e.m. Comparisons were made by RM One-way ANOVA, followed by *post-hoc* Tuckey's test; **** $p<0.0001$; *** $p<0.001$; ** $p<0.01$; ns = not significant as $p>0.05$.

Taste discrimination and stimulus specificity

Next (**Figure 15A**), it was tested whether the suppression was specific to C. A naïve cohort of 12 snails was subjected to the C-HS procedure and then it was tested the response to another appetitive stimulus (i.e., sucrose). A Friedman test followed by a Dunn's multiple comparisons *post-hoc* test indicated that the HS procedure significantly suppressed the feeding response to C but not to sucrose [Friedman statistic=29.97, P<0.0001]. The feeding response elicited by sucrose was not statistically different from that elicited by C pre. Moreover, the response to sucrose shown here was comparable to the responses elicited by sucrose in naïve snails (See the Material and methods). While no significant differences were found between the number of rasps performed in sucrose at 3h post-HS with respect to the initial C response (z=0.16, p>0.05), the feeding behaviour elicited by C at 4h post-HS was significantly suppressed compared to what is observed in either C pre-HS test or to the sucrose response (z=4.03, p =0.0003). Thus, the negative hedonic shift was only evident to C and not to sucrose. In addition, the positive hedonic value to sucrose did not alter the negative shift in hedonic value to C as a result of C preceding the HS.

In another naïve cohort of snails (N= 12; **Figure 15B**) the feeding behaviour post-HS was first investigated in C (i.e., C 3h), and then in sucrose (i.e., sucrose post-4h). An RM One-way ANOVA [F (1.958,21.54) = 80.43, P<0.0001, R2=0.87] followed by Tukey's *post-hoc* test revealed that after C was paired with the HS, it no longer elicited a feeding response (q=21.34, p<0.0001 vs C pre) but sucrose did. It was found no significant differences between the number of rasps elicited by sucrose at 4h post-HS when compared to the initial C response (q=0.26, p>0.05). Thus, it was concluded that the association (i.e., the negative hedonic shift to C) created between C and the HS was not generalized to another food substance.

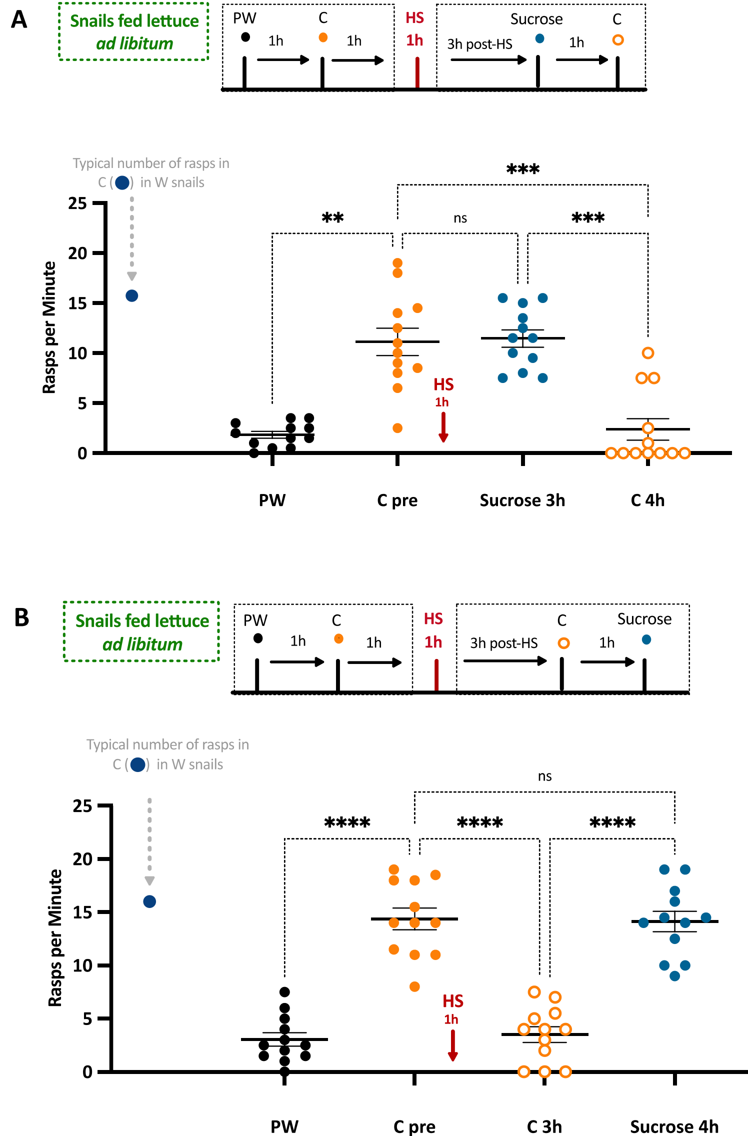


Figure 15. After the one-trial conditioning, C-HS procedure snails still have the capacity to discriminate between C and sucrose.

The timeline of each experiment is presented above the data. The average number of rasps elicited by L in naïve animals (i.e., not exposed to the HS) is presented in the graph for comparison.

(**A**) In a cohort of snails (N=12) spontaneous rasping was counted for 2 min in PW and, 1h later, in C (C pre). These snails never experienced C before, thus C was a novel appetitive stimulus. Animals subsequently experienced the HS for 1h and, 1h later, were exposed to sucrose (Sucrose 3h). One hour later, snails were re-exposed to C (C 4h). The C-HS procedure did not suppress the feeding response to sucrose but did to C. Comparisons were made by Friedman test, followed by Dunn's multiple comparisons test.

(**B**) In a cohort of snails (N=12) spontaneous rasping was counted for 2 min in PW and, 1h later, in C (C pre), which was a novel taste. These snails never experienced C before as they were fed lettuce *ad libitum*. Following this, snails experienced the HS. The feeding behaviour 3h post-HS to C was then assessed (C 3h) and 1h later the response to sucrose was recOrded (sucrose 4h). The C-HS procedure again did not suppress the feeding response to sucrose but did to C. Comparisons were made by RM One-way ANOVA, followed by *post-hoc* Tuckey's test.

The solid line is the mean, and the error bars are the s.e.m. **** $p<0.0001$; *** $p<0.001$; ** $p<0.01$; ns = not significant as $p>0.05$.

Interval between stimuli

In the experiments presented above (**Figures 13-15**) there was a 1h interval between the snails' initial exposure to C and then the HS. The effects of 3 longer intervals (4h, 24h, and 48h) between C and HS were also evaluated. During these intervals, snails continued to have *ad libitum* access to food (lettuce) in their home aquaria. In **Figure 16A-C** it was used an inter-stimulus interval (ISI) of 4h, 24h, and 48h, respectively. However, regardless of the interval length duration, similar results were found (i.e., there was no significant interaction effect between ISI's and different treatments of stimulus-induced feeding response; Two-way RM ANOVA: Treatments*ISI: [$F (6,87) = 1.26$, $P=0.28$].

In each of the naïve cohorts regardless of the interval between the first presentation of C and the experience of the HS a negative hedonic shift occurred to C 3h and 24h post-HS [$F (2.59,75.29) = 137.3$, $P<0.0001$; *post-hoc*: C3h: $q=18.2$, $p<0.0001$; C 24h: $q=15.80$, $p<0.0001$]. However, it was found an overall significant effect of the ISI [$F (2,29) =5.58$, $P=0.008$].

Thus, to more easily compare the hedonic response elicited by C with the three-time intervals used (4h, 24h, and 48h) between the presentation of C and the HS it was employed the 'marks-scheme' described in the Methods section. These data are presented in **Figure 16**. As can easily be seen the number of A decreases from 64% to 30% as the interval increases from 4h to 48h. The 'best' outcome was achieved with the shorter interval compared to the longest interval. Interestingly, it was only observed C grades in the 24h and 48h ISI and F grades only in the 48h ISI. Thus, despite the number of 'poorer performers' increasing with the length of the interval between C and HS. Even with the longest interval of 48h between the first experience of C and receiving the HS, a significant negative hedonic shift occurred.

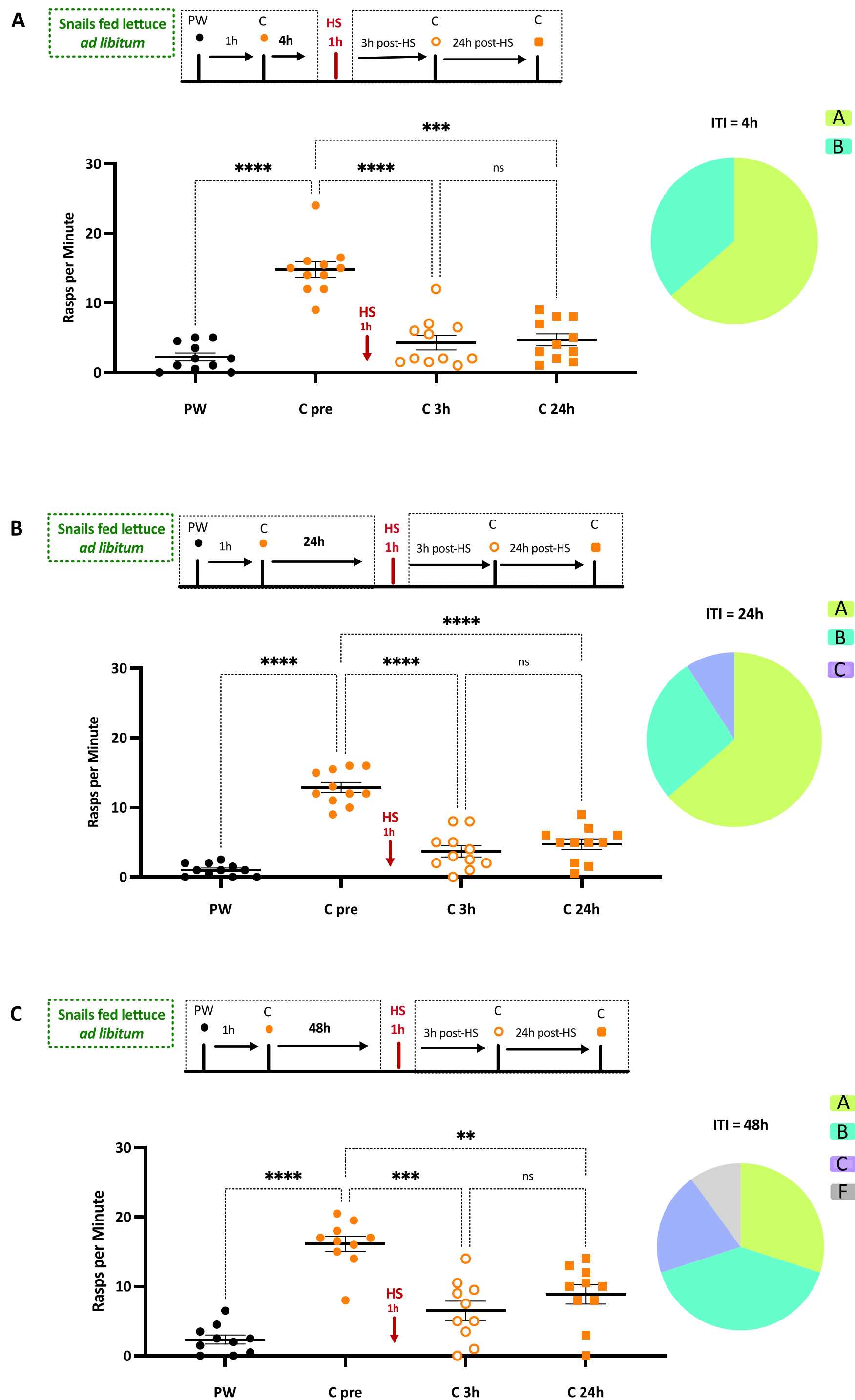


Figure 16. Lengthening the interval between the initial presentation of C and the HS.
The timeline of each experiment is presented above the data. The response to PW and then to C before the presentation of the HS are shown. The interval between the initial presentation of C (C pre) and the HS was 1h (**A**, N=11), 24h (**B**, N=11) and 48h (**C**, N=11) respectively. In all three cohorts, the response to C 3h (C 3h) and 24h (C 24h) was significantly reduced compared to C pre. The solid line is the mean, and the error bars are the s.e.m. Comparisons were made by RM two-Way ANOVA. ****p<0.0001; ***p<0.001; ** p<0.01.
Pie charts. The same data have been replotted using a marks scheme. Snails were given grades based on their individual performance. Grades were calculated as follows: a 50% reduction or greater is an A (green), a B is a reduction of 35–49.99% (light blue), a C is a 20–34.99% reduction (purple), and an F is a reduction of less than 20% (grey).

Garcia effect in food-deprived snails

Then, it has been hypothesized that altering the internal state of the snails by a period of food deprivation before their initial exposure to C, would mitigate the negative hedonic shift to C as a result of the pairing. Thus, a new naïve cohort of snails (N=9, **Figure 17**) following their initial exposure to C was food-deprived (i.e., no lettuce in their home aquarium) for 48 h before they experienced the HS. The feeding response to C was tested 3h after the HS and an overall significant effect of the treatment was found (Friedman statistic = 27.87; P<0.0001). At 3h post-HS there was no negative hedonic shift to C (z=0.596, p>0.05, *post-hoc* Dunn's test). These snails were then returned to their home aquarium where they had *ad libitum* access to lettuce for 24h. They were no longer in a food-deprived state. Interestingly, their hedonic response to C at 24h post-HS was significantly reduced compared to the previous C exposures (C pre vs C 3h: z = 2.83, p = 0.04; C pre vs C 24h z = 3.43, p = 0.006, respectively). Thus, a change in the internal state of these snails from a food-deprived to a non-food-deprived state enabled the negative shift in the hedonic value of C to become observable. These data suggest that when snails are 2-day food-deprived, the homeostatic drive to eat prevailed over the negative hedonic shift to C brought about by the pairing of C with the HS.

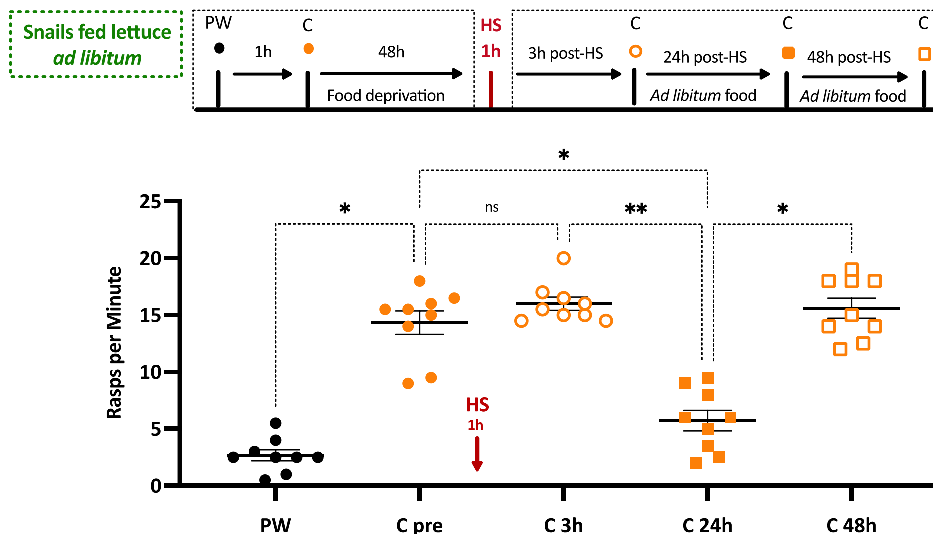


Figure 17. Garcia effect in food-deprived snails.

The timeline for the experiment is presented above the data. In a cohort of snails (N=9) spontaneous rasping was first counted in PW for 2 min (PW) and was followed by C (C pre), which was a novel taste. An interval of 48h then ensued before the presentation of the HS. During this 48h interval snails were food-deprived. Three hours post-HS, snails were re-exposed to C (C 3h) and the number of rasps was not significantly different from C pre. Animals were then returned to their home aquarium for 24h during which time had *ad libitum* access to food (lettuce). The feeding response to C was then recorded 24h and 48h post-HS (C 24h and C 48h, respectively). The response to C 24h post-HS (C 24h) was suppressed compared to C pre and C 3h, and came back to pre-heat shock levels at C 48h post-HS. The solid line is the mean, and the error bars are the s.e.m. Comparisons were made by Friedman test, followed by Dunn's test. **p<0.01; *p<0.05; ns= not significant as p>0.05.

Food novelty

In earlier studies (Garcia *et al.* 1966) a novel food substance was 'paired' with the stimulus (e.g., LiCl or radiation) that caused nausea some hours later. Here it was studied whether a familiar taste would not cause the Garcia effect to form when paired with the HS. Thus, a cohort (N=11) was fed carrots only for 7 days before C was paired with the HS (**Figure 18**). The feeding response was analysed using RM One-way ANOVA [F (1.762,17.62) = 70.2, P<0.0001, R2=0.87]. It was found rasping in PW was significantly lower than C pre (q=17.33, p<0.0001), and C 3h post-HS (q=13, p<0.0001). C 3h post-HS continued to elicit a feeding response like what occurred before being paired with the HS (q=0.67, p=0.884). No significant differences emerged in the number of rasps elicited by C 3h after HS compared to C before the HS. Thus, when C was not a novel taste the Garcia effect did not occur.

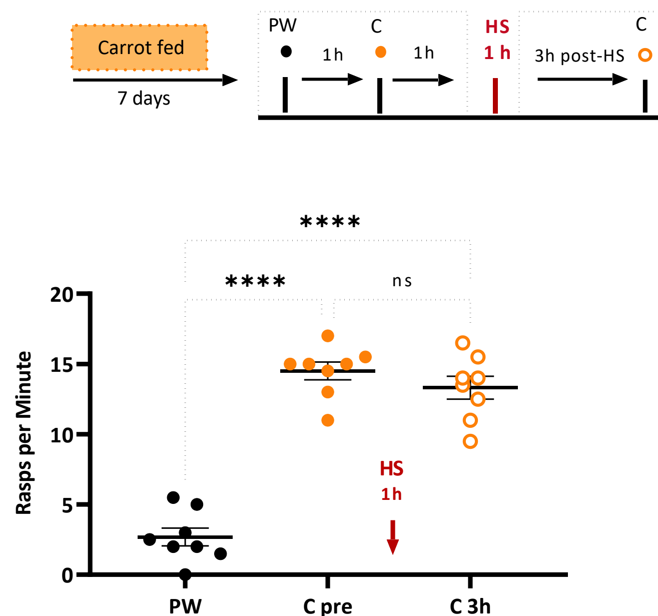


Figure 18. The Garcia effect does not occur when C is not a novel food taste.
The timeline for the experiment is presented above the data. A cohort of snails (N=11) was fed with only C diet for 7 days. before C-HS procedure. The following day spontaneous rasping was counted for 2 min in PW (PW) and, 1h later, in C (C pre). Snails received HS subsequently. At 3h post-HS (C 3h) the feeding behaviour was not significantly suppressed with respect to C pre. The solid line is the mean, and the error bars are the s.e.m. Comparisons were made by RM One-way ANOVA, followed by *post-hoc* Tuckey's test. ****p<0.00001; ns = not significant as p>0.05.

Gene expression analysis

It was next attempted to elucidate possible molecular mechanisms by which the HS brings about food suppression to C. Previous studies revealed that (1) in the CNS, HS causes a rapid up-regulation of the mRNA levels of both HSP40 and HSP70 that reaches a peak of expression within 2-4h of exposure to the thermal stress (Foster *et al.* 2015); (2) experiencing the thermal stressor prior to or following a single 0.5h training session for operant conditioning of aerial respiration resulted in an enhanced LTM formation (Teskey *et al.* 2012); (3) the HS-mediated enhancement of memory can be blocked by a flavonol, quercetin (Q - Sunada *et al.* 2016).

It was therefore investigated whether HSP70 and HSP40 would play a role in the molecular pathway activated by the HS during the association that occurs when the presentation of C precedes the HS. To accomplish this, it was studied the transcriptional activity of HSP70 and HSP40 induced by the application of Q alone, before or after the HS. It was first verified that a DMSO concentration of 0.1% (vehicle in which Q was dissolved) did not alter the transcriptional activity of *Lym* HSP70 or *Lym* HSP40 in the CNS. A group of naïve snails was exposed to PW (N=6) or DMSO (N=6) for 1 h and the CNS was extracted for gene expression analysis 4h later. No differences between vehicle (DMSO) and PW snails in the expression levels of *Lym* HSP70 (unpaired t-test, $t=0.25$, $p>0.05$, **Figure 19A**) and *Lym* HSP40 ($t=0,28$, $p>0.05$, **Figure 19B**) were found.

Similar results were obtained in snails exposed to PW (N=6) or DMSO (N=6) for 1 h and then subjected to the HS (HS Alone). The CNS was sampled for gene expression analysis 3h after the termination of thermal stress. No differences in the expression levels of *Lym* HSP70 ($t=0.65$, $p>0.05$, **Figure 19C**) and *Lym* HSP40 ($t=0,09$, $p>0.05$, **Figure 19D**) emerged between vehicle (DMSO) control and naïve (PW) control groups.

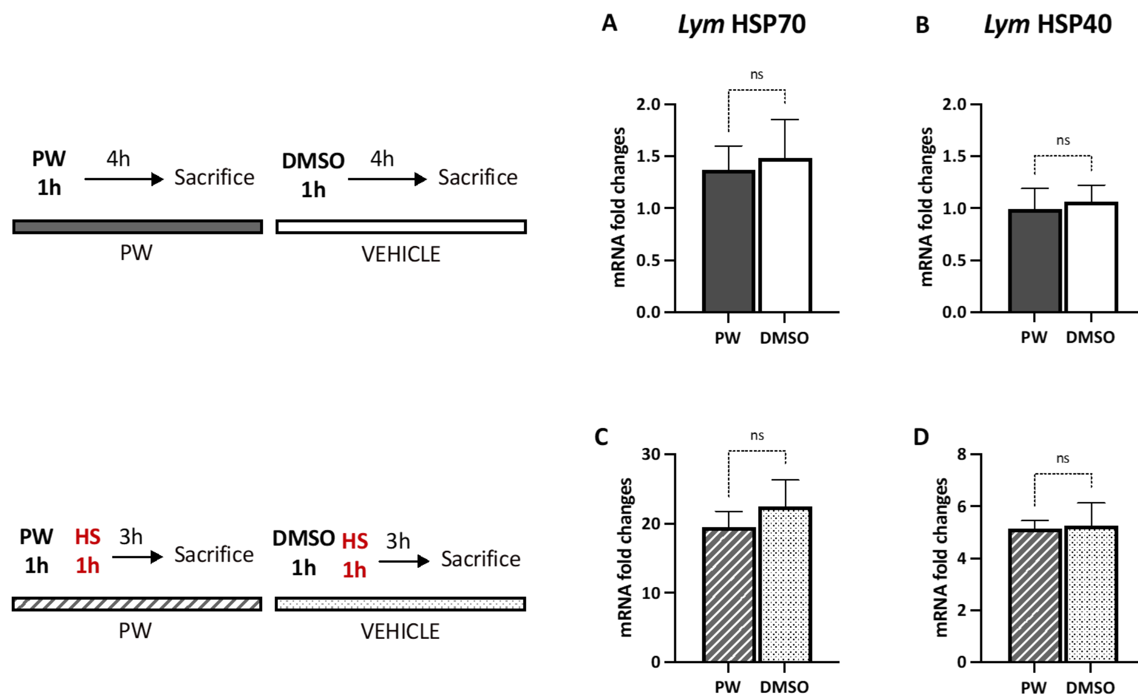


Figure 19. The exposure to DMSO 0.1% has no effect on the expression levels of *Lym* HSP70 and *Lym* HSP40 in the CNS of *L. stagnalis*.

Four cohorts of adult snails were employed: the first cohort was exposed to PW (grey bars); the second cohort was exposed to DMSO (vehicle – white bars) for 1h; the third cohort was exposed to PW for 1h and then to the HS for 1h (dashed bars); the fourth cohort was exposed to DMSO for 1h, before experiencing the HS for 1h (dotted bars). The expression levels of *Lym* HSP70 (**A-C**) and *Lym* HSP40 (**B-D**) in the central ring ganglia, with *Lym* TUB as endogenous control, were measured by Real-time PCR. N=6 for each group. The solid line is the mean, and the error bars are the s.e.m. Data were analysed with an unpaired t-test. ns = not significant as p>0.05.

Thus, it was investigated the differences in the transcriptional profiles of HSP70 and HSP40 in CNS of snails following different treatments (**Figure 20**). Five cohorts of snails (N=6 in each cohort) were employed in which snails were:

1. exposed to DMSO for 1h but did not experience the HS (i.e., vehicle cohort);
2. immersed in DMSO for 1h and then was subjected to the HS procedure but were not exposed to quercetin (i.e., HS alone cohort);
3. immersed in Q for 1h but did not experience the HS (i.e., Q alone cohort);
4. exposed to Q for 1h before the HS presentation (i.e., Q pre-HS cohort);
5. exposed to Q for 1h immediately after the HS presentation (i.e., Q post-HS cohort).

A One-way ANOVA revealed a main effect of the different procedures on the mRNA levels of LymHSP70 [F (4,25) = 221.4, $P<0.0001$, $R^2=0.97$ - **Figure 20A**]. *Post-hoc* Tukey's tests showed that the expression levels of HSP70 were significantly induced only in snails of the 'HS alone' (q=28.06 vs 'vehicle'; q=28.39 vs 'Q alone'; q=27.72 vs 'Q Pre-HS'; $p<0.0001$) and 'Q post-test (q=26.22 vs 'vehicle'; q=26.54 vs 'Q alone'; q=25.87 vs 'Q pre-HS'; $p<0.0001$) groups.

The increased expressions levels due to the HS was prevented by the exposure to Q: *Lym* HSP70 mRNA levels of the groups 'Q alone' and 'Q pre-HS' were not statistically different from that of the vehicle (q=0.33, $p>0.05$ and q=0.34, $p>0.05$, respectively). A similar trend was observed for *Lym* HSP40 [One-way ANOVA, F (4,25) = 43.77, $P<0.0001$, $R^2=0.87$ - **Figure 20B**]: the exposure to the HS resulted in an overall significant increase in *Lym* HSP40 expression levels in snails of the 'HS alone' (q=14.03 vs 'vehicle'; q=14.69 vs 'Q alone'; q=12.43 vs 'Q pre-HS'; $p<0.0001$) and 'Q post-HS' (q=9.99 vs 'vehicle'; q=10.65 vs 'Q alone'; q=8.399 vs 'Q pre-HS'; $p<0.0001$) groups with respect to the other ones. Again, this up-regulation was prevented by the exposure to Q alone (q=0.66, $p<0.0001$) and before the HS (q=1.6, $p<0.001$), resulting in no statistical differences with the vehicle.

Because no differences were observed in the expression levels of *Lym* HSP70 and *Lym* HSP40 between snails exposed to quercetin alone and before the HS procedure (q=0.66 for HSP70 and q=2.25 for HSP40; $p>0.05$), all the transcriptional effects observed in snails of the 'Q pre-HS' group were considered as quercetin mediated. Thus, the up-regulation of the two HSPs induced by the heat shock stressor can be prevented by exposure to quercetin for 1h before HS.

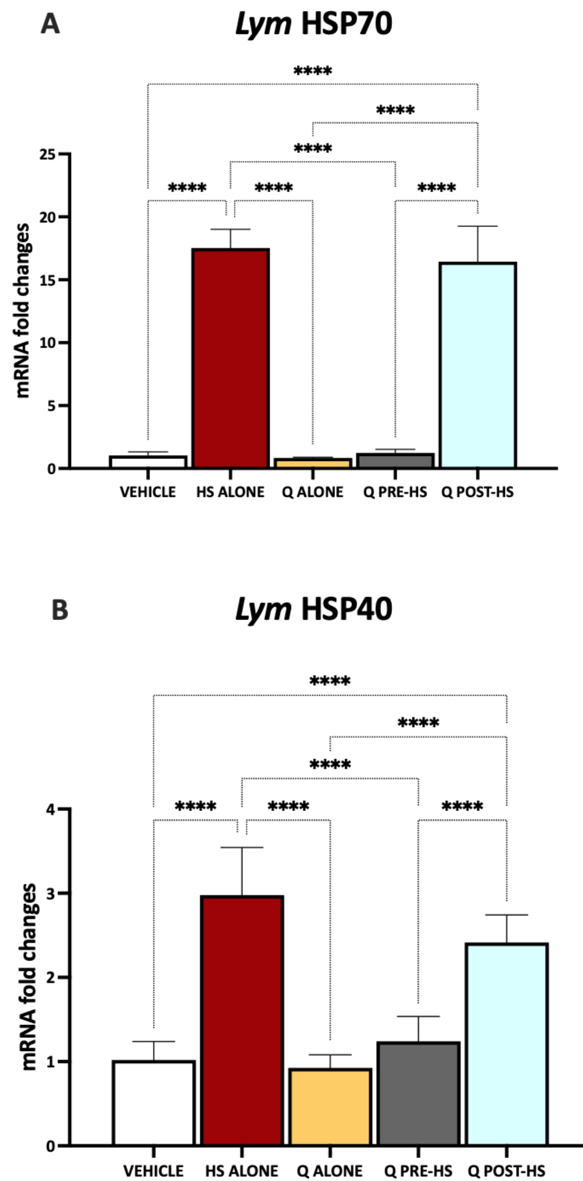


Figure 20. HSP70 and HSP40 elaboration in the CNS under different conditions.

The timeline of each experiment is presented above the data. Five cohorts of snails (N=6 for each cohort) were employed: (1) the first cohort was exposed to DMSO for 1h and did not experience the HS (vehicle – white bars); (2) the second cohort was exposed to DMSO for 1h and then was subjected to the HS procedure (HS alone – red bars); (3) the third cohort was exposed to quercetin (Q) for 1h but did not experience the HS (Q alone – yellow bars); (4) the fourth cohort was exposed to Q for 1h before the HS presentation (Q PRE-HS – grey bars); (5) the fifth cohort was exposed to quercetin for 1h immediately after the HS presentation (Q POST-HS – light blue bars). The heat shock induced a strong up-regulation of HSP70 and HSP40, which can be prevented by exposing snails to quercetin for 1h before the HS. The expression levels of *Lym* HSP70 (**A**) and *Lym* HSP40 (**B**) in the central ring ganglia, with *Lym* TUB as endogenous control, were measured by Real-time PCR. The solid line is the mean, and the error bars are the s.e.m. Comparisons were made by RM One-way ANOVA, followed by *post-hoc* Tuckey's test; ****$p<0.00001$.

Quercetin and the Garcia effect

Based on these molecular data, it has been hypothesized that the up-regulation of HSPs induced by HS plays a role in the negative hedonic shift to C because of the C-HS pairing (**Figure 21**). To test this hypothesis, it was included exposure to quercetin in the behavioural paradigm. It was first confirmed that Q, in the absence of the heat stressor, did not significantly alter the feeding behaviour elicited by C. Thus, a cohort of naïve snails (N=12) was exposed to PW and then 1h later to C and the number of rasps was counted. One hour later, snails were immersed in Q for 1h and the feeding behaviour was tested in C 3h later. An RM One-way ANOVA [F (1.938, 21.31) = 23.85, P<0.0001, R2=0.68; **Figure 21A**) followed by Tukey's *post-hoc* test revealed the following: (1) there was no difference in the rasping behaviour to C by exposure to Q (i.e., C was not different from C 3h: q=0.78, p=0.84); (2) there was a significant increase in feeding behaviour in C before and C 3h later compared to that in PW (q=7.7, p=0.0005 and q=8.45, p=0.0002 for C and C 3h respectively). Thus, Q on its own does not alter the feeding response elicited by C.

Next, (**Figure 21B**) a naïve cohort of snails (N=12) was first exposed to C 1h before HS and immediately after were immersed in Q for 1h. A main effect of the treatment was found (Friedman statistic=32.40, P<0.0001). In particular, the feeding behaviour elicited by C at 3h and 24h post-HS was significantly suppressed (z=3.23, p=0.001 and z=3.68, p=0.002 *post-hoc* Dunn's test, respectively). The feeding suppression was not significantly different between the post 3h and post 24h C sessions (z=0.45, p>0.05). Thus, Q exposure after the HS does not prevent the negative hedonic shift to C following the C-HS pairing.

On the other hand, when the Q exposure occurred immediately before the HS, the feeding behaviour in C at 3h and 24h post-HS was not suppressed [RM ANOVA, F (2.911, 32.02) = 20.43, P<0.0001, R2=0.65, **Figure 21C**). No differences were observed between the feeding behaviour in C pre-HS and at 3h and 24h post-HS (q=3.86, p=0.08 and q=1.42; p =0.75, respectively).

These data are consistent with the hypothesis that up-regulation of HSP70 and HSP40 plays a role in the long-lasting association between pre-exposure to a food substance and the HS stressor that results in the negative hedonic shift to C. However, it cannot be excluded that Q may act on two parallel outputs, one is the memory, and the other is HSP40 and HSP70.

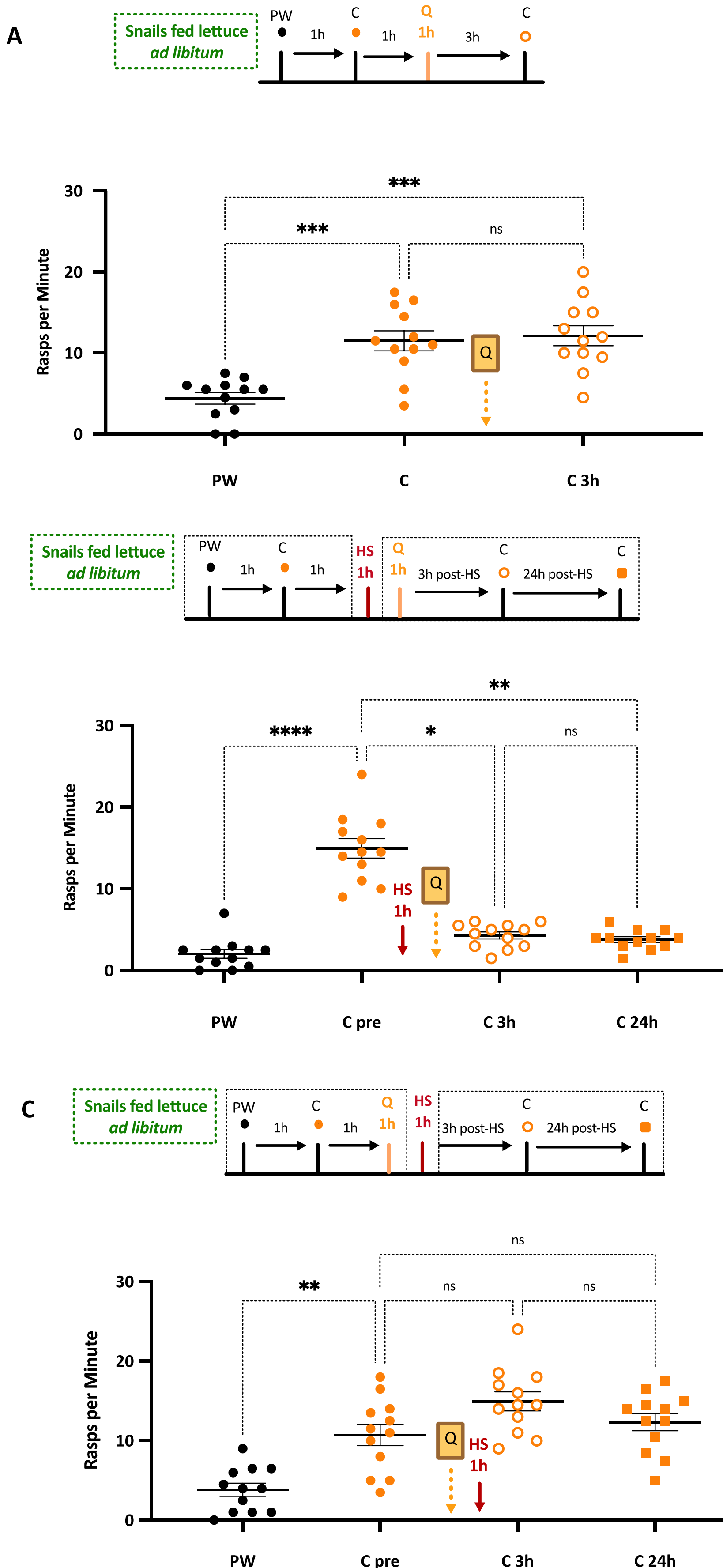


Figure 21. Quercetin and the Garcia effect.

The timelines for each experiment are presented above the data. The snails used in these experiments never experienced C.

(A) In a cohort of snails (N=12) spontaneous rasping was counted in PW (PW) and 1h later in C (C pre). Subsequently, snails were immersed in quercetin (Q) for 1h and 3h later were re-exposed to C (C 3h). Q exposure did not alter the feeding behaviour in C.

(B) In a cohort of snails (N=12) spontaneous rasping was first counted in PW (PW) and, 1h later, by C (C pre). Then, snails experienced the HS and were then immersed in Q for 1h. 3h and 24h later, snails were re-exposed to C (C 3h and C 24h). The exposure to Q after the HS did not block the Garcia effect as the response to C at both intervals was suppressed.

(C) In a cohort of snails (N=12) spontaneous rasping was first counted in PW (PW) followed by C (C pre). One hour later, snails were immersed in Q for 1h and immediately later were exposed to the HS. The Garcia effect was not formed as the response to C 3h (C 3h) and 24h (C 24h) after HS was not suppressed.

The solid line is the mean, and the error bars are the s.e.m. Comparisons were made by RM One-way ANOVA, followed by *post-hoc* Tuckey's test. ****$p < 0.0001$; ***$p < 0.001$; ** $p < 0.01$; * $p < 0.05$. ns= not significant as $p > 0.05$.

AIM 3

Comparison of the cognitive properties of different strains of L. stagnalis using an environmentally relevant stressor

Background

L. stagnalis represents a multipurpose model organism in which to address a large range of different biological questions, problems, and phenomena related to the environment and the nervous system. Among them, attention should be paid to the effects of the current global warming on multiple behavioural and physiological traits of organisms in nature.

Although many studies demonstrated that the rise in temperatures is severely impacting the behavioural ecology of animals, the role of thermal stress on neuronal plasticity and memory formation is not well understood. Behaviours like feeding, respiration, and locomotion are poorly understood, limiting the possibility to predict the effects of global warming on animal resilience and adaptive behavioural traits. As a poikilothermic ectotherm, *L. stagnalis* is dependent on the thermal conditions of its environment. Because the thermal tolerance and sensitivity to heat stress show variation across populations of the same species experiencing different thermal regimes, differences can be found between wild animals and their laboratory-reared counterparts.

Although the upper lethal thermal limit of inbreeding snails is ∼32–33 °C (Vaughn 1953; Salo *et al.* 2017), the acute exposure to a heat stressor (HS, 30°C for 1h) as might be experienced in mid-summer in the natural environment, either before, during or immediately after operant conditioning of the aerial respiratory behaviour, enhances the ability of laboratory-inbred snails to form LTM.

In freshly collected snails, instead, the same stressor does not induce LTM, as these animals do not perceive the heat shock as a stressor (Hughes *et al.* 2017).

The different thermo-environmental conditions induce different behavioural responses to the same HS in snails, suggesting that memory formation is modified by the level of stress perceived and experienced during learning and a memory consolidation period. In the previous chapter, it has been shown that a single acute exposure to an HS paired with a novel taste, induces a Garcia-like effect

in laboratory-reared snails W snails. In the current study, instead, the focus has been placed on the effects induced by a daily presentation to an HS before the Garcia effect procedure

The importance of this study relies on the fact that the current global warming is increasing not only the mean temperature, but is also leading to higher fluctuations in daily temperatures (Barton 2010), which, in turn, may impact the cognitive abilities of animals. It has been investigated whether the repeated exposure to a thermal stressor, which mimics the thermal fluctuations occurring in wild environments, before the Garcia effect procedure, could result in an increased or decreased sensitivity to the heat.

Furthermore, it has been compared the heat sensitivity and the cognitive performances between laboratory-reared and wild snails.

In particular, it has been studied whether:

1. the daily exposure to the heat stressor for one week before the Garcia effect procedure could increase the thermal stress tolerance of W snails or acclimate them to the point that the HS no longer induced the avoidance response to the specific taste to which it had been paired;
2. freshly collected snails would exhibit a Garcia effect following a single and/or a repeated HS presentation;
3. breeding and raising the first generation (F1) of the freshly collected snails under constant laboratory temperature conditions could transform them to resemble the behavioural phenotype exhibited by the W snails. In that way, it has been possible to define if the heat stress sensitivity of F1 snails is more dependent on their parental inheritance (nature) or the current local environment in which they are raised (nurture).

Material and methods

Animals

Laboratory reared inbred *L. stagnalis* (i.e., W snails) were bred and raised in the snail facility at the University of Calgary from a strain of *L. stagnalis* originally obtained from Vrije Universiteit in Amsterdam. Adult (2 months old) snails (with a shell length of 2.5–3.0 cm) were maintained at a density of one snail per liter in artificial PW under eumoxic (i.e., normal O2 levels; PO2 > 9975 Pa) conditions. Artificial PW consisted of deionized water containing 0.26 g/l Instant Ocean (Spectrum Brands, Madison, WI, USA), with added calcium sulfate dihydrate to create what we refer to as a 'standard calcium level' of 0.8 g/L (Dalesman and Rundle 2010). Animals were maintained at room temperature (20–22 °C) on a schedule of 16 h:8 h light: dark and had ad libitum access to romaine lettuce.

The wild snails were freshly collected from Delta Lake, Saskatoon, Saskatchewan (53°47'01.78" N, 102°20'07.35" W; elevation 265m) and have been defined as the Delta snails. The freshly collected strain used (2.5–3.0 cm shell length) was maintained at room temperature (∼20°C) for at least 2 weeks after being collected in the spring and summer and had *ad libitum* access to lettuce in their home eumoxic aquaria. Finally, the F1 offspring from the Delta snails were also used. The eggs were laid in September 2020 in the laboratory of the University of Calgary and developed and matured under laboratory conditions.

Carrot slurry (C)

See Aim 2. The number of rasps per minute elicited by C in naïve W snails (N=15) is mean±se: 18.9 ±2.7, whereas in Delta snails (N=15) is mean±se: 19.47±3.2.

Lettuce slurry (L)

Snails were exposed to a lettuce slurry (L), which is an appetitive and well-known food stimulus that elicits a robust feeding behaviour. This was done by preparing an L slurry by putting two medium leaves of romaine lettuce (approximately 20 g) in a blender along with 500 mL of PW. Following blending and straining of the mixture, lettuce–PW slurry without any observable pieces of lettuce was obtained. The snails used in these experiments had been fed *ad libitum* romaine lettuce in their home aquaria. In naïve W snails (N=15) the average number of rasps per minute in L is mean±se: 15.6 ±2.19, whereas in Delta snails (N=15) is mean±se: 14.53 ±2.3.

Heat stressor (HS)

See the Material and Methods of Aim 2.

Garcia effect behavioural procedure

A modified Garcia effect behavioural paradigm than the one presented in Aim 2 was used in this study. Snails were first acclimated for 3 min in C. After that, the experimental session began, and the number of rasps was counted for 2 min. One hour later, snails were exposed to the HS for 1h or 15 min (depending on the behavioural procedure). Animals were then returned to their home aquaria for 24h, where they had *ad libitum* access to lettuce. The rasping behaviour was again determined for 2 min in C at different time intervals.

Repeated heat-shock procedure

Snails were exposed to heat stress (HS - 30°C) for 1h every day for a week. Following each HS exposure, animals had ad libitum access to romaine lettuce. At the end of the repeated HS presentation, snails were maintained in their home aquarium for 3 days at relatively constant water temperature and were fed ad libitum romaine lettuce.

Repeated handling procedure

Every day for one week, control snails were moved from their home aquarium and were placed for 1h in a beaker containing clean, room-temperature (RT - 20-22°C) pond water. Following each handling procedure, snails had ad libitum access to food (i.e., lettuce). At the end of the repeated transfer, snails were maintained in their home aquaria for 3 days and were fed *ad libitum* romaine lettuce.

Statistical analysis

All behavioural data were first analysed for normality assumption using Kolmogorov–Smirnov one-sample test for normality (K-S distance and P). Parametric data were then further analysed using a repeated-measures analysis of variance (RM One-way ANOVA) followed by a Tukey's *post-hoc* test. Significant differences between the 2 groups were examined by the Student t-test. All tests were defined significantly at $p<0.05$. R squared (R2) values have been reported for all ANOVA models and q scores for *post-hoc* tests wherever applicable. All statistical analyses were performed using GraphPad Prism v. 9.00e for MAC® (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Effect of the repeated exposure to an HS on the feeding behaviour of laboratory-reared snails

In Aim 2 it has been demonstrated that to achieve the Garcia effect a novel taste must be used. This result has been confirmed here, as the Garcia effect was not formed when the lettuce slurry (L), which is not a novel taste as lettuce is the exclusive food of laboratory snails' diet, was paired with the HS. As shown in **Figure 22**, at 24h post-HS, L continued to elicit a feeding response like that occurring before being paired with the HS ($t=0.54$, $df=11$, $p=0.59$). This result is important because the rasping response to L before and after the HS has been used as a valid indicator for monitoring the snails' feeding behaviour in the following experiments.

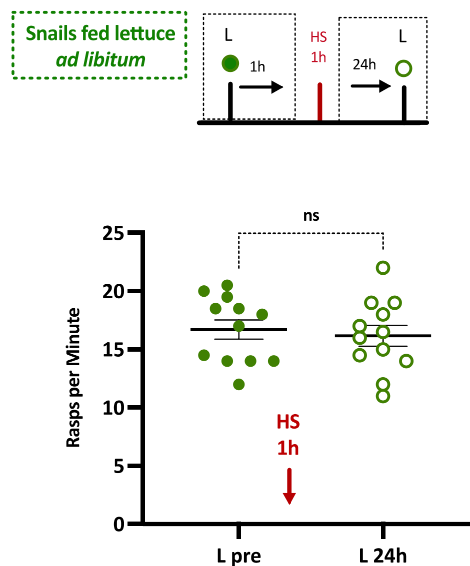


Figure 22. The Garcia effect does not occur in W snails when lettuce (which is not a novel food) is used.
The timeline for the experiment is presented above the data. The rasping behaviour was counted in L (L pre) for 2 minutes. Snails (N=11) received the HS subsequently. At 24h post-HS (L 24h), the feeding behaviour was not significantly suppressed with respect to L pre-HS. The comparison was made by paired t-test. ns = not significant as $p>0.05$.

Next, it was determined whether the repeated exposure of laboratory-reared W snails to an HS could acclimate them to the heat stress to the point that when they experienced 30°C after C, the heat-induced nausea or sickness necessary to form the Garcia effect would be prevented. To do that, the following control experiments were performed to define the maximum duration of the repeated HS procedure and, if necessary, the time (i.e., number of days) required for the feeding behaviour to be restored. As heat is a strong stressor for laboratory-reared W snails, it was first necessary to determine any adverse effects of repeated heat (if present).

First, the number of rasps in L before and during the repeated HS procedure was recorded. Based on the results shown in Figure 22, it has been assumed that any reduction in the number of rasps elicited by L from the second HS exposure onward is indicative of stress or sickness. As it was apparent (**Figure 23**), there were significant differences in the feeding responses in this experiment [RM One-way ANOVA, F (2.849,31.33) = 69.20, P<0.0001, R2=0.86]. On Day 1, following the initial observation of rasping in L, snails were exposed to the HS for 1h and then were returned to their home aquarium for 24h. On Day 2 (i.e., 24h post-HS), the rasping behaviour was again monitored in L and, consistent with Figure 20, no differences in the number of rasps compared to the previous day were found (q=1.93, p=0.86). W snails were subsequently exposed to the HS and their rasping behaviour was observed 24h later (i.e., Day 3). This procedure was repeated in the following days (i.e., Day 4, Day 5, Day 6, and Day 7). On Day 8 it was just measured the rasping behaviour in L. Snails were exposed to the HS for 7 days. From Day 3 to Day 8, there was a significant decrease in the number of rasps in L compared to that observed on Day 1 (i.e., before the first HS exposure) (Day 1 vs Day 3: q=7.66; Day 1 vs Day 4: q=21.66; Day 1 vs Day 5: q=20.59; Day 1 vs Day 6: q= 33.16; Day 1 vs Day 7: q=19.92; Day 1 vs Day 8: q=0.28; all p<0.0001, Tukey's *post-hoc*). Moreover, there was a significant reduction in the feeding response to L moving from Day 2 to Day 3 (q=7.25, p=0.0054), and from Day 3 to Day 4 (q=6.87, p=0.008). From Day 4 onwards, no differences in the number of rasps were found. It was also noticed that from Day 4 animals were eating increasingly less lettuce even when they were placed in their home aquarium (personal observation). Given this prolonged inappetence and to protect the snails' survival, it has been established a maximum duration of 7 days for the repeated HS procedure.

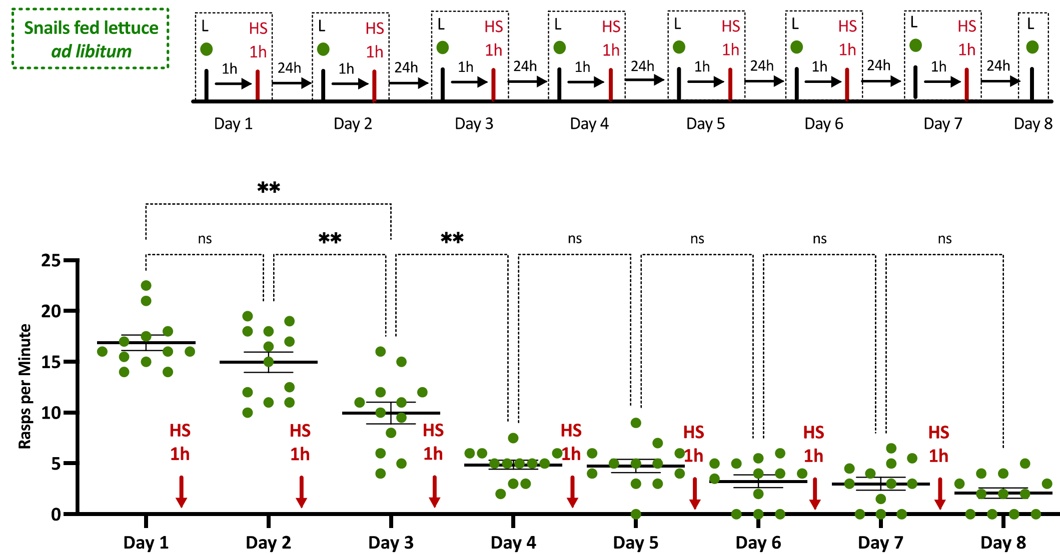


Figure 23. The repeated exposure to an HS for 7 days gradually suppresses the feeding behaviour in W snails. The timeline for the experiment is presented above the data. The number of rasps was first counted in L for 2 min (Day 1) and, 1h later, W snails (N=12) were exposed to an HS. At 24h post-HS (Day 2), the feeding behaviour was measured, and it was not significantly suppressed with respect to Day 1. However, from Day 3 to Day 8 the feeding behaviour was suppressed. The solid line is the mean, and the error bars are the s.e.m. Comparisons were made by RM One-way ANOVA, followed by Tukey's *post-hoc* test. **p<0.01; ns: not significant as p>0.05.

Because handling can stimulate stress in laboratory animals and may introduce confounding factors (Gouveia and Hurst, 2019), control experiments have been performed to overcome any potential behavioural interferences deriving from the repeated transfer of animals from their home aquarium to a new environment (i.e., beakers with clean PW) (**Figure 24**). Thus, a new cohort of W snails (N=10) was moved from their home aquarium and placed for 1h in a beaker with clean 20-22°C PW, which is the average temperature of their home aquarium. This procedure was repeated for 7 days. The snails' feeding behaviour in L was daily recorded. No significant differences in the rasping responses induced by the repeated handling of the animals occurred in this experiment [F (3.338, 36.72) = 0.85, P=0.49, R2=0.07]. Based on these results, the severe reduction in rasps observed from Day 2 to Day 7 in Figure 21 was due to extreme stress or sickness behaviour induced by the repeated HS exposure.

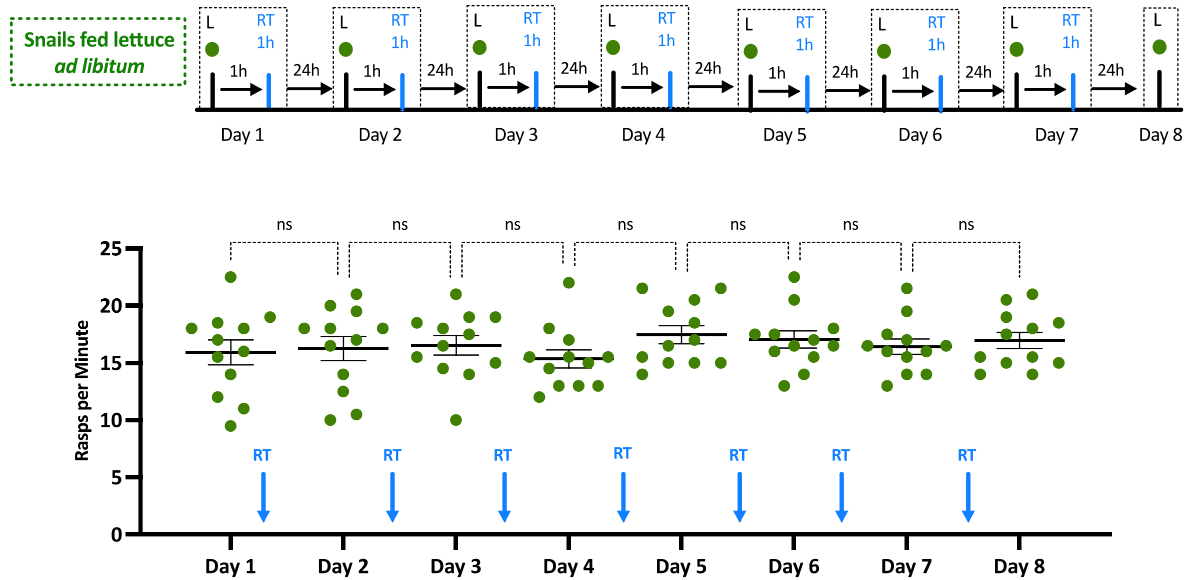


Figure 24. The repeated exposure to HS experimental setup without heat stimulus does not affect the feeding behaviour of W snails.

The timeline for the experiment is presented above the data. The number of rasps was first counted in L for 2 min (Day 1). Twenty-four hours later W snails (N=12) were moved from their home aquarium to a new environment (i.e., beakers with clean PW for 1h). This procedure was repeated for the following 6 days. No differences in feeding behaviour were found from Day 1 to Day 8. Comparisons were made by RM One-way ANOVA, followed by Tukey's *post-hoc* test. The solid line is the mean, and the error bars are the s.e.m. ns = not significant as $p>0.05$.

In light of these results, it was examined how long the sickness behaviour persisted following the repeated HS procedure. A naïve cohort of W snails (N=13) was subjected to the repeated HS procedure and in the following days, it was quantified how long it took for the snails to attain their standard feeding response to L (**Figure 25**). Significant differences in the feeding responses overall were found [RM One-way ANOVA, $F(3.24, 39.98) = 65.28$, $p<0.0001$, $R2=0.84$]. In particular, twenty-four hours after the last HS presentation (Day 1), animals were tested in L, and it was found a severe reduction in the number of rasps compared to that elicited by L in naïve snails not exposed to the repeated HS procedure (See Materials and methods). However, over the days in which the animals were not exposed to the HS, there was a gradual return to the standard feeding behaviour.

On Day 2, it was found an increase in feeding behaviour induced by L with respect to what was observed the day before (L Day 1 vs Day 2: $q=4.974$, $p=0.04$, Tukey's *post-hoc* test). The day after, the number of rasps in L was statistically higher than that recorded the day before ($q= 7.6$, $p=0.002$) and returned to the level recorded in naïve snails. Thus, following the 7-days repeated HS procedure, three days without any HS exposure were required for the feeding behaviour to be recovered.

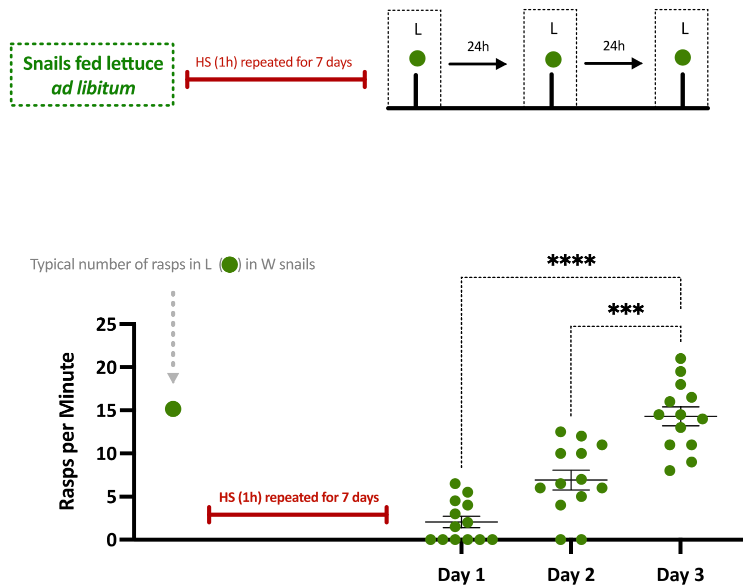


Figure 25. Three days without any HS are necessary for removing the inappetence-state induced by the repeated HS exposure in W snails.

The timeline for the experiment is presented above the data. The average number of rasps elicited by L in naïve animals (i.e., not exposed to the HS) is presented in the graph for comparison. Snails (N=10) were exposed to an HS for 1h. This procedure was repeated for the following 7 days. The rasping behaviour was recorded in L on Day 1, Day 2 and Day 3 post-repeated HS presentation. Snails returned to their normal feeding response by Day 3 post repeated HS. The solid line is the mean, and the error bars are the s.e.m. Comparisons were made by RM One-way ANOVA, followed by Tukey's *post-hoc* test. ****$p<0.0001$; ***$p<0.001$.

Garcia effect in laboratory-reared snails that previously experienced a repeated HS

Based on the results of the previous experiments, it was next investigated whether the exposure to an HS for 1h could induce the Garcia effect in W snails that had repeatedly experienced the HS for 7 days. Thus, a new cohort of W snails (N=10) was subjected to the repeated HS procedure for one week and then had 3 days of rest. After this HS-free period, the rasping behaviour was recorded in C (**Figure 26**). An overall significant effect of the treatments [RM One-way ANOVA, $F(2.92,26.25) = 82.32$, $P<0.0001$, $R^2=0.9$] was found, such that when snails were exposed to C for the first time the response was comparable to the responses elicited by C in naïve snails (See the Material and methods), suggesting that the repeated HS procedure and the following recovery time did not alter the response to C. It was also investigated whether the effects of heat shock (HS) or Garcia effect on feeding extended beyond 24h. Thus, C was presented 24h, 48h, 72h, and 96 after the HS. When snails were exposed to 24h, 48h, and 72h after the HS, their feeding response was significantly decreased compared to that recorded 1h before the HS (C pre vs C 24h: $q=15.77$; C pre vs C 48h: $q=15.36$; C pre vs C 72h: $q=18.82$, all $p<0.0001$, Tukey's *post-hoc* test).

However, the number of rasps elicited by C 96h after HS was significantly greater than the response to C recorded the previous three days (C 96h vs C 24h: $q=15.85$, $p<0.0001$; C 96h vs C 48h: $q=15.08$, $p<0.0001$; C 96h vs C72h: $q=12.24$, all $p=0.0002$). Therefore, it was concluded that the repeated HS presentation did not affect the ability of laboratory-reared inbred snails to undergo the Garcia effect. The paired presentation of the novel taste with the HS, still conditioned the feeding behaviour and this stimulus-specific suppression persisted for 72h as LTM. Thus, after the repeated HS procedure a single C-HS exposure was able to extend the feeding response to C from 24h to 72h. In other words, snails now showed a longer-lasting memory.

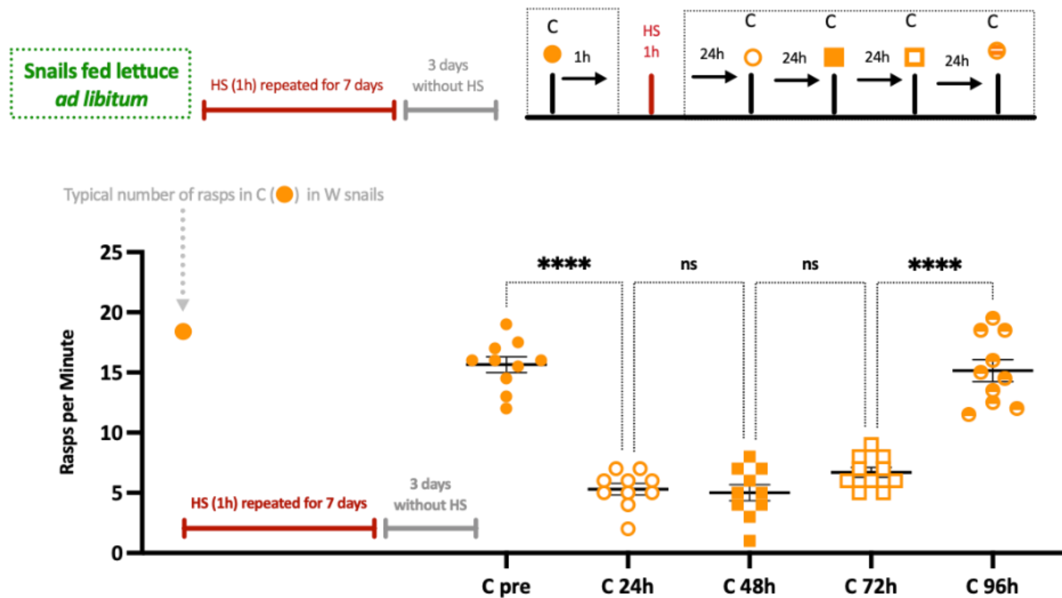


Figure 26. A Garcia effect lasting 72h is induced in W snails that previously experienced an HS for 7 days.
The timeline for the experiment is presented above the data. The average number of rasps elicited by C in naïve animals (i.e., not exposed to the HS) is plotted in the figure for comparisons. A cohort of W snails (N=10) was exposed to the HS for 1h every day for 7 days. After 3 days of rest, the number of rasps was counted in C (C pre) and then snails experienced the HS for 1h. When animals were exposed to C 24h (C 24h), 48h, (C 48h), 72h (C 72h), and 96h (C 96h) after the HS, a 72h-lasting Garcia effect was observed. The solid line is the mean, and the error bars are the s.e.m Comparisons were made by RM One-way ANOVA, followed by Tukey's *post-hoc* test. ****p<0.0001; ns: not significant as p>0.05.

Next, a naïve cohort of W snails (N=12) was used to ascertain whether only with the repeated presentation of the handling stress LTM would be enhanced. Thus, snails were daily transferred and maintained for 1h in a beaker with clean 20°C PW. This procedure was repeated for 7 days. Then, snails were given 3 days of rest (**Figure 27**) before the Garcia effect procedure was performed. Consistent with previous data (Aim 2), it was found that following the paired C-HS presentation the feeding response to C was suppressed for 24h but not 48h nor 72h [RM One-way ANOVA, F (1.987, 21.86) = 78.35, P<0.0001, R^2=0.88]. In particular, the feeding response elicited by C 24h after the HS exposure was significantly lower than that observed in C pre (q=15.22, p<0.0001), whereas the number of rasps at 48h post-HS was significantly higher than C 24h (q=15.68, p<0.0001). Thus, the repeated transfer of animals did not interfere with their ability to show the Garcia effect, but did not enhance LTM duration.

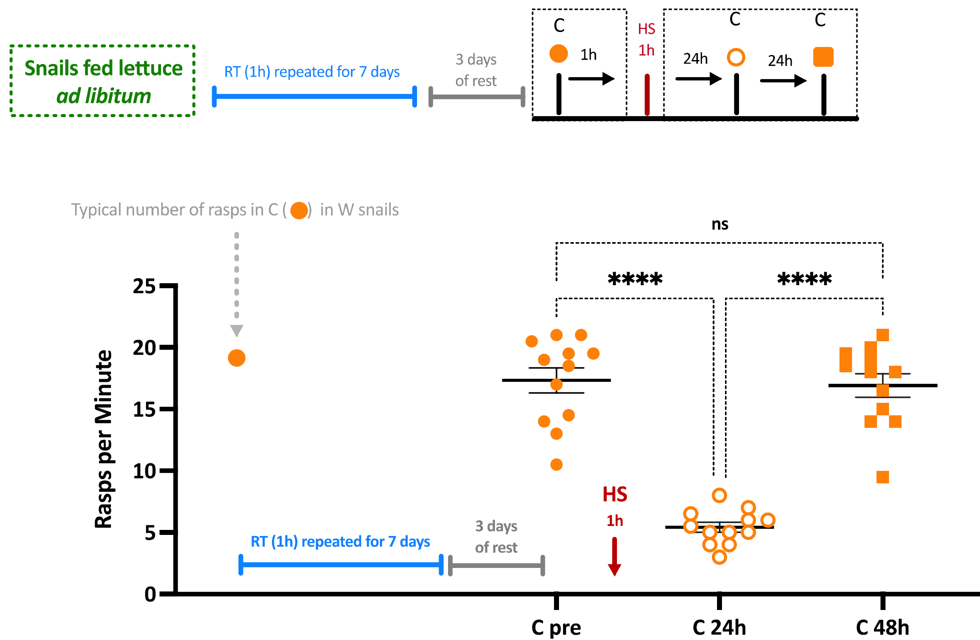


Figure 27. The repeated exposure of W snails to the experimental setup does not enhance the duration of the Garcia effect.

The timeline for the experiment is presented above the data. The average number of rasps elicited by C in naïve animals (i.e., not exposed to the HS) is plotted in the figure for comparison. A cohort of snails (N=12) was placed in a beaker with clean room-temperature PW for 1h every day for 7 days. After 3 days of rest, the spontaneous rasping was counted in C (C pre). Snails subsequently experienced the HS for 1h and 24h and 48h later were exposed to C (C 24h and C 48h). This procedure induced a standard Garcia effect lasting for 24h as LTM. The solid line is the mean, and the error bars are the s.e.m. Comparisons were made by RM One-way ANOVA, followed by Tukey's *post-hoc* test. ****$p<0.0001$; ns: not significant as $p>0.05$.

Concurrently a control group was run for the same procedure but L, which was not a novel taste, was paired with the HS (**Figure 28**). Consistent with Figure 22, the increased feeding behaviour induced by L was not reduced following the HS when animals were tested 24h later (paired t-test: $t=0.767$, $df=11$, $p=0.46$). Thus, even after the repeated HS procedure, a novel taste must be used for the Garcia effect to be formed.

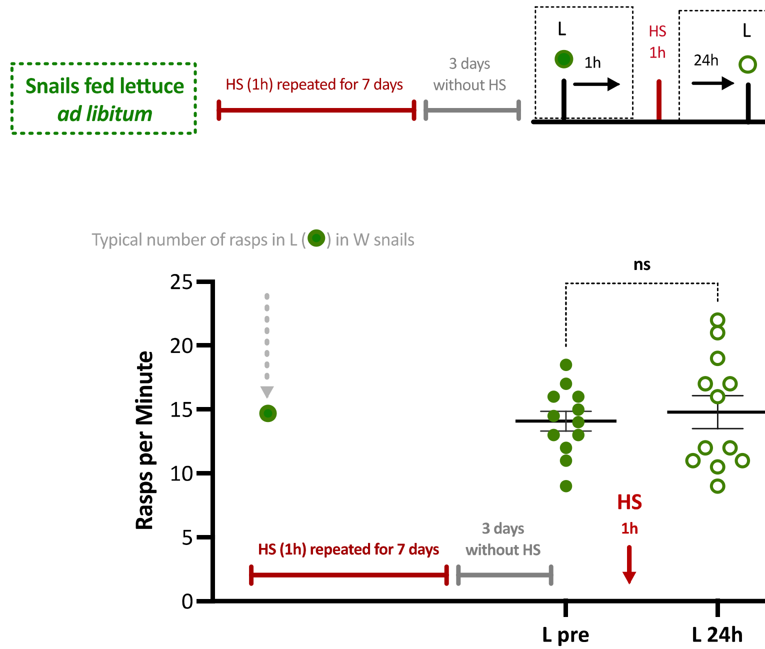


Figure 28. The Garcia effect does not occur in W snails when lettuce (which is not a novel food) is used after repeated HS for 7 days. The timeline for the experiment is presented above the data. The average number of rasps elicited by L in naïve animals (i.e., not exposed to the HS) is plotted in the figure for comparison. A cohort of snails (N=12) was exposed to HS for 1h every day for 7 days. After 3 days of rest, the spontaneous rasping was counted in L (L pre). Snails experienced the HS for 1h and 24h later were exposed to L (L 24h). This procedure did not induce a Garcia effect. The solid line is the mean, and the error bars are the s.e.m. ns: not significant as $p>0.05$. The comparison was made by RM One-way ANOVA, followed by Tukey's *post-hoc* test. ns: not significant as $p>0.05$.

Effect of the exposure to a 15 min HS in laboratory-reared snails that previously experienced repeated HS

Previous studies from mammals indicated that the repeated exposure to an acute stressor over time modifies the stress vulnerability and activates molecular and behavioural strategies aimed at increasing stress resilience. Here, it has been investigated whether the repeated exposure to the HS increased laboratory inbred snails' stress sensitization to the point that a shorter exposure to that stressor (i.e., 15 min) would be sufficient to trigger its initial onset. To do that, it was first verified that a 15 min-lasting exposure to HS did not induce per se the Garcia effect in snails.

Thus, it was examined the feeding response to C in a naïve cohort of snails (N=11) 1h before and 24h after the 15-min exposure to the HS. The increased feeding behaviour elicited by C was not reduced when snails were tested 24h after the 15-min HS ($t=0.98$, $df=10$, $p=0.35$) (**Figure 29**). Thus, the sole exposure to an HS for 15 min did not alter the hedonic value of C and the Garcia effect did not occur.

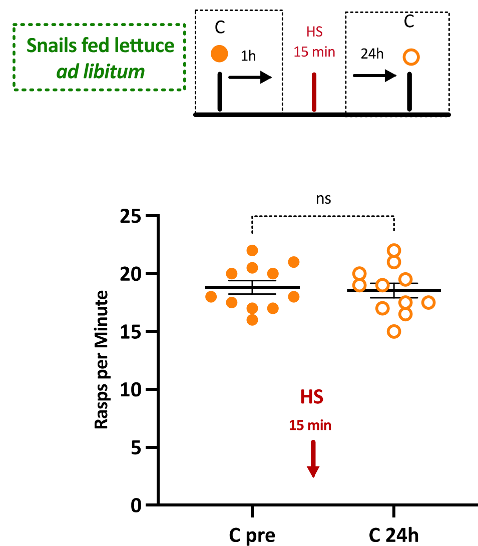


Figure 29. The exposure to the HS for 15 min does not induce a Garcia effect in W snails. The timeline for the experiment is presented above the data. In a cohort of W snails (N=11) the rasping behaviour in C was recorded 1h before (C pre) and 24h after (C 24h) the exposure to an HS for only 15 min (instead of 1h). The Garcia effect was not formed. The solid line is the mean and the error bars are the s.e.m. The comparison was made by paired t-test. ns: not significant as $p>0.05$.

Then, it was investigated whether 15 min of HS were enough to induce the Garcia effect in snails that already experienced the repeated HS in the preceding days. A new cohort of W snails (N=11) was first exposed to the HS for 1h every day for a week. In the following three days snails rested in their home aquarium and had *ad libitum* access to lettuce. Then, the Garcia effect with 15-min of HS exposure was performed (**Figure 30**). Significant differences in rasping behaviour were found in this procedure [RM One-way ANOVA, $F(2.594,25.94) = 117.4$, $P<0.0001$, $R2 = 0.92$].

The exposure to C before the HS elicited a rasping behaviour comparable to that recorded in naïve snails (See the Material and Methods). These snails were subsequently exposed to 15 min of HS, and 24h later their rasping behaviour was recorded. The significant reduction in the number of raps ($q= 22.7$, $p<0.0001$, *post-hoc* Tukey's test) suggested that in snails that experienced the repeated HS for 7 days the exposure to 15 min of HS was enough to induce a Garcia effect. It was also determined how long the negative hedonic shift to C persisted. Animals were tested C at 48h and 72h after the 15-min HS exposure and a significant reduction in the number of rasps compared to C pre was found (C pre vs C 48h: $q=19.04$, $p<0.0001$; C pre vs C 72h: $q=25.14$, $p<0.0001$). However, when animals were tested in C 96h after the HS, the feeding response was significantly higher than all the previous exposures to C following the HS (C 24h vs C 96h: $q=15.98$, $p<0.0001$; C 48h vs C 96h: $q=13.63$, $p< 0.0001$; C 72h vs C 96h: $q=15.28$, $p<0.0001$). Snails returned to normal feeding at C 96h (C pre vs C 96h: $q=3.39$, $p=0.20$). It was therefore concluded that in snails that experienced the HS for 1h every day for one week, 15 minutes of exposure to the HS was sufficient to suppress feeding response elicited by carrot up to 72h.

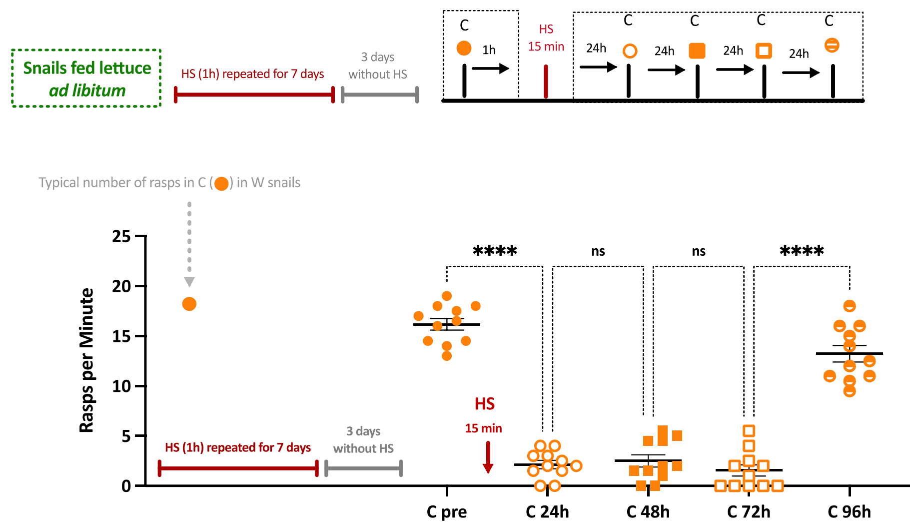


Figure 30. The exposure to the HS for 15 min induces a Garcia effect in W snails that previously experienced an HS for 7 days. The timeline for the experiment is presented above the data. The average number of rasps elicited by C in naïve animals (i.e., not exposed to the HS) is plotted on the figure for comparison. A cohort of W snails (N=11) was exposed to the HS for 1h every day for 7 days. After 3 days of rest, the spontaneous rasping was counted in C (C pre) and 1h later snails experienced the HS for 15 min. The number of rasps in C was recorded at 24h (C 24h), 48h, (C 48h), 72h (C 72h), and 96h (C 96h) post-HS. The solid line is the mean and the error bars are the s.e.m. Comparisons were made by RM One-way ANOVA, followed by Tukey's *post-hoc* test. ****$p<0.0001$; ns: not significant as $p>0.05$.

Garcia effect in freshly collected snails and their first-generation offspring

Moving from laboratory-inbred snails to wild Delta strain snails, it was first investigated whether in a naïve cohort of freshly collected (F0) Delta snails (N=11) the Garcia effect could be formed. The number of rasps was recorded 1h before and 24h the HS. The elicited feeding behaviour induced by C was not suppressed following the thermal exposure (t=1.25, df =10, p=0.24) (**Figure 31A**). Then, it was tested the first generation (F1) of freshly collected Delta snails, which has been developed and raised in laboratory conditions. To accomplish this task, a naïve cohort of F1 Delta snails (N=10) was given the Garcia effect procedure (i.e., C preceded HS by 1h) and at 24h post-HS the robust feeding behaviour elicited by C was not suppressed (t=1.075, df =10, p=0.3). Thus, contrary to lab-bred snails, the Garcia effect was not shown by F1 wild strains (**Figure 31B**).

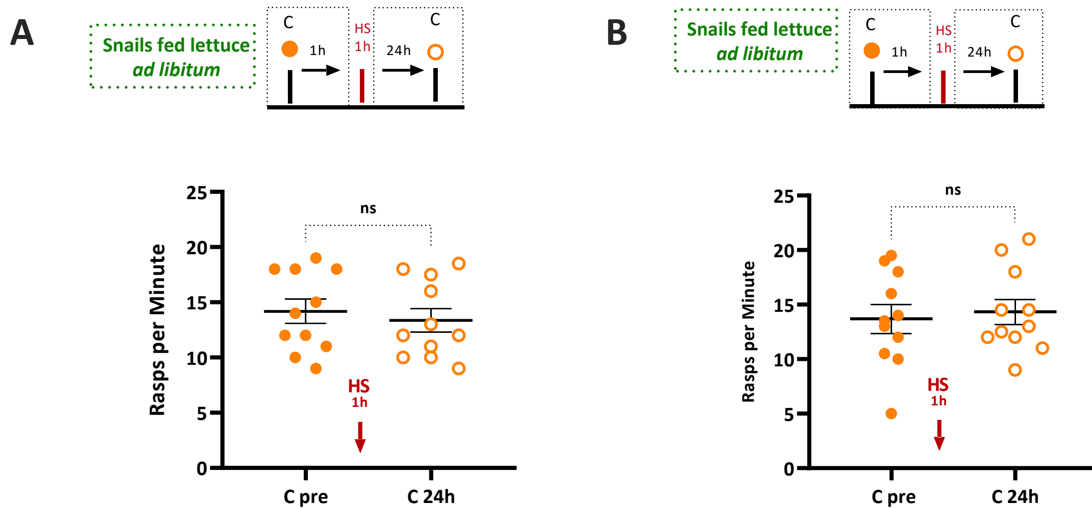


Figure 31. The Garcia effect in F0 and F1 of freshly collected Delta snails. The timeline of each experiment is presented above the data. N=11. The solid line is the mean, and the error bars are the s.e.m. Comparisons were made by paired t-test. ns: not significant as p>0.05. Freshly collected Delta snails **(A)** and their first offspring **(B)** were exposed to C (C pre) and 1h later received HS. At 24h (C 24h) the number of rasps was recorded, and it was not different from C pre-HS. Thus, in Delta snails the Garcia effect is not formed. ns: not significant as p>0.05.

Effect of the repeated HS exposure before the Garcia effect procedure in the F1 of freshly collected snails

The effect of the repeated HS exposure before the Garcia effect procedure was studied in Delta snails F1 offspring. These snails were bred and raised in relatively constant temperature ranges, but their freshly collected parents might have experienced thermal fluctuations in the wild. This allowed me to investigate whether, compared to their parents, the heat stress tolerance of F1 snails would have been lower as they were raised under constant temperatures. If so, it would be expected that after the repeated HS procedure (which increased the heat sensitivity in W snails) the HS would induce a Garcia effect.

To test this hypothesis, it was first studied the effect of the daily exposure to the HS on snails' feeding behaviour and the number of rasps in L before and during the 7-days repeated HS procedure was recorded (**Figure 32**).

No significant differences in the feeding responses induced by the repeated HS exposure occurred in this experiment [RM One-way ANOVA, F (2.894,26.05) = 1.09, P=0.36, R^2=0.11], showing that the repeated HS exposure did not make animals sick or stressed.

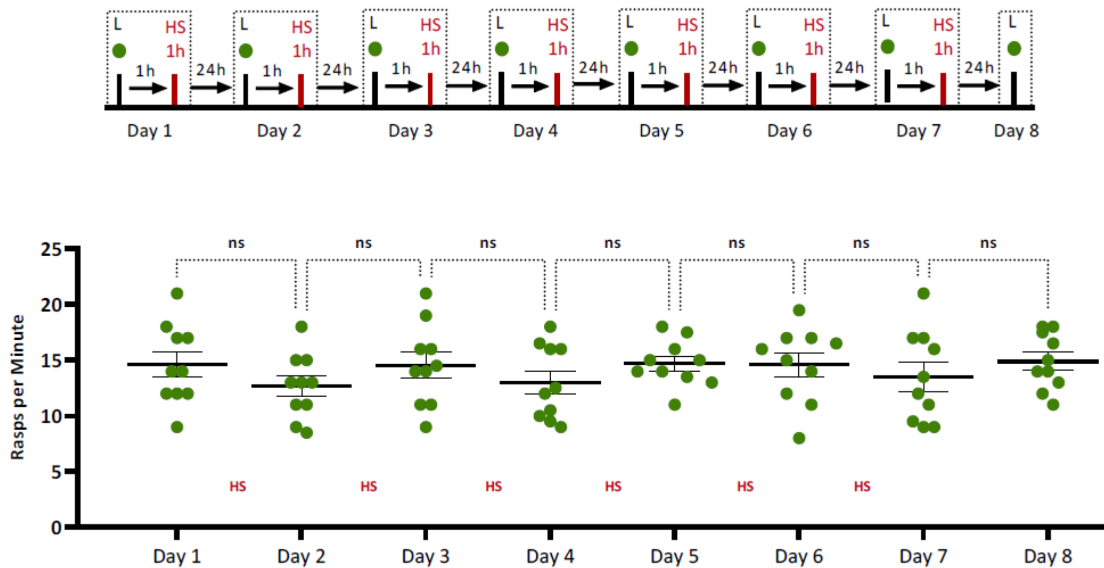


Figure 32. The daily exposure to an HS for 7 days does not suppress the feeding behaviour in F1 Delta snails. The timeline for the experiment is presented above the data. The number of rasps was counted in L for 2 min (Day 1) before F1 Delta snails (N=10) received an HS 1h later. The same procedure was repeated for 7 days. The feeding behaviour in L was not affected by the repeated HS procedure. The solid line is the mean and the error bars are the s.e.m. Comparisons were made by RM One-way ANOVA, followed by Tukey's *post-hoc* test. ns: not significant as p>0.05.

Finally, a naïve cohort of F1 Delta-snails (N=10) was subjected to the repeated HS procedure for one week and after 3 days of rest, the rasping behaviour was recorded in C. Twenty-four hours later, snails were exposed to the HS (1h) (**Figure 33**). The number of rasps at C 24h post-HS was not different from that recorded in C pre (paired t-test: t=0.07, df =9, p=0.94), and these animals were not transformed by the repeated HS procedure. Thus, F1 Delta snails were not transformed by developing in a relatively constant laboratory temperature environment and the repeated HS procedure.

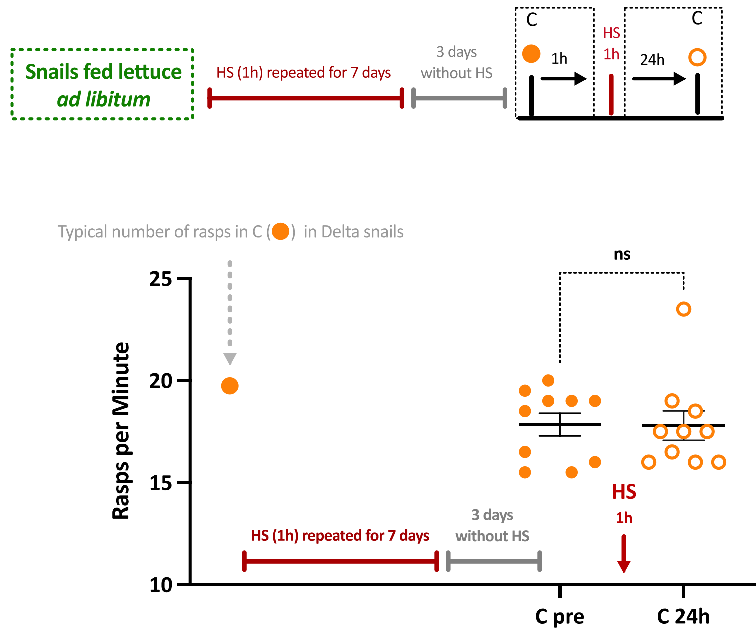


Figure 33. The repeated HS exposure does not induce a Garcia effect in F1 Delta snails. The timeline for the experiment is presented above the data. The average number of rasps elicited by carrot in naïve animals (i.e., not exposed to the HS) is plotted for comparison. A cohort of F1 Delta snails (N=10) was exposed to the HS for 1h every day for 7 days. After 3 days of rest, the rasping behaviour was recorded in C (C pre). Snails subsequently experienced the HS for 1h and 24h later were exposed to C (C 24h). This procedure did not result in a Garcia effect. The solid line is the mean and the error bars are the s.e.m. The comparison was made paired t-test. ns: not significant as $p>0.05$.

Thus, lab-bred W-snails showed increased sensitivity to heat stress after the repeated heat exposure for 7 days, leading to enhanced LTM for Garcia effect with only 15min of heat exposure instead of standard 1h. On the other hand, freshly collected wild snails did not show Garcia effect. Additionally, the F1 generation of wild snails raised and maintained under laboratory conditions still retain their heat stress tolerance similar to their parents and did not show a Garcia effect under standard learning paradigm or even after repeated heat stressor.

AIM 4

Study of the memory-enhancing effect of the flavonoid quercetin in L. stagnalis' CNS

Background

Flavonoids and cognitive functions

A key feature of LTM is that a newly encoded memory initially exists in a fragile state and, over time, becomes increasingly resistant to disruption. With retrieval or reactivation, a consolidated memory can re-enter a labile state until it is re-stabilized through a process of reconsolidation (Nader 2003; Alberini 2009; Bisaz *et al.* 2014; Squire *et al.* 2015). During both consolidation and reconsolidation processes, memory is susceptible to modification by environmental factors, such as stress and lifestyle choices (McKenzie and Eichenbaum 2011). One key lifestyle choice that impacts the strengthening or the awakening of the memory trace is diet (Swinton *et al.* 2018). Growing evidence suggests that a group of dietary-derived phytochemicals, known as flavonoids, may improve LTM formation, consolidation, storage, and retrieval (Letenneur *et al.* 2007; Haleagrahara *et al.* 2011; Fruson *et al.* 2012; Knezevic and Lukowiak 2014; Socci *et al.* 2017; Ayaz *et al.* 2019). A multitude of studies reported that regular dietary consumption of flavonoids and flavonoid-rich foods can improve various cognitive dysfunctions and dementia-like alterations in different animal models (Spencer 2007; Yevchak *et al.* 2008; Macready *et al.* 2009; Mastroiacovo *et al.* 2015; Socci *et al.* 2017; Bakoyiannis *et al.* 2019). The positive cognitive effects of flavonoids in mammals have been attributed to the protection of neural functioning, stimulation of neuronal regeneration, and increased blood flow to the brain (Yevchak *et al.* 2008; Macready *et al.* 2009; Mastroiacovo *et al.* 2015; Socci *et al.* 2017).

Potential memory-enhancing effects of quercetin: role of CREB and serotonin

Quercetin (Q, 3,3′,4′,5,7-pentahydroxyflavone) is one such flavonoid and is widely distributed in fruits and vegetables, like apples, berries, onions, asparagus, capers, and red leaf lettuce (Manach *et al.* 2004, 2005). Studies from mammals indicate that Q has antioxidant properties and protects neuronal cells from neurotoxicity caused by free radicals (Robak and Gryglewski 1988; Cushnie and Lamb 2005; Eggler *et al.* 2008; Ahmad *et al.* 2016). The effects of Q have been studied also in invertebrate models like *C. elegans* and *D. melanogaster* leading to a prolonged life span and reduced oxidative stress or protection against toxins. However, to date, there is a paucity of studies about the memory-enhancing effect of Q on healthy individuals and animal models. Most of the studies on the role of Q on cognitive functions have been performed on animal models of neurodegenerative diseases (Hosokawa *et al.* 1990; Williams *et al.* 2004; Cushnie and Lamb 2005; Ansari *et al.* 2009; Jayasena *et al.* 2013).

These studies showed a Q-induced up-regulation of the cyclic AMP response element-binding protein 1 (CREB1) (Babaei *et al.* 2018). This transcriptional factor regulates memory formation, consolidation, and reconsolidation in a wide range of animal models, from invertebrates to mammals (Stevens 1994; Yin and Tully 1996; Bozon *et al.* 2003; Alberini 2009). Other studies demonstrated that Q ameliorates cognitive performances by increasing the serotonin (5HT) levels in stressed mice. The central role played by CREB and the serotoninergic pathway in mediating LTM formation, makes them valid pharmacological targets for improving memory functions, in both diseased and healthy individuals (Tully 1984; Barco *et al.* 2003; Khan *et al.* 2009). In this context, *L. stagnalis* represents a valid animal model in which to decipher and elucidate the molecular and behavioural effects induced by Q. *L. stagnalis* responds to different bioactive compounds that have been shown to enhance memory formation (e.g., epicatechin). These compounds can easily penetrate through the integument of the snail and, passing into the open circulatory system, can easily and quickly reach and alter CNS activity (Fruson *et al.* 2012; Rivi *et al.* 2020; Itoh *et al.* 2021). Moreover, by using solid paradigms of learning and memory, it is possible to study the effects of this flavonoid when it is encountered at different times in the learning, memory formation, and recall continuum.

The serotoninergic pathway and CREB1 in the CNS of L. stagnalis

In *L. stagnalis*, the orthologous of CREB1 (*Lym* CREB1) has been cloned and characterized (Sadamoto *et al.* 2004), and plays a conserved key role in LTM formation (Ribeiro *et al.* 2003). CREB1 levels are regulated by many molecular cascades, including the 5HT pathway. As a neurotransmitter, 5HT not only regulates many important physiological processes such as body temperature, sleep, appetite, and motor activity but also modulates higher brain functions, including cognition. In *L. stagnalis*, 5HT enhances LTM following the operant conditioning of aerial respiration with exposure to a predator scent (Il-Han *et al.* 2010), whereas the administration of a 5HT receptor antagonist before the memory test brings about a reversible amnesia in taste-aversive learning (Nikitin *et al.* 2008).

5HT is synthesized by the orthologous of the enzyme tryptophan hydroxylase (*Lym* TPH) (Koert *et al.* 2001). In the cytoplasm of the presynaptic serotonergic neurons, it is packaged into vesicles and, upon fusion with the cell membrane, it is released into the synaptic cleft. Here, 5HT binds its receptors *Lym* HTR1 and *Lym* HTR2 (Geraerts *et al.* 1992), activates many intracellular pathways, leading to the upregulation of *Lym* CREB1. Finally, the recapture of 5HT is regulated by a specific transporter, *Lym* 5HTT (Sadamoto 2010) (**Figure 34**).

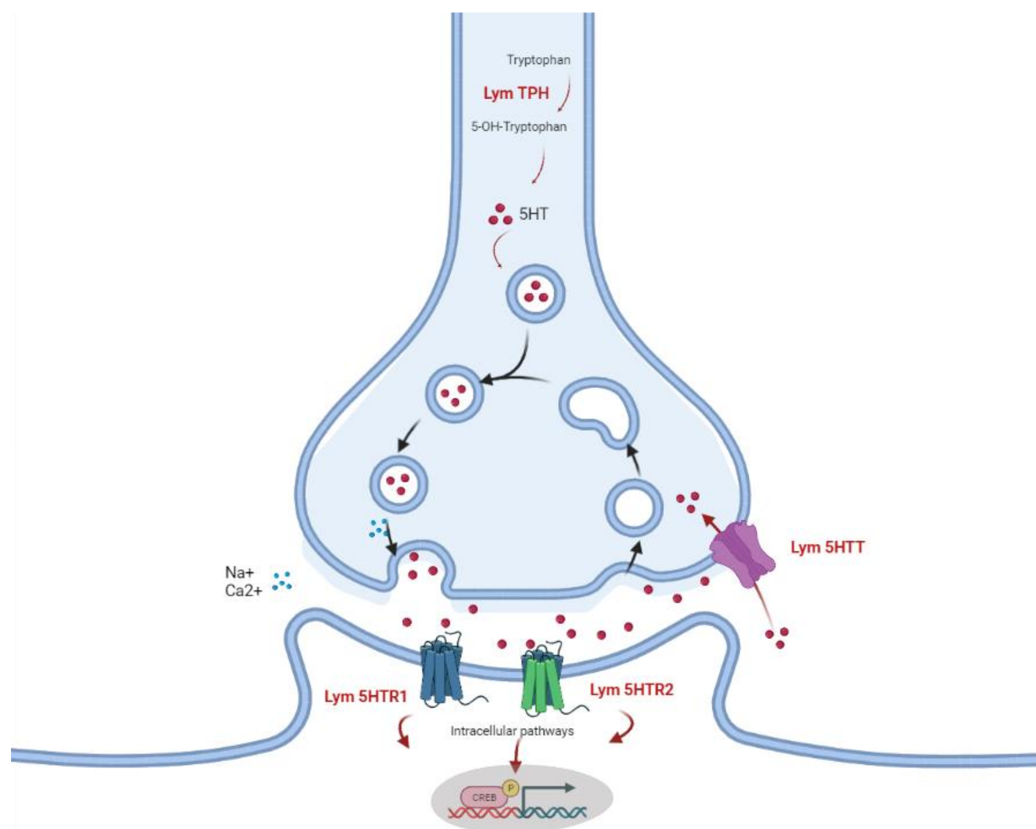


Figure 34. Selected transcripts involved in serotonergic transmission in *L. stagnalis*.
Serotonin (5HT) is synthesized by the orthologous of the enzyme tryptophan hydroxylase (*Lym* TPH) After its released into the synaptic cleft, 5HT binds its receptors *Lym* HTR1 and *Lym* HTR2 and activates many intracellular pathways, which led to the upregulation of the orthologous of CREB1 (*Lym* CREB1). 5HT is recaptured by a specific transporter, *Lym* 5HTT or SERT.

Operant conditioning of the aerial respiratory behaviour as an ideal paradigm for studying the effects of Q on learning and memory

In Aim 2 it has been shown that Q application before an HS blocks the upregulation of the HSPs and, as consequence, a Garcia-like effect is not formed. The same flavonoid also prevents the HS to enhance LTM formation following a single 0.5h training session for the operant conditioning of aerial respiratory behaviour (Sunada *et al.* 2016).

However, the treatment with Q after the HS does not interfere with LTM formation for both the Garcia effect and the operant conditioning. Moreover, when applied alone or following the thermal stressor, Q does not affect the HSPs expression levels. Thus, there are two situations in which the exposure of *L. stagnalis* to Q results in two opposite effects on LTM formation: (1) when Q is applied before the HS, the upregulation of HSP70 and HSP40 is blocked and the LTM for the operant conditioning and Garcia effect is not observed, whereas (2) experiencing Q alone or after the HS, does not interfere with the HSPs levels and does not block LTM.

No previous studies investigated the effect of experiencing Q without any additional stressor on LTM formation.

Studies from *L. stagnalis* demonstrated that another flavonoid, (−)-Epicatechin enhances LTM following operant conditioning of aerial respiratory behaviour (Fruson et al. 2012; Knezevic and Lukowiak 2014; Fernell et al. 2016). However, these two flavonoids show different mechanisms of action. For example, the paired exposure to epicatechin and the HS results in a memory enhancement, which is stronger (i.e., lasting for at least 48 h) than that produced by the thermal stress and epicatechin alone (i.e., 24 h) and epicatechin has no effect in blocking the action of the HS.

Thus, there can be variation in the action of similar bioactive compounds as well as variation in how a single compound act under different learning paradigms.

With the aforementioned data in mind, in this study, I focused my attention on the operant conditioning paradigm and tested for the first time in an animal model (i.e., adult, healthy snails) whether the exposure to Q without any stressors would have an enhancing, detrimental, or no effect on LTM formation, consolidation, and recall.

Material and methods

Animals

Behavioural experiments were performed using laboratory, inbred *L. stagnalis*, the W strain, bred and raised in the snail facility at the University of Calgary. Molecular experiments were performed using laboratory, inbred *L. stagnalis*, bred and raised in the snail facility at the University of Modena and Reggio Emilia.

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

See the Material and Methods of Aim 2.

In this experiment, total RNA was extracted from a single ganglion and 6 samples were collected for each group. The accession number, the size (bp) of the PCR product obtained by amplification of the cDNA (mRNA) are given for each target (**Table 7**).

Table 7. Nucleotide sequences of the forward and reverse primers used for Real-Time PCR

GENE BANK ACCESSION NR	TARGET	Product length	Forward primer	Reverse primer
AB041522.1	*L. stagnalis stagnalis* cAMP responsive element binding protein, ***Lym* CREB1**	180 bp (49-229)	GTCAGCAGGGAATGGTCCTG	AACCGCAGCAACCCTAACAA
L06803.1	*L. stagnalis stagnalis* 5HT receptor 1, ***Lym* HTR1**	126 bp (893-1019)	ACTATCTCATCCTGTCCTTG	GATATCCACATGTCACACAC
U50080.1	*L. stagnalis stagnalis* 5HT receptor 2, ***Lym* HTR2**	115 bp (884-999)	ACACCTGGAGTATTCTCATC	GAAGTAGTTGGTCACGTTCT
AF129815.1	*L. stagnalis stagnalis* Serotonin transporter, ***Lym* 5HTT**	177 bp (726-903)	ATACCGTACCTTGTCATGTT	TGTTGTAGTACCAGGAGACA
AF129815.1	*L. stagnalis stagnalis* Tryptophan hydroxylase, ***Lym TPH***	179 bp (238-417)	AGGATACAGTCTACCGACAG	TGAGTTCACGGAAAACTATT
X15542.1	Snail βtubulin ***Lym βTUB***	127 bp (92-219)	CGCCTCTGTGAACTCCATCT	GAAATAGCACCGCCATCC
DQ278441.1	*L. stagnalis stagnalis* Elongation factor 1-α,***Lym*** **EF1α**	144 bp (13-157)	CTGGGAGCAAAGTCAAGCAT	TTCGCTCATCAATACCACCA

Quercetin solution

See the Material and Methods of Aim 2.

Aerial respiratory behaviour

See the Material and Methods of Aim 4.

A 0.5h training session procedure for operant conditioning of the aerial respiratory behaviour

See the Material and Methods of Aim 4

'Single poke' procedure for the operant conditioning of the aerial respiratory behaviour

Snails were placed in the hypoxic environment for a 10-min acclimation period without receiving any negative reinforcement stimulus. During the following 0.5h of exposure to hypoxic PW they received a poke only the first time they began to open the pneumostome. Hence the term "'single poke' procedure' (Karnik *et al.* 2012).

Statistical analyses

For gene expression analyses, mRNA levels were analysed as in Aim 2.

For the OC experiments, all the experimental treatments were independent of each other and have specific stimulus exposure timelines. Thus, the data could not be combined in a meaningful manner for any grouped statistical analyses. It was thus performed an individual paired-sample t-test for each experiment. In all analysis reported here, a type I error rate of 0.05 was used. All statistical analyses were performed using GraphPad Prism v. 9.00e for MAC® (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Transcriptional effects of quercetin on the expression levels of Lym CREB and the serotoninergic pathway

Snails (N=6 per group) were randomly assigned to two treatment groups: (1) exposed to DMSO for 1h (i.e., CTRL snails); and (2) exposed to Q for 1h (i.e., Q). Three hours after the Q or DMSO exposure, snails were euthanized and mRNA levels of *Lym* CREB (**Figure 35A**), *Lym*5HTR1 (**Figure 35B**), *Lym* 5HTR2 (**Figure 35C**), *Lym* TPH (**Figure 35D**), and *Lym* 5HTT (**Figure 35E**) between the two groups were quantified. A 3h interval was selected as it was known from previous studies that Q inhibits HSPs elaboration 3h post-HS exposure. An unpaired t-test revealed that the expression levels of *Lym* CREB1 were significantly induced in snails exposed to Q with respect to the control (i.e., DMSO) ($t=-5.1$, $p=0.001$). Similar results were obtained for *Lym* 5HTP ($t=3.42$, $p=0.004$), *Lym* 5HT1 ($t=3.73$, $p=0.002$), and *Lym* 5HT2 ($t=4.44$; $p=0.0007$). The exposure to Q, instead, had no transcriptional effects on the expression levels of *Lym* 5HTT ($t=1.26$, $p=0.24$).

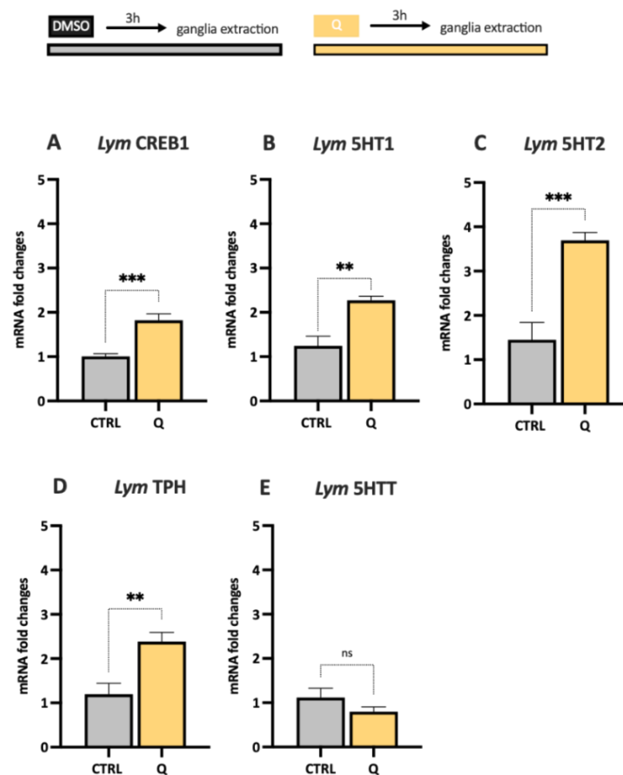


Figure 35. Transcriptional effects of Q on the expression levels of *Lym* CREB, *Lym*5HT1, *Lym* 5HT2, *Lym* TPH, and *Lym* 5HTT.

Adult snails (N=6 for each group) were exposed for 1h to DMSO (CTRL - grey bars) or quercetin (Q – yellow bars). Three hours later, animals were sacrificed, and the central ring ganglia were extracted. Q significantly upregulates (**A**) *Lym* CREB1, (**B**) *Lym* 5HT1, (**C**) *Lym* 5HT2, and (**D**) *Lym* 5TPH in the cohort exposed to Q compared to DMSO. No differences in the expression levels of *Lym* 5HTT (**E**) between Q and CTRL were found. Data are represented as means ± s.e.m. ***$p<0.001$; **$p<0.01$; ns = not significant as $p>0.05$.

Effect of Q on snails' aerial respiratory behaviour

Having shown that CREB1 and the serotoninergic pathway are upregulated at 3h post Q exposure, and considering their role in enhancing learning and memory, it has been investigated whether the exposure to Q without any additional stressor within 3h of critical memory phases would have effects on *L. stagnalis*' ability to form LTM. In this study, it has been hypothesized that any enhancement observed during learning and memory consolidation and reconsolidation can be due to the elevated levels of *Lym* CREB1 and the 5HT pathway. It was first ascertained that the aerial respiratory behaviour was not significantly altered by Q. Total breathing time (TBT) was measured in W snails (N=10) in the hypoxic environment. The first measurement (Obs 1) was made 20h before the snails experienced Q while the second observation was made 3h after Q exposure. TBT before and following exposure to Q was not significantly different (t=0.72, df =9, p=0.49) (**Figure 36**).

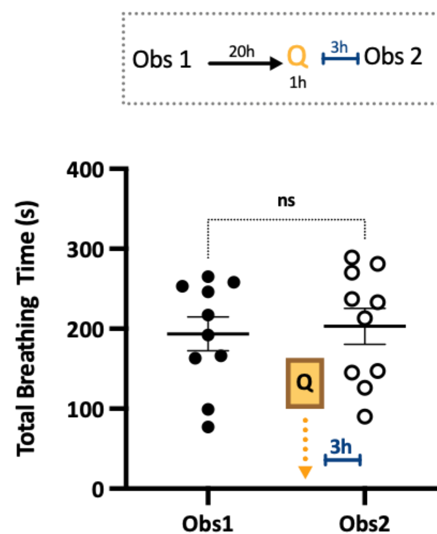


Figure 36. Quercetin exposure does not affect aerial respiration in W-strain snails.
The timeline for the experiment is presented above the data. The total breathing time (TBT) between the first observation period (Obs1) was compared with the second one performed 24h later, 3h after the exposure to Q (Obs2). The solid line is the mean, and the error bars are the s.e.m. The comparison was made by paired t-test. ns = not significant as p>0.05.

Effect of Q exposure 3h before or after a 0.5h training session on LTM formation

First, it has been confirmed previous results (Lukowiak et al., 2020) showing that in W-strain snails a 0.5 h training session does not result in LTM formation (**Figure 37**). A naïve cohort of W-strain snails (N=8) was trained with a 0.5 h TS. LTM was tested 24h later. These snails did not exhibit LTM as the number of attempted pneumostome openings between the training session and the memory test was not statistically different ($t=1.94$, $df = 9$; $p=0.084$).

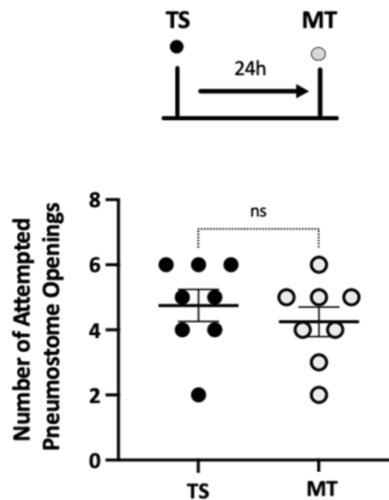


Figure 37. Snails given a single 0.5h training session in hypoxic PW do not form LTM.
The timeline for the experiment is presented above the data. A naïve cohort of snails (N=8) received a 0.5h training session (TS). LTM was tested 24h later (MT). LTM was not formed as no differences in the number of attempted pneumostome openings between the TS and the MT were found. The comparison was made by paired t-test. ns = not significant as $p>0.05$.

In order to determine if Q-exposure had an enhancing effect on LTM, two cohorts of naïve W snails (N=11 for each group) were exposed to Q 3h before or 3h after the training session (**Figure 38**) and memory was tested 24h later. When the Q-exposure occurred 3h before the training session, LTM was formed, as the number of attempted pneumostome openings in the memory test was significantly reduced compared to the TS ($t=7.9$, $df =10$; $p<0.0001$) (**Figure 38A**). Similar results were obtained when the Q exposure occurred 3h after the 0.5h training session ($t=8.74$, $df =10$, $p<0.0001$). (**Figure 38B**). Thus, a single training session could now result in LTM formation; caused by the exposure to Q 3h before or after training.

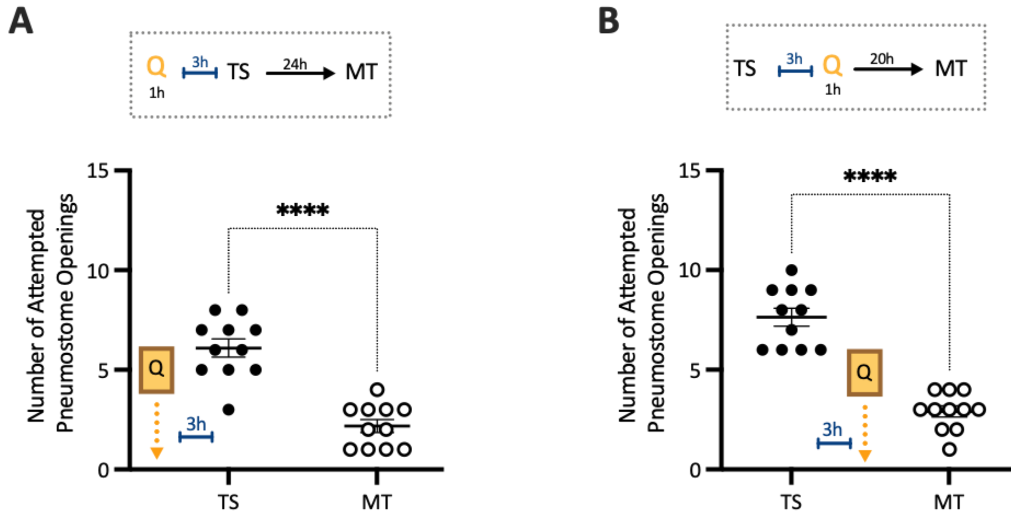


Figure 38. The exposure to quercetin 3h before or after a 0.5h training session enhances LTM.
The timeline for each experiment is presented above the data. Q-exposure occurred 3h before **(A)** or after **(B)** a single 0.5h training session (TS) and an LTM lasting for at least 24h was formed, as the number of attempted pneumostome openings recorded during the memory test (MT) was significantly reduced compared to the TS. The solid line is the mean, and the error bars are the s.e.m. Comparisons were made by paired t-test. **** $p<0.0001$. N=11 for each group.

Control experiments were done to test the effect of the vehicle used to dissolve Q (i.e., DMSO) on LTM enhancement (**Figure 39**). The exposure to DMSO 3h before the 0.5h training session did not result in LTM when animals (N=10) were tested 24h later ($t=0.81$, df $=9$, $p=0.43$) (**Figure 39A**). Similar results were obtained when DMSO exposure occurred 3h after the 0.5h training session ($t=0.82$, df $=9$, $p=0.43$; N=10) (**Figure 39B**).

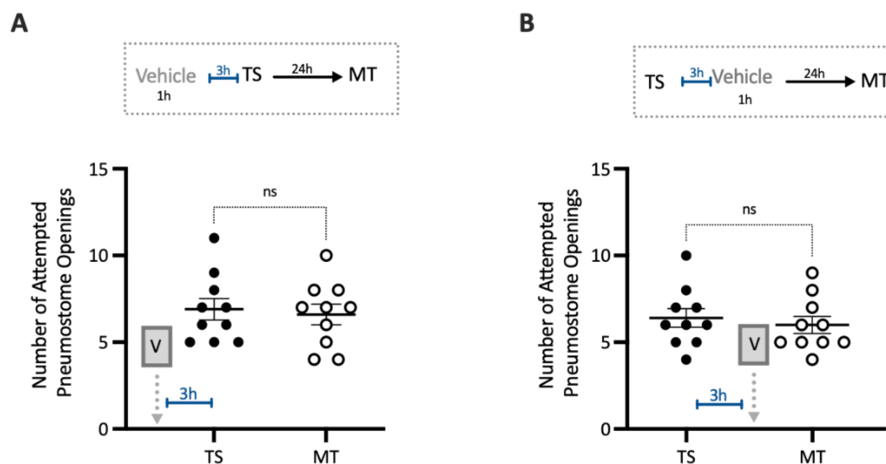


Figure 39. The vehicle that Q is dissolved in does not enhance LTM formation.
The timeline for each experiment is presented above the data. Vehicle-exposure occurred 3h before **(A)** or after **(B)** a single 0.5h training session (TS) did not result in LTM formation (MT), as no differences in the number of attempted pneumostome openings were found. The solid line is the mean, and the error bars are the s.e.m. The solid line is the mean, and the error bars are the s.e.m. Comparisons were made by paired t-test. ns = not significant as $p>0.05$. N=10 for each group.

Effect of the single poke procedure and Q exposure on LTM

Having demonstrated that Q-exposure both 3h before and after a 0.5h training session enhances LTM formation, the effects of Q exposure on memory reconsolidation were then investigated. Previous studies using W snails demonstrated that the process that causes ITM formation following a single 0.5h training session is sufficient to allow a second 0.5h training performed 48h after the first one to produce LTM (Parvez 2005; Parvez *et al.* 2006). Although the memory phenotype is not apparent 24h after the 0.5h training session, a residual memory trace is present and serves as a foundation upon which a subsequent training procedure can induce LTM (Parvez 2005; Parvez *et al.* 2006). Here, it was first attempted to determine whether 24h after the initial 0.5h training session a 'single poke' to the first attempted pneumostome opening was sufficient to cause LTM when tested 24h after the 'single poke'. A naïve cohort of W snails (N=11) was trained and 24h later were subjected to the 'single poke' procedure. When animals were tested 24h after the 'single poke' procedure (i.e., 48h post-training), the respiratory behaviour was not significantly different with respect to the 0.5h training session performed 48h before (t=1.79, df =10, p=0.1). Thus, a 'single poke' by itself had no significant effects on the residual memory trace in enabling LTM to be formed (**Figure 40**).

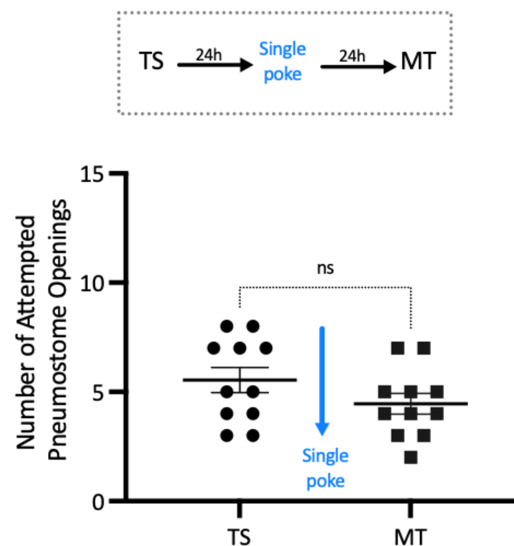


Figure 40. Snails receiving the 'single poke' procedure 24h before the 0.5h training session do not form LTM.
The timeline for the experiment is presented above the data. A naïve cohort of W snails (N=11) was trained with a single 0.5h training session (TS). 24h later animals received the 'single poke' procedure and 24h later were tested for LTM (memory test - MT). LTM was not formed as the number of attempted openings in the MT was not significantly lower than the number recorded in the TS. The solid line is the mean, and the error bars are the s.e.m. The comparison was made by paired t-test. ns = not significant as p>0.05.

Then it was asked whether the combined exposure to Q and the 'single poke' procedure performed 24h after a 0.5h training session procedure would result in LTM when memory was tested 24h later (**Figure 41A**). Thus, a naïve cohort of W snails (N=11) was first trained in a 0.5h training session and 20h later was exposed to Q for 1h. Three hours later (i.e., 24h after the training session), animals were placed in the hypoxic environment and received a poke only the first time they began to open the pneumostome (i.e., the 'single poke' procedure). LTM was then tested 24h later (i.e., 48h after training session – memory test). In these snails LTM was demonstrated 24h after the 'single poke'. the number of attempted pneumostome openings during the memory test was significantly reduced with respect to the training session (t=3.02, df =10, p=0.012). Thus, combining Q-exposure 3h before the ''single poke' procedure' resulted in an LTM when tested 24h after the 'single poke'.

This result led to the hypothesis that Q-exposure 3h after the 'single poke' procedure would also result in enhanced LTM formation. A naïve cohort of animals (N=12) was tested using the same above behavioural procedure, but in these animals, the exposure to Q occurred 3h after the 'single poke' procedure, which was performed 24h after the 0.5h training session (**Figure 41B**). When snails were tested 24h after the 'single poke' procedure, their respiratory behaviour was significantly reduced with respect to the 0.5h training session performed 48h before (t=8.25, df =11, p<0.01), indicating that LTM was formed due to a 'single poke' coupled 3h later to experiencing Q.

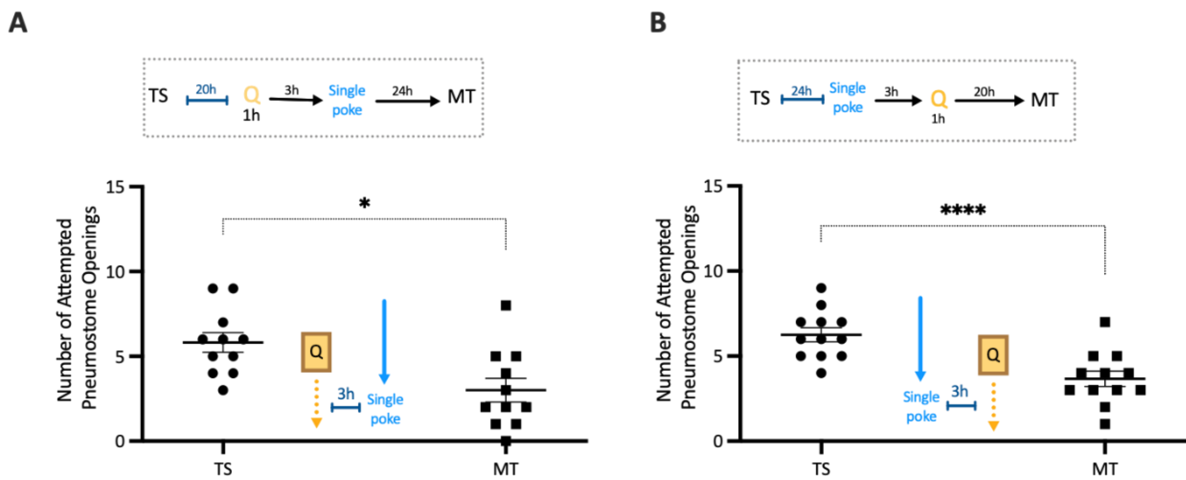


Figure 41. The 'single poke' procedure, quercetin exposure, and enhanced LTM formation.
The timeline for each experiment is presented above the data.
(A) A naïve cohort of snails (N=11) received a single 0.5h training (TS) and 20h later was exposed to Q for 1h. 3h later, snails received the 'single poke' procedure. LTM was tested 24h later (memory test - MT). LTM was formed as the number of attempted openings in the MT was significantly less than in the TS.
(**B)** A naïve cohort of snails (N=11) received a 0.5h training session and 24h later was subjected to the 'single poke' procedure. 3h later snails were exposed to Q for 1h and 20h later, LTM was tested (memory test - MT). LTM formed as the number of attempted openings in the MT was significantly less than in the TS. The solid line is the mean, and the error bars are the s.e.m. Comparisons were made by paired t-test. * p<0.05; **** p<0.0001.

Finally, it has been shown in a cohort of W snails (N=10) trained as above that when Q-exposure was delayed by 20h after the 'single poke' procedure, LTM was not present (**Figure 42**). The number of attempted pneumostome openings between the 0.5h training session and the memory test performed 48h later was not significantly different (t=0.3, df =9, p=0.77). These data show that the 'single poke' procedure acts on a residual trace present 24h after the TS and when combined with Q-exposure 3h before or after, enhanced LTM formation.

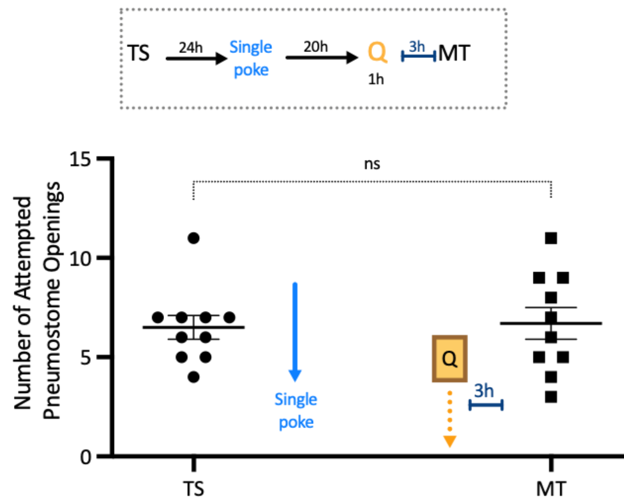


Figure 42. Q-exposure 20h after the 'single poke' procedure does not result in enhanced LTM formation.
The timeline for the experiment is presented above the data. Twenty-four hours after the 0.5h training session (TS), naïve W snails (N=10) were subjected to the 'single poke' procedure. Q exposure occurred 20h later, 3h before LTM was tested (memory test - MT). LTM was not formed as the number of attempted openings in the MT was not significantly lower than the TS. The solid line is the mean, and the error bars are the s.e.m. The comparison was made by paired t-test. ns = not significant as p>0.05.

Effect of the single poke procedure and Q exposure before a 0.5h training session and LTM formation

Continuing with the effects of the 'single poke' procedure, it was asked what effect, if any, the 'single poke' would have if the W-strain snails received it before a 0.5h training session. In a naïve cohort of snails (**Figure 43A**; N=10) the 'single poke' procedure was performed 24h before the 0.5h training session and LTM was not formed when snails were tested 24h later. A paired t-test revealed that the number of attempted pneumostome openings between the 0.5h training session and the memory test performed 48h later was not significantly different (t=0.58, df =9, p=0.59). Thus, LTM was not present. Then it was investigated whether Q exposure 24h before a 0.5h training session resulted in enhanced LTM formation. Thus, a naïve cohort of snails (N=10) was exposed to Q 24h before the TS and was then tested for LTM 24h later (**Figure 43B**). A paired t-test revealed that the number of attempted pneumostome openings during the memory test was not significantly different from the number in the training session (t=1.33, df =9, p=0.21). Thus, neither the 'single poke' procedure nor Q-exposure 24h before a 0.5h training session resulted in enhanced LTM formation.

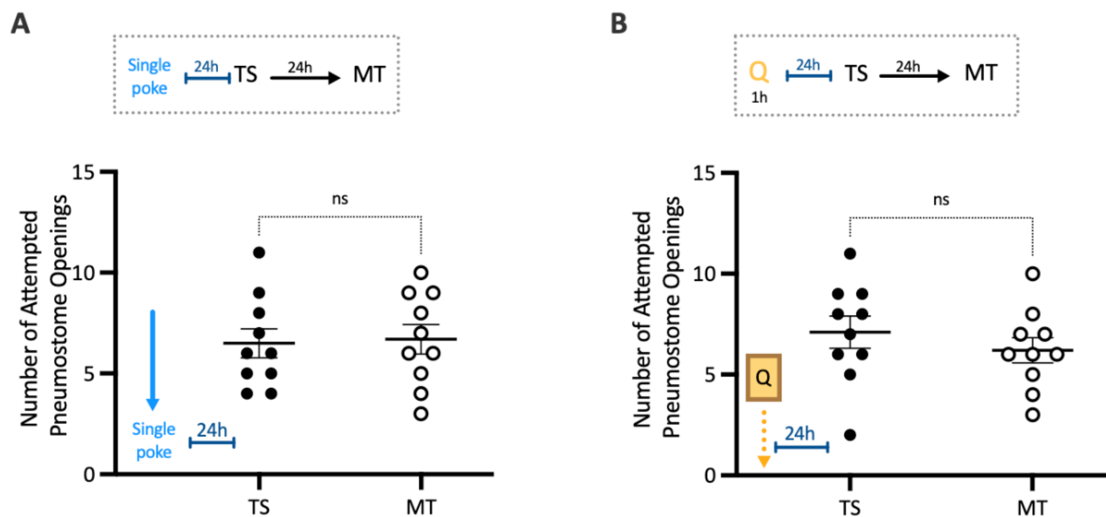


Figure 43. The 'single poke' procedure or Q-exposure 24h before a 0.5h training session does not result in LTM formation.

The timeline for each experiment is presented above the data.

(A) Snails (N=10) were trained 24h after receiving the 'single poke' procedure and LTM was tested 24h later (memory test - MT). LTM was not formed as the number of attempted openings during the memory test was not significantly different than the number in the training session (TS).
(B) Snails (N=10) were exposed to Q for 1h and 24h later received a 0.5h training session (TS). LTM was tested 24h later. LTM did not form as the number of attempted openings in the memory test was not significantly different than that recorded in the TS.

The solid line is the mean, and the error bars are the s.e.m. Comparisons were made by paired t-test. ns = not significant as p>0.05.

Then, it has been asked if a combination of the 'single poke' procedure and Q-exposure 24h before a 0.5h training session would result in enhanced LTM formation. These data are presented in **Figure 44**. A naïve cohort of snails (N=10) was first exposed to Q for 1h and 3h later was subjected to the 'single poke' procedure (**Figure 44A**). Animals were then returned to their home aquaria for 20h before being trained with a 0.5h training session. These snails were then tested for LTM 24h later and a significant reduction in the number of attempted pneumostome openings compared to the training session was found ($t=7.23$; $df = 11$; $p<0.0001$). Thus, LTM was present. These data show that Q-exposure, combined with the single poke procedure 24h before a single 0.5h training session resulted in LTM formation.

Next presented a naïve cohort of snails (N=10) was subjected to the single poke procedure first and then 3h later was exposed the snails to Q for 1 h (**Figure 44B**). These snails then were trained 20h later with a 0.5h training session and were tested for LTM 24h later. The sequential presentation of the single poke and then Q exposure resulted in LTM. That is, the number of attempted pneumostome openings during the memory test was significantly reduced compared to the number in the training session ($t=2.84$, $df=9$; $p=0.02$).

Thus, the 'single poke' procedure and Q-exposure 3h later was sufficient to cause LTM formation following a 0.5h training session 20h later. Not shown here, are control experiments where the vehicle is used rather than Q along with the single poke procedure; in all cases enhanced LTM formation was not observed.

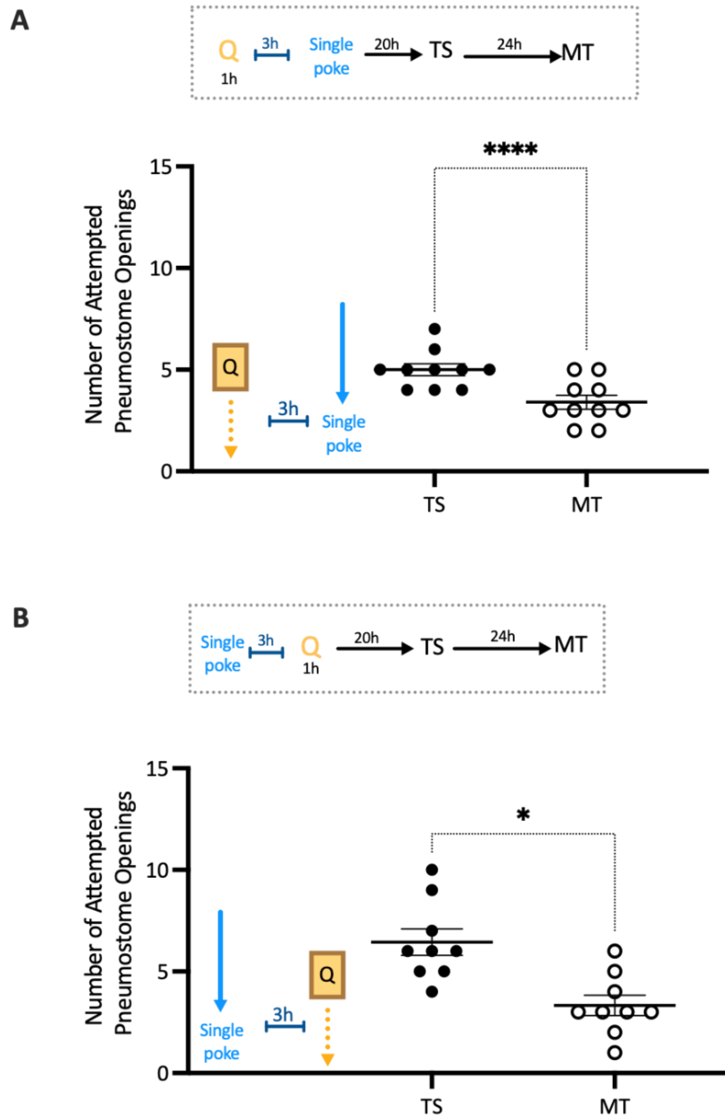


Figure 44. The 'single poke' procedure combined with Q-exposure 3h before a 0.5h training session results in enhanced LTM formation.

The timeline for each experiment is presented above the data.

(A) A naïve cohort of W snails (*N*=10) was exposed to Q for 1h and 3h later received the 'single poke' procedure. Twenty hours later, animals were trained with a single 0.5h training session (TS) and LTM was tested at 24h (memory test - MT). LTM was formed as the number of attempted openings during the MT was significantly less than in the TS.

(B) A naïve cohort of snails (*N*=9) received the 'single poke' procedure and 3h later was exposed to Q for 1h. Twenty hours later, animals were trained with a single 0.5h training session (TS) and LTM was tested 24h later (memory test - MT). LTM was formed as the number of attempted openings recorded in the MT was significantly less than in the TS.

The solid line is the mean, and the error bars are the s.e.m. Comparisons were made by paired t-test. **** $p<0.0001$; * $p<0.05$.

To sum up, when applied within 3h of critical periods of memory, Q regulates four different phases:

1. The exposure to Q 3h before a single 0.5h training session, improves the learning event;
2. Similar enhancing effects can be obtained by exposing animals to Q 3h before or after a 'single poke' procedure preceding by 24h a training session, improves the learning event;
3. The exposure to Q 3h after a single 0.5h training session enhances the consolidation processes after acquisition;
4. The exposure to Q 3h before the 'single poke procedure', which was performed 24h after a single 0.5h training session, enhances memory recall;
5. The exposure to Q 3h after the 'single poke procedure', which was performed 24h after a single 0.5h training session, memory reconsolidation.

In all these phases Q-exposure enhanced LTM persistence.

Discussion

The experiments performed during my PhD and reported in the present thesis highlighted at least three major advantages of using *L. stagnalis* as a model for Translational Neuroscience.

(1) *L. stagnalis* is capable of a variety of behaviours that can be conditioned by various sensitive, solid, easily reproducible, and simple conditioning paradigms which allow to uncover the memory abilities of this model system and to study high-order forms of learning. Moreover, by focusing on the animal's responses to internal and external stimuli at various times during training, it is possible to study the temporal dimension of memory formation and storage.

(2) *L. stagnalis* has a relatively simple nervous system, consisting of only ≈25 000 neurons, which are big in size and easily identifiable. It is therefore possible to study various pathways from the behavioural outputs to the molecular level. This reductionistic but not simplistic approach represents a rapid and cost-effective option for elucidating the causal, behavioural, and molecular changes underlying the complex mechanisms occurring in the mammalian brain.

(3) *L. stagnalis* can be used as a model system in a wide range of applied biological fields, including ecotoxicology, developmental biology, and immunology, providing insights into how can respond appropriately to environmental challenges, such as global warming and pharmacologically active compounds.

My studies involved several methods, that included qPCR, behavioural tests, and *in silico* search of new genes, allowing me to collect new evidence of the potentialities of this model in Neuroscience.

To date, the molecular level analyses in *L. stagnalis* have been limited by the lack of an annotated and characterized genome and transcriptome (Tascedda *et al.* 2015).

Development of a method for characterizing conserved pathways in the CNS of L. stagnalis

In this thesis, a strong effort was made to develop a quick, but solid and highly reproducible method for the characterization of important and conserved molecular pathways involved in physiological and pathological processes in *L. stagnalis* CNS.

The first step consisted of the generation of a database containing all *L. stagnalis*'s contigs (i.e., the series of overlapping DNA sequences used to make a physical map that reconstructs the original DNA sequence). Then, the contigs have been matched with the genes coding for the targets of interest in the annotated genome of another snail, *B. glabrata*, whose genome has already been characterized. Next, it has been chosen a pathway to be first identified *in silico* and then characterized by gene expression analyses.

Characterization, induction, and regulation of the enzymes of the KP in the CNS of L. stagnalis

The choice fell on the kynurenine pathway (KP) because of the high level of conservation between vertebrates and invertebrates, its pharmacological inducibility, and its relevance to the field of Neuroscience (Savitz 2020). Under physiological conditions, the KP is the primary route for tryptophan catabolism and the starting point for the synthesis of the ubiquitous co-enzyme nicotinamide adenine dinucleotide, which, in turn, fulfills the cellular energy requirements (Braidy and Grant 2017). As the KP plays a key role in immune function and energy metabolism, aberrations of its molecular cascade have been identified diverse human diseases and disorders, including inflammation/immune disorders, endocrine/metabolic conditions, Alzheimer's disease, amyotrophic lateral sclerosis, Huntington's disease, cancer, depression, and schizophrenia (Albuquerque and Schwarcz 2013; Dantzer 2016; Braidy and Grant 2017b; Allen *et al.* 2018; Garrison *et al.* 2018; Castro-Portuguez and Sutphin 2020). Given these premises, it seemed worthwhile to characterize the KP in an invertebrate model which is a promising candidate for Translational Neuroscience research, like *L. stagnalis*.

To date, the KP has been studied only in two invertebrates: the worm *C. elegans* and the fly *D. melanogaster*. *In C. elegans*, the alteration of the KP causes neurodegeneration and ageing (van der Goot *et al.* 2012), whereas in *D. melanogaster* a correlation between elevated levels of 3-hydroxy kynurenine and synaptic plasticity was found (Savvateeva *et al.* 2000). Apart from these two studies, this is the first study in which the KP is characterized and induced in a molluscan model.

Once the pathway has been chosen, the contigs of *L. stagnalis* have been matched with the genes coding for the enzymes of the KP in the annotated genome of *B. glabrata*. This has led to a univocal identification of putative transcripts of KP enzymes in *L. stagnalis* CNS. As all the putative transcripts identified contained an open reading frame (ORF - i.e., a portion of a DNA molecule that, when translated into amino acids, contains no stop codons), it was then possible to generate *in silico* the nucleotide sequences and the corresponding aminoacidic sequences of each KP enzyme in *L. stagnalis*. These sequences, in turn, have been aligned with the deposited orthologues of other model organisms, including *Homo sapiens*.

The alignment and the phylogenetic trees confirmed the high level of homology between vertebrates and invertebrates among the transcripts involved in the tryptophan catabolism via kynurenine. These results are fundamental in order to better understand the extent to which CNS functions are conserved and consequently how generalizable experimental findings are across species. The transcripts identified *in silico* were used to design highly-efficient and species-specific primers for all putative KP-enzymes, which allowed to amplify by PCR the DNA region of interest prior to Sanger sequencing. The sequencing analyses revealed a high level of identity with the sequences predicted by the bioinformatic analysis. Thus, the proven validity of the multidisciplinary approach developed and adopted in this project, can be extremely useful for overcoming the lack of an annotated genome and transcriptome in *L. stagnalis*.

The final step was the pharmacological regulation of the putative enzymes of the KP. The snails were therefore injected with LPS which, from data in the literature, can modify the functionality of the pathway. Significant molecular effects between treated animals compared to controls through expression studies by qRT-PCR were investigated in the ganglia of animals sacrificed 2, 6, or 24 hours after the injection. This study demonstrated that specific transcription was strongly induced after the injection with LPS in the ganglia of *L. stagnalis*.

First, it was found a significant increase in the mRNA expression levels of *Lym* IDO and *Lym* TDO at 2h and 6h post LPS injection. IDO and TDO are the initial and rate-limiting enzymes in the KP and regulate the production of active metabolites. These enzymes are structurally distinct but catalyze the same reaction, the conversion of tryptophan to N-formyl kynurenine.

In mammals, TDO mainly acts under physiological circumstances (Marszalek-Grabska *et al.* 2021), whereas IDO-dependent tryptophan metabolism is strongly activated in response to proinflammatory stimuli (i.e., including LPS) (Nishi *et al.* 2018).

As in *L. stagnalis* these enzymes are both activated by a proinflammatory stimulus, it is possible to speculate that their mechanisms of activation and action are generalized, whereas the increasing complexity of the vertebrate immune and nervous systems might have led to selective pressures favouring the specialization of these enzymes in different tissues and under different conditions. Interestingly, in *L. stagnalis* CNS the mRNA levels of the orthologue of ACMSD were upregulated as an immediate response to LPS treatment, and this upregulation was still present 6h after the injection, mirroring the up-regulation of IDO and TDO. This is the first time that the expression levels of this enzyme were measured following an LPS injection.

However, as ACMSD competes with the non-enzymatic spontaneous cyclic reaction of the neurotoxic branch of the KP (Allen *et al.* 2018), it is possible to hypothesize that, following the treatment with LPS, the upregulation of *Lym* ACMSD may be the result of a modulatory response to the increased mRNA levels of IDO and TDO, committing the pathway to switch towards the production of picolinic acid.

Consistent with this hypothesis, is the fact that the LPS treatment at the concentration used in this study (6.25 µg/ml) did not induce the upregulation of the orthologue of the neurotoxic KP enzymes downstream of IDO (KMO, KYNU, and HAAO). In particular, while *Lym* KMO and *Lym* HAAO expression was not affected at either time point in response to LPS treatment, *Lym* KYNU, instead, was downregulated only at 6h post-LPS treatment.

On the opposing metabolic branch, the expression levels of the orthologues of KAT enzymes (*Lym* KYAT and *Lym* AADAT) were found to be differentially regulated by the LPS injection. While *Lym* KYAT remained unaffected, *Lym* AADAT was significantly downregulated 6h and 12h after LPS treatment. Consistently with previous studies from rodents (Parrott *et al.* 2016), the LPS treatment induced the downregulation of AADAT, which, in turn, leads to the synthesis *de novo* of the kynurenic acid, a neuroprotective metabolite (Ganong and Cotman 1986).

Studies aimed at characterizing the metabolites of the KP in *L. stagnalis* are currently in progress and could help the understanding the role of the kynurenine pathway in neurotransmission and neuroinflammation in this model system.

Moreover, future studies will help to investigate complex effects of an LPS peripheral administration on metabolite kinetics, which might be lost if studied only a few time points.

The experiments conducted in this project highlighted the validity of the experimental approach developed for identifying and characterizing conserved transcripts in the genome of *L. stagnalis*, opening numerous scenarios in the use of this model in translational neuroscience. In this pilot study, in fact, an innovative, solid, quick, and highly reproducible approach for characterizing *L. stagnalis*'s genome and transcriptome has been developed and validated. This has been a steppingstone of molecular studies in this model, allowing to efficiently study and characterize other molecular pathways of interest in Neuroscience.

Moreover, it has been provided for the first time the biochemical characterization of the kynurenine pathway highly conserved and generates biologically active intermediates, which are physiologically active and affect diverse physiological systems. With increasing recognition that kynurenine and its metabolites can affect and even control a variety of classic neurotransmitter systems, directly and indirectly, interest in this area is expanding rapidly. In this context, the study of the molecular and behavioural effects induced by the manipulation of the KP in *L. stagnalis*' CNS represents an appealing strategy both to assist the current unsolved questions and to drive the field forward. This model not only can help the discovery of additional physiological and pathological roles for kynurenines, but also may contribute to the development of new therapies based on these roles.

Definition and characterization of a behavioural protocol for inducing a Garcia effect-like phenotype in L. stagnalis

Moving from the bioinformatics/molecular to the behavioural experiments, in this project has been reported the first demonstration of a Garcia-like effect in invertebrates. This higher-order learned behaviour consists of a negative hedonic shift to a novel taste consumed before becoming nauseous (i.e., a visceral illness) (Garcia *et al.* 1966). Data presented in my project are all consistent with the 'requirements' presented by the Garcia group as the distinctive traits of the Garcia effect (Garcia *et al.* 1974): the aversion is taste-specific, is acquired rapidly, and persists for long periods. In particular, a single paired presentation of a novel appetitive taste (i.e., carrot slurry, C) with a heat stressor (HS), is sufficient to induce a long-lasting (i.e., at least 24h) negative hedonic shift to C. If C is not a novel taste or it is presented after the HS, the Garcia effect is not formed. The HS on its own does not cause a feeding inhibition, but the negative shift in the hedonic value of C is the result of the association made by the snails when the presentation of C precedes by some hours (i.e., till 48h) the HS. Moreover, the negative hedonic shift is specific to the taste paired with the stressor and not to other tastes (i.e., sucrose), suggesting that the Garcia effect may be a defence mechanism to protect organisms against future ingestion of a food taste which became associated with a visceral illness (e.g., nausea) from which they recovered. Interestingly, in many mammalian studies examining the duration of the interval between the presentation of the novel food taste and the experiencing the visceral sickness, it was found that the longer the interval the less lasting was the acquired negative hedonic shift (Nachman and Ashe 1973; Domjan and Bowman 1974; Andrews and Braveman 1975). In humans, for example, the Garcia effect has been reported with the interval between taste and nausea separated in time for up to 7 hours (Logue *et al.* 1981).

Data obtained in *L. stagnalis* mirror those from mammalian preparations. This can be explained by the fact that in nature some toxins/poisons have slow uptake times across the gut mucosa and thus the onset of the nauseating effects following ingestion will be delayed. This fact further captures the value of the Garcia effect as defensive behaviour conferring positive survival value to the animal.

In this study, was also investigated the ability of food-deprived *L. stagnalis* to acquire and exhibit the Garcia effect. Following their exposure to the novel C, snails were subjected to 2 days of food deprivation before they experienced the HS. When tested 3h post-HS to C, the Garcia effect was not apparent. The fact that the memory phenotype was not exhibited in food-deprived snails has an important biological meaning. Although generally, animals avoid poisonous substances for their safety, snails that are severely food-deprived (i.e., 48h) must eat something to survive, even if that food source is a reliable predictor for subsequent nausea.

The Garcia affect memory phenotype re-emerged following a 24h period in which snails had *ad libitum* access to food (lettuce), suggesting that severely food-deprived animals learned and formed memory, but their memory performances were suppressed by food deprivation as they needed to compensate for energetic demand.

Thus, a change in the central state of the snail determines whether it 'allows' expression of the formed memory. This mechanism has been elegantly defined by Ito *et al.* (2015) as "*necessity knows no law*" behaviour, to indicate that memory may only be expressed after the resolution of the conflict between the necessity to eat and the memory of the aversive consequences associated with the appetitive stimulus (Ito *et al.* 2017).

Moreover, the molecular events induced in the CNS by the HS have been investigated. The focus has been placed on two heat shock proteins (HSPs), HSP40 and HSP70, as previous studies demonstrated that acute exposure to 30°C for 1h was sufficient to increase their expression levels above constitutive levels, peaking between 2 and 4h after the end of thermal stress (Foster *et al.* 2015). Here, the expression profiles for HSP40 and HSP70 were obtained 3h after exposure to the HS and a 15-fold increase for HSP70 and about 4-fold increase for HSP40 were found. Similar up-regulation of HSPs in response to a comparable heat shock stressor has been reported in other mollusks, including *Pomacea canaliculata* and pacific oysters (*Crassostrea gigas*) (Dong and Dong 2008; Song *et al.* 2014).

Other studies demonstrated that exposure to the HS before the operant conditioning of aerial respiration causes an enhancement of LTM formation (Teskey *et al.* 2012; Sunada *et al.* 2016). On the other hand, this memory enhancement can be prevented by exposing snails to the flavonol Q before the HS (Sunada *et al.* 2016). In this thesis, it was found that Q exposure for 1h before the HS prevents the heat-induced up-regulation of HSP40 and HSP70.

Thus, in *L. stagnalis*, as in other model organisms, Q blocks HSPs induction or up-regulation (Hosokawa *et al.* 1990; Wang *et al.* 2008; Storniolo *et al.* 2015).

I, therefore, hypothesized that Q's ability to alter the up-regulation of HSPs caused by the HS prevents the memory-enhancing effect of the HS on operant conditioning LTM.

However, Q blocks HSPs up-regulation only when it is applied before snails experience the HS: its presentation alone or immediately after the HS does not block the increase of HSP70 and HSP40 expression levels.

Initially characterized for their response to thermal stressors (hence their name), HSPs play a central role in the prevention of protein misfolding and HSP70 may importantly be involved in synaptic plasticity and memory formation (Lindquist and Craig 1988; Igaz *et al.* 2004; Ambrosini *et al.* 2005; Hung *et al.* 2010; Song *et al.* 2014; Lackie *et al.* 2017; Porto *et al.* 2018).

For example, following aversive and fear conditioning in rodents, HSP70 is induced in the hippocampal CA1 region (Pizarro *et al.* 2003; Igaz *et al.* 2004). Recently, Porto and colleagues (2018) reported that in rats HSP70 is rapidly induced and modulates the MAPK signalling pathway during memory consolidation in hippocampal neurons (Porto *et al.* 2018).

Interestingly, an increase in the MAPK phosphorylation in *L. stagnalis* buccal and cerebral ganglia (i.e., that are involved in the plasticity of the feeding behaviour) is essential for food-reward conditioning (Ribeiro 2005). Thus, in future studies, it will be investigated whether a molecular link between HSP70 and MAPK also exists in *L. stagnalis* nervous system and if it is involved in the Garcia effect.

At the behavioural level, it was shown that the exposure to Q before the HS altered the ability of snails to acquire or express the Garcia effect. It was hypothesized that Q's ability to alter the up-regulation of HSPs caused by the HS prevents the acquisition of the Garcia effect. However, it is likely that other pathways, in addition to the HSPs, are involved in the acquisition of the Garcia effect.

Differently from the 'classical' conditioned taste aversion, the acquisition of the Garcia effect depends on animals experiencing a visceral sickness (e.g., nausea). As has been pointed out in mammals, the symptoms of lithium intoxication, which is used in many studies to induce visceral illness, are similar to those produced by a heat shock (Davey and Biederman 1996). Thus, it was not too surprising to see that such a heat shock stimulus could be used to induce a Garcia-like effect in rodents. In humans, heat stress can result from hours later in nausea and vomiting (Becker and Stewart 2011). As for laboratory-reared snails, a prolonged (> 3h) exposure to temperatures of 30°C or above is lethal (McDonald 1973), it is possible to assume that the HS does result in some sort of visceral illness (reaction/sickness) in this strain.

Different, however, is the effect of heat in freshly-collected snails. While the exposure to a single HS for 1h following the presentation of a novel taste results in a long-lasting Garcia effect in laboratory-reared snails maintained under standardized temperature conditions for generations, in freshly collected *L. stagnalis* and their first-generation offspring raised in laboratory standardized temperature conditions, does not. The difference in the memory phenotypes produced by heat is likely to be due to differences among the strains in their heat stress response.

Therefore, F0 and F1 Delta may not perceive the HS as a stressor and, as a consequence of that, they do not become bait-shy to the novel taste.

This is likely because wild snails have repeatedly experienced temperature fluctuations (from 4°C to above 35°C in summer) during their natural history, thus becoming more tolerant and resilient to heat.

Moreover, these data illustrate that the heat sensitivity of F1 Delta snails is more dependent on their innate heat tolerances rather than the local environment in which they were raised. Thus, F1 Delta snails maintain the parental phenotype and are not stressed by heat shock like the lab-bred ones even though both were raised and maintained under the same environmental conditions. This is not surprising as many species develop adaptive responses to local environmental conditions across generations and parental generations (Feder and Hofmann 1999).

Heat tolerance across animal *taxa* is influenced by multiple genes including the heat shock proteins that are highly conserved and are upregulated after heat stress (Morimoto 1993). Thus, the Garcia effect represents a valid learning paradigm for investigating if organisms can alter their behaviours to face ecological stressors (Angilletta Jr. 2009), and whether they would develop adaptive traits over generations through natural selection to match local conditions (Hoffmann *et al.* 2003; Narum *et al.* 2013).

As the heat shock up-regulates HSP70 and HSP40 in *L. stagnalis* CNS and this up-regulation plays a necessary role in the acquisition of the Garcia effect, it is possible to speculate that the Garcia effect may not occur in wild snails because of their higher basal heat tolerance which makes the heat shock used in the current study not strong enough to induce the upregulation threshold of the HSPs. Additional studies will be performed in the future to elucidate the heat-induced effects on other traits closely linked to fitness, from respiration rate to feeding and locomotion, as well as predator-prey interactions, to emphasize the ecological impacts that heat can have at the species to ecosystem level.

Nature versus nurture in heat stress induced learning between inbred and outbred populations of L. stagnalis

Having demonstrated that the exposure to a single HS can induce a long-lasting Garcia effect in *L. stagnalis*, it has been investigated whether lab-reared snails can be acclimatized to the thermal stress following repeated exposure to heat. Moreover, the environmental stressor used in this study, a repeated heat shock, allowed to answer some questions about thermal tolerance and the effects of heat stress on cognitive functions. Across animal *taxa*, the current increase in environmental temperature can affect physiological, psychological, behavioural, and cognitive functions. Firstly, it was tested the thermal plasticity of lab-bred snails exposed daily to a heat stressor like the one used here for the Garcia effect procedure. It was hypothesized that snails might acclimate to this repetitive heat stress. In fact, previous studies demonstrated that acclimation or reduction of heat shock response is beneficial to balance the cost of producing HSPs to face a stressor that has a high energy demand and can impair growth and reduce fitness (Sørensen *et al.* 2003). To test this hypothesis, I first defined the maximum duration for the repeated heat shock procedure. In fact, on the third day of the repeated heat exposure, there was a severe suppression of the feeding behaviour, which was accentuated from Day 4 onward when it was noticed that snails did not eat the lettuce in their home aquaria.

The data on the repeated HS procedure is in line with the results from rodents for which the most reported result is that repeated stress reduces food intake and body weight in a manner directly related to the stress duration. However, following the heat procedure, three days without any heat exposure were necessary and sufficient for the feeding behaviour to be recovered. On the third-day post-repeated heat procedure, the feeding behaviour was restored, and no differences were observed when compared to naive animals. This data indicated that the repeated heat-shock exposure strongly affected the snails' feeding behaviour.

Thus, to preserve the snails' survival, a maximum duration of 7 days was set for the repeated heat shock procedure. For the same reason, snails were not exposed to temperatures higher than their upper thermal tolerance limit of 30°C. This behavioural procedure allowed me to demonstrate that laboratory-inbred snails do not acclimate to the repeated exposure of a heat stressor. Quite the opposite, experiments reported in Aim 3, show that the daily exposure to an HS increased snails' thermal sensitivity. Following the repeated heat presentation, in fact, the paired presentation of C and 30°C resulted in an aversion lasting up to 72h (instead of 24h).

Thus, following the repeated HS procedure, LTM was 48h longer than that formed by snails that experienced heat for the first time during the Garcia effect procedure. Additionally, it was found that, after the daily exposure to a thermal stressor, a shorter time of heat exposure (15 min instead of 1h) after the C presentation was sufficient for a 72H-lasting LTM to be formed. To note, the exposure to an HS for 15 minutes *per se* without previous repeated heat exposure was not sufficient to induce the Garcia effect in lab-reared snails. Together, these data demonstrate that following the repeated exposure to a thermal shock, the heat stressor can ultimately result in an identical behavioural phenotype (i.e., LTM lasting for 72h) even if it is applied for 1h or 15 minutes. These results are consistent with previous studies from mammals indicating that the repeated exposure to an acute stressor over time modifies stress vulnerability and increases stress sensitization. Thus, it is possible to speculate that the repeated heat shock presentation might reduce the threshold of activation of HSPs to the extent that only 15 minutes of heat exposure become sufficient to induce a long-lasting Garcia effect. This study also demonstrated that even after the repeated heat shock procedure a novel taste must be paired with the heat stressor for the Garcia effect to be induced, as proven by the fact that when a familiar taste (i.e., lettuce) was used, the Garcia effect was not shown. Moreover, having excluded any interferences due to the handling of animals from their aquarium to the beaker containing clean room-temperature water, it is possible to assume that the behavioural outcomes resulting from these experiments were heat-specific. The heat sensitivity of this inbred strain appears to result from the acclimation of snails to the constant lab temperatures, finally selecting out their broad thermotolerance range.

Previous studies from ectotherm animals, including the kelp crab *Taliepus dentatus* (Storch *et al.* 2009), the killifish *Fundulus heteroclitus* (Fangue *et al.* 2006), the intertidal snail *Nucella canaliculate* (Kuo and Sanford 2009), and *D. melanogaster* (Hoffmann *et al.* 2003), compared the thermal adaptations to temperature extremes among conspecific populations living at different latitudes or altitudes. Most of these studies focused on the effect of heat on organismal survival, however, to understand how organisms respond to climate changes, the question should be addressed on multiple traits (Forsman 2015).

In this study, it has been tested the effect of a repeated HS on a single trait, feeding, because it is closely linked to fitness and provides an ideal system for studying the physiological basis of a plastic behaviour (Benjamin 2008).

Additional studies on the effects HS have on traits like aerial respiration, and locomotion, are required for a 'whole organism' comprehension of the ecological and physiological impacts of the current global warming.

Predicting which organisms are going to be 'winners' or 'losers' under the ongoing climate-change is one of the most important challenges of the Anthropocene (Somero 2010). However, attention should be paid to making predictions on the thermal sensitivity among populations of the same species, especially if data are extrapolated from laboratory to wild conditions.

As shown in Aim 3, freshly collected snails and their F1 generation raised and maintained under the same laboratory conditions as W snails do not show a Garcia effect phenotype even after repeated HS. By providing insight into the complex interactions between organisms' thermal physiology and their environments, this study illustrates how different selection pressures influence the heat sensitivity between laboratory-reared strain and the progeny of a wild population.

Understanding how and why these differences occur is important in both Neuroscience and Ecology. In this context, *L. stagnalis* represents a multipurpose model organism in various biological disciplines, from neurobiology to ecotoxicology.

Q is capable of enhancing LTM formation if encountered at different times in the learning, memory formation, and memory recall continuum

Finally, in this project, I investigated the effects of a bioactive compound, the flavonoid quercetin on learning and memory formation. Numerous studies reported that regular dietary consumption of flavonoids and flavonoid-rich foods can improve various cognitive dysfunctions and dementia-like alterations in different animal models (Williams *et al.* 2004; Vauzour *et al.* 2007; Spencer 2007; Yevchak *et al.* 2008; Macready *et al.* 2009; Mastroiacovo *et al.* 2015; Socci *et al.* 2017; Bakoyiannis *et al.* 2019). However, to date, only a few studies elucidated the possible memory-enhancing effect of Q on healthy individuals.

In Aim 2 it was demonstrated that Q blocks the upregulation of HSP70 and HSP40 induced by an HS, and, as consequence, a Garcia-like effect was not formed. However, as shown in Aim 4, in addition to blocking HSP expression, Q also upregulates multiple signalling molecules such as CREB1 and the serotoninergic pathway.

The role of serotonin and CREB1 in learning and memory was revealed for the first time in the '70s by Kandel and colleagues. Studies in *A. californica* demonstrated that 5HT leads to a local increase in cAMP and the activation of the PKA, which in turn, activates CREB1 (Kandel *et al.* 2014). The role of CREB1 in LTM formation has been demonstrated in many *taxa*, including *L. stagnalis*.

This study demonstrated for the first time that the exposure to Q for 1h results, 3h later, in the upregulation of the orthologous genes of CREB 1, 5HTR1, 5HTR2, and TPH in L. *stagnalis* CNS, suggesting a conserved role of Q in regulating signalling pathways underlying LTM across multiple associative learning paradigms and *taxa*.

Thus, I hypothesized that the exposure to Q within 3h of critical memory phases would enhance the *L. stagnalis*' ability to learn and form LTM following a single 0.5h training session for the operant conditioning of the aerial respiratory behaviour. Normally when W snails are trained with a single training, the memory only persists for 3h as ITM. The fact that, following a Q exposure, a learning paradigm that exclusively results in ITM formation (i.e., in laboratory-reared snails a single 0.5h training session is not sufficient for operant conditioning LTM to be formed) can be efficiently converted into LTM that necessarily involves altered gene expression and protein synthesis, indicates that although the memory types are distinct, they can be flexibly converted from one to another. Moreover, the conversion of ITM to LTM by Q in *L. stagnalis* in a relatively short period highlights the capability of acute experience-dependent changes that can play a role in learning and memory. However, it must be emphasized that for Q-exposure to cause enhanced LTM it was necessary to be encountered in a very limited time window of opportunity (approximately 3h). Outside of that window, Q-exposure did not result in enhancement of memory formation. When Q exposure occurs 24h learning, snails do not form LTM. A similar trend has been observed when snails were exposed to Q 3h before the memory test at 24h (i.e., 20h after the training session). Previous studies demonstrated different compounds (i.e., KCl, green tea, and cannabinoids), when applied before or after the 0.5h training session, enhances LTM formation (Orr *et al.* 2009; Sunada *et al.* 2016; Batabyal and Lukowiak 2021).

Consistent with previous literature, this study illustrated that a 1h exposure to Q, 3h before or after the 0.5 training session caused enhancement of LTM. Using the same inbred laboratory-reared strain of *L. stagnalis*, Parvez et al., (2005, 2006) found that there was a residual memory trace 24h after training, following a single 0.5h training session, even though the LTM phenotype was absent (Parvez 2005; Parvez *et al.* 2006). Thus, using this residual memory trace period, it has been demonstrated that Q-exposure during that period can alter LTM formation. When the 'single-poke procedure' was applied following a 0.5h training session and it did not result in LTM. However, when the snails experienced Q 3h before or after the single poke procedure (i.e., during the residual memory trace interval) LTM was formed 24h after the single poke and Q-experience. Data such as those show that once a memory is consolidated, the exposure to Q together with a stimulus associated with learning (i.e., a physical poke), enhances memory recall and may re-enact many of the same molecular events occurring during the initial memory consolidation phase (Sara 2000). It was further showed that the exposure to Q 3h before or after the single poke procedure enables a 0.5h training session, given 20h later, to result in LTM formation. These data suggest that when combined with the single poke, Q activates molecular cascades whose effects persist for sufficient time to boost the learning event so that pathways necessary for LTM formation can be triggered.

Importantly, the enhanced abilities of snails exposed to Q to form, consolidate, and recall LTM is not due to any effect of this flavonoid on homeostatic aerial respiratory behaviour as there were no changes in aerial respiration following Q exposure and in the number of pneumostome opening attempts during the training sessions among the cohorts exposed to Q or the vehicle.

Abundant data from both vertebrates and invertebrates, including *L. stagnalis*, show a key role of CREB1 in LTM formation after various learning paradigms (Sangha 2003; Alberini 2009).

Both memory consolidation and reconsolidation use similar molecular pathways that converge on the activation of CREB1 (Tronson and Taylor 2007; Alberini 2009; Nader and Einarsson 2010; McKenzie and Eichenbaum 2011). The up-regulation of CREB at 3h post-Q exposure is consistent with the hypothesis that among the targets which are differentially boosted by Q during the 3h-lasting window there are CREB and the members of the serotoninergic pathway.

These factors may be differently involved and regulated by Q during the various phases in the process of transforming learning into LTM and its recall, depending on the time of Q application. This upregulation by Q-exposure at 3h nicely coincides with the 3h timeframe required for Q to be effective in causing enhancement of LTM formation.

While not proving that this is the mechanism by which Q-exposure causes the seen LTM enhancement the data are consistent with that hypothesis. Thus, there are two situations in which the exposure of *L. stagnalis* to Q results in two opposite effects on LTM formation: (1) when Q is applied before the HS, the upregulation of HSP70 and HSP40 is blocked and the LTM for the operant conditioning and the Garcia effect was not observed, whereas (2) experiencing Q alone within 3 hours of critical periods of memory, enhances the learning event, the consolidation processes after the acquisition, the memory recall, and reconsolidation. This LTM enhancement was attributed to Q causing upregulation of CREB1 and the 5HT pathway.

These data provide the groundwork for future analysis of how Q-exposure acts at the neuronal and molecular level on the mechanisms involved in enhancing memory formation, storage, and recall, paving the way for interesting translational neuroscience studies (Tascedda *et al.* 2015).

Finally, this study illustrates that extrinsic factors significantly influence memory formation. In the contemporary scenario where most of the attention in neuroscience is focused on intrinsic factors such as differential gene expression, cellular dynamics, and molecular biochemistry to explain frontier cognitive phenomena, the conversion of ITM to LTM induced by an extrinsic dietary flavonoid such as Q has been highlighted the role that external factors simultaneously play with intrinsic factors to shape complex cognitive processes. The unique combination of the operant conditioning paradigm coupled with natural memory enhancing bioactive compound and the elegant nervous system of *L. stagnalis* has come together to reiterate the importance of experience-dependant changes that play a role in learning and memory.

Future perspectives

The results presented in this thesis opened many questions and demonstrated that *L. stagnalis* can be used as a 'multi-tasking' model for translational neuroscience (**Figure 45**).

The bioinformatic-molecular approach developed to characterize *L. stagnalis*' genome and transcriptome will be followed by the search for specific functioning antibodies in snails to assess, by Western blotting, the translation of genes demonstrated to be expressed in the nervous tissue of *L. stagnalis*. Among these genes, there will be those that in mammals are involved in learning, memory, stress response, and inflammation, expanding the results presented in this thesis. The results of these studies will be paired with immunocytochemical analysis to localize at the cellular level in the ganglion of *L. stagnalis* the proteins demonstrated to be synthesized. Moreover, the different molecular pathways will be analysed by high-performance liquid chromatography coupled to a high-resolution mass/mass spectrometer (HPLC-ESI-HRMS/MS) in the nerve tissue of snails. This technique combines the separation power of HPLC of high molecular weight metabolites with the ability of the mass spectrometer to identify and confirm the identity of the molecules under investigation.

Moreover, there will be performed studies of pharmacological regulation of different pathways involved in neuroplasticity, inflammation, and stress, treating the snails with compounds (i.e., quercetin), or pharmacological tools (i.e., LPS) and search for a significant molecular effect between the treated animals compared to controls through expression studies by qRT-PCR and analysis of protein levels by Western blotting.

The Garcia effect procedure developed in this study represents an ideal learning paradigm for studying the behavioural and molecular basis of learning and memory as a single trial is sufficient for a robust and long-lasting memory to occur. Using this conditioning protocol, it will be possible to search for a correlation between molecular effect and behaviour. Finally, it will be possible to turn off, through the technique of CRISPR/Cas9, the key pathway genes found to be most sensitive to drug treatment (estimated based on correlation studies) and/or that from the data in the literature is essential in the functionality of the pathway itself, in turn, implicated in diseases with cognitive impairments.

The CRISPR/Cas9 genome editing method has recently been applied in *L. stagnalis* to knock out the gene responsible for coiling direction during development (Abe and Kuroda 2019), opening significant opportunities for functional genomics to investigate the role of the genes characterized.

The last step will be the evaluation of the phenotypic, molecular, and behavioural consequences of switching off the selected genes, to search for causal relationships between genetic alterations of the pathway, molecular changes and function at the central level and behaviour.

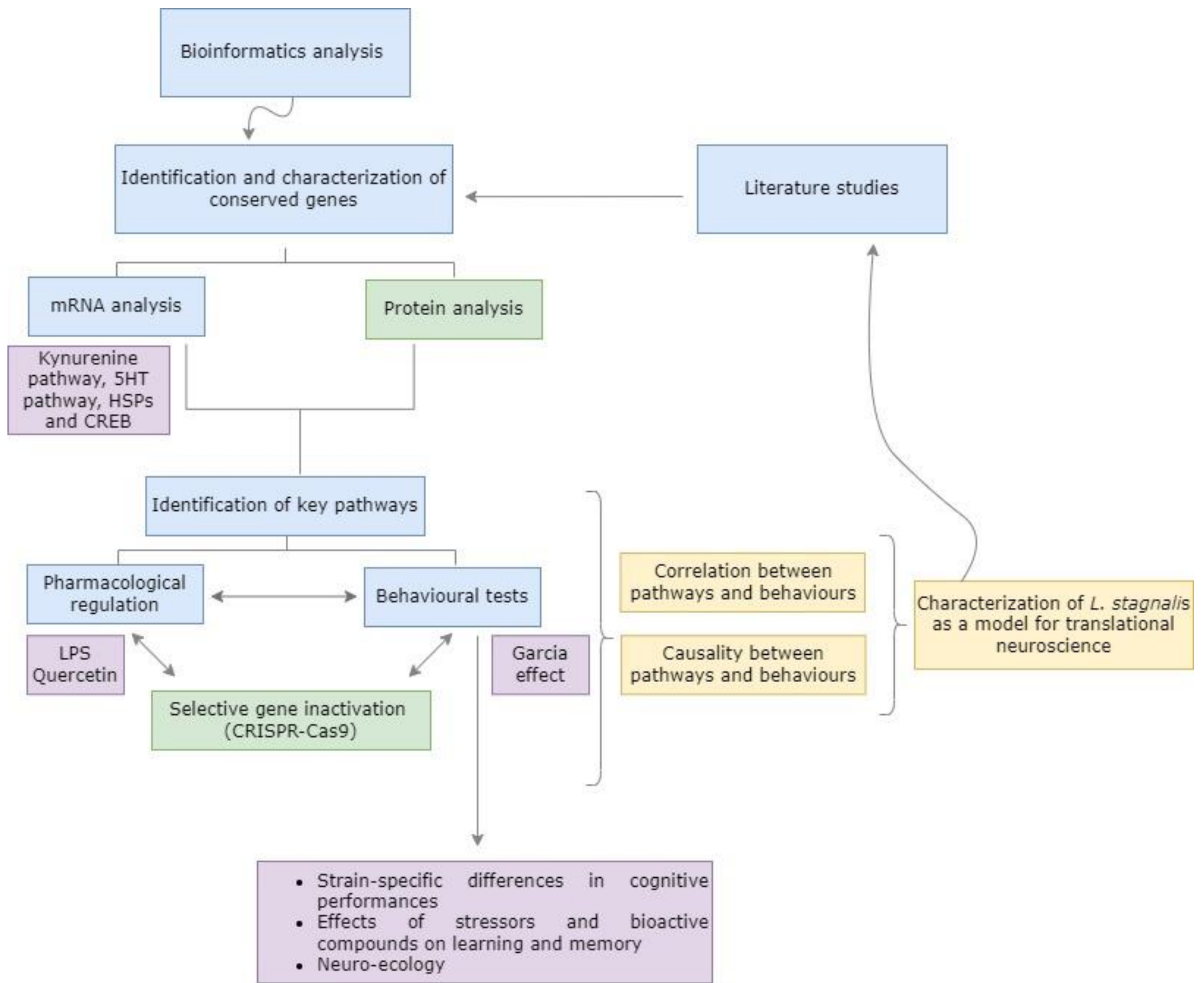


Figure 45. Schematic representation of the current and future studies using *L. stagnalis* as the model organism of choice for translational neuroscience's purposes. Boxes in blue indicate the aims reached in this thesis. Into the purple boxes are indicated the procedures adopted, the drugs administrated, and the targets studied in this project. Green boxes indicate the future studies that will be performed, and the yellow ones are the final steps required to translate data obtained in *L. stagnalis* in rodent models.

List of Abbreviations

5HT	Serotonin
5HTT	Serotonin Transporter
ACMSD	2-Amino 3-Carboxymuconate 6-Semialdehyde Decarboxylase
AFMID	Kynurenine Formamidase
C	Carrot slurry
C/EBPs	CCAAT-Enhancer-Binding Proteins
CaMKII	Ca2+/Calmodulin-Dependent Protein Kinase II
CGC	Cerebral Giant Cell
CREB	cAMP-Response Element-Binding Proteins
CS	Conditional Stimulus
CNS	Central Nervous System
CTA	Conditioned Taste Aversion
DMSO	Dimethyl Sulfoxide
Elongation factor 1α	Ef1α
GO	Gene Ontology
HAAO	Hydroxyanthranilate 3,4-Dioxygenase
Heat-shock protein	Hsp
HS	Heat Shock Stressor
IDO	Indoleamine 2,3 Dioxygenase
IGV	Integrative Genomic Browser
ISI	Inter-Stimulus Interval
ITM	Intermediate-Term Memory
KMO	Kynurenine 3-Monooxygenase
KP	Kynurenine Pathway
KYAT and AADAT	Kynurenine Aminotransferases
KYNU	Kynureninase

L	Lettuce slurry
LPS	Lipopolysaccharide
LTM	Long-Term Memory
MAPK	Mitogen-Activated Protein Kinases
MT	Memory Test
NAD+	Nicotinamide Adenine Dinucleotide
NMDA	N-Methyl-D-Aspartate
NO/cGMP	Nitric Oxide/Cyclic GMP
ORF	Open Reading Frame
PKA	cAMP-Protein Kinase A
PW	Pond Water
Q	Quercetin
RPeD1	Right Pedal Dorsal 1
RT	Room-Temperature
Sal	Snail Saline
STM	Short-Term Memory
TBT	Total Breathing Time
TDO	Tryptophan 2,3-Dioxygenase
TPH	Tryptophan Hydroxylase
TS	Training Session
TSA	Transcriptome Shotgun Assembly
US	Unconditional Stimulus
V	Vehicle - DMSO
b TUB	β-tubulin

References

- Abe M, Kuroda R (2019) The development of CRISPR for a mollusc establishes the formin Lsdia1 as the long-sought gene for snail dextral/sinistral coiling. Development 146:dev175976. https://doi.org/10.1242/dev.175976
- Ahmad S, Ullah F, Sadiq A, et al (2016) Chemical composition, antioxidant and anticholinesterase potentials of essential oil of Rumex hastatus D. Don collected from the North West of Pakistan. BMC Complement Altern Med 16:29. https://doi.org/10.1186/s12906-016-0998-z
- Alberini CM (2009) Transcription factors in long-term memory and synaptic plasticity. Physiol Rev 89:. https://doi.org/10.1152/physrev.00017.2008
- Albuquerque EX, Schwarcz R (2013) Kynurenic acid as an antagonist of α7 nicotinic acetylcholine receptors in the brain: Facts and challenges. Biochemical Pharmacology 85:1027–1032. https://doi.org/10.1016/j.bcp.2012.12.014
- Allen AP, Naughton M, Dowling J, et al (2018) Kynurenine pathway metabolism and the neurobiology of treatment-resistant depression: Comparison of multiple ketamine infusions and electroconvulsive therapy. Journal of Psychiatric Research 100:24–32. https://doi.org/10.1016/j.jpsychires.2018.02.011
- Allison DJ, Ditor DS (2014) The common inflammatory etiology of depression and cognitive impairment: a therapeutic target. J Neuroinflammation 11:151. https://doi.org/10.1186/s12974-014-0151-1
- Ambrosini MV, Mariucci G, Tantucci M, et al (2005) Hippocampal 72-kDa heat shock protein expression varies according to mice learning performance independently from chronic exposure to stress. Hippocampus 15:413–417. https://doi.org/10.1002/hipo.20069
- Andrews EA, Braveman NS (1975) The combined effects of dosage level and interstimulus interval on the formation of one-trial poison-based aversions in rats. Animal Learning & Behavior 3:287–289. https://doi.org/10.3758/BF03213446
- Angilletta Jr. MJ (2009) Thermal adaptation: a theoretical and empirical synthesis. Oxford University Press, Oxford
- Ansari MA, Abdul HM, Joshi G, et al (2009) Protective effect of quercetin in primary neurons against Abeta(1-42): relevance to Alzheimer's disease. J Nutr Biochem 20:269–275. https://doi.org/10.1016/j.jnutbio.2008.03.002
- Aonuma H, Kaneda M, Hatakeyama D, et al (2016) Relationship between the grades of a learned aversive-feeding response and the dopamine contents in Lymnaea. Biology Open 5:1869–1873. https://doi.org/10.1242/bio.021634
- Arif H, Aggarwal S (2021) Salicylic acid (Aspirin). In: StatPearls. StatPearls Publishing, Treasure Island (FL)
- Ayaz M, Sadiq A, Junaid M, et al (2019) Flavonoids as prospective neuroprotectants and their therapeutic propensity in aging associated neurological disorders. Front Aging Neurosci 11:. https://doi.org/10.3389/fnagi.2019.00155
- Babaei F, Mirzababaei M, Nassiri-Asl M (2018) Quercetin in food: possible mechanisms of its effect on memory. Journal of Food Science 83:2280–2287. https://doi.org/10.1111/1750-3841.14317
- Badawy AA-B, Namboodiri AMA, Moffett JR (2016) The end of the road for the tryptophan depletion concept in pregnancy and infection. Clinical Science 130:1327–1333. https://doi.org/10.1042/CS20160153
- Bakoyiannis I, Daskalopoulou A, Pergialiotis V, Perrea D (2019) Phytochemicals and cognitive health: Are flavonoids doing the trick? Biomedicine & Pharmacotherapy 109:1488–1497. https://doi.org/10.1016/j.biopha.2018.10.086
- Baranyi A, Meinitzer A, Breitenecker RJ, et al (2015) Quinolinic acid responses during interferon-α-induced depressive symptomatology in patients with chronic hepatitis c infection - a novel aspect for depression and inflammatory hypothesis. PLoS ONE 10:e0137022. https://doi.org/10.1371/journal.pone.0137022
- Barco A, Pittenger C, Kandel ER (2003) CREB, memory enhancement and the treatment of memory disorders: promises, pitfalls and prospects. Expert Opin Ther Targets 7:101–114. https://doi.org/10.1517/14728222.7.1.101
- Barton BT (2010) Climate warming and predation risk during herbivore ontogeny. Ecology 91:2811–2818. https://doi.org/10.1890/09-2278.1
- Bassi GS, Kanashiro A, Santin FM, et al (2012) Lipopolysaccharide-induced sickness behaviour evaluated in different models of anxiety and innate fear in rats. Basic Clin Pharmacol Toxicol 110:359–369. https://doi.org/10.1111/j.1742-7843.2011.00824.x

- Batabyal A, Lukowiak K (2021) Configural learning memory can be transformed from intermediate-term to long-term in pond snail Lymnaea stagnalis. Physiol Behav 239:113509. https://doi.org/10.1016/j.physbeh.2021.113509
- Becker JA, Stewart LK (2011) Heat-related illness. AFP 83:1325–1330
- Benjamin PR (2008) Lymnaea. Scholarpedia 3:4124. https://doi.org/10.4249/scholarpedia.4124
- Benjamin PR, Kemenes I (2013) *Lymnaea* neuropeptide genes. Scholarpedia 8:11520. https://doi.org/10.4249/scholarpedia.11520
- Bermúdez-Rattoni F (2004) Molecular mechanisms of taste-recognition memory. Nat Rev Neurosci 5:209–217. https://doi.org/10.1038/nrn1344
- Biesmans S, Meert TF, Bouwknecht JA, et al (2013) Systemic immune activation leads to neuroinflammation and sickness behavior in mice. Mediators Inflamm 2013:271359. https://doi.org/10.1155/2013/271359
- Bisaz R, Travaglia A, Alberini CM (2014) The neurobiological bases of memory formation: from physiological conditions to psychopathology. Psychopathology 47:347–356. https://doi.org/10.1159/000363702
- Blom JMC, Ottaviani E (2017) Immune-neuroendocrine interactions: evolution, ecology, and susceptibility to illness. Med Sci Monit Basic Res 23:362–367. https://doi.org/10.12659/msmbr.907637
- Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30(15):2114-2120. https://doi:10.1093/bioinformatics/btu170
- Bozon B, Kelly Á, Josselyn SA, et al (2003) MAPK, CREB and zif268 are all required for the consolidation of recognition memory. Phil Trans R Soc Lond B 358:805–814. https://doi.org/10.1098/rstb.2002.1224
- Braidy N, Grant R (2017) Kynurenine pathway metabolism and neuroinflammatory disease. Neural Regen Res 12:39. https://doi.org/10.4103/1673-5374.198971
- Brown, RE (2020). Donald O. Hebb and the Organization of Behavior: 17 years in the writing. Mol Brain 13, 55. doi:10.1186/s13041-020-00567-8.
- Castro-Portuguez R, Sutphin GL (2020) Kynurenine pathway, NAD+ synthesis, and mitochondrial function: Targeting tryptophan metabolism to promote longevity and health span. Experimental Gerontology 132:110841. https://doi.org/10.1016/j.exger.2020.110841
- Cushnie TPT, Lamb AJ (2005) Antimicrobial activity of flavonoids. International Journal of Antimicrobial Agents 26:343–356. https://doi.org/10.1016/j.ijantimicag.2005.09.002
- Dantzer R (2009) Cytokine, sickness behavior, and depression. Immunol Allergy Clin North Am 29:247–264. https://doi.org/10.1016/j.iac.2009.02.002
- Dantzer R (2016) Role of the kynurenine metabolism pathway in inflammation-induced depression: preclinical approaches. In: Dantzer R, Capuron L (eds) Inflammation-associated depression: evidence, mechanisms and implications. Springer International Publishing, Cham, pp 117–138
- Darlington LG, Forrest CM, Mackay GM, et al (2010) On the biological importance of the 3-hydroxyanthranilic acid: anthranilic acid ratio. Int J Tryptophan Res 3:51–59
- Davey VA, Biederman GB (1996) Conditioned antisickness: Heat as an internal stimulus in conditioning taste aversion and aversion failure in rats. Journal of Experimental Psychology: Animal Behavior Processes 22:235–243. https://doi.org/10.1037/0097-7403.22.3.235
- Domjan M, Bowman TG (1974) Learned safety and the CS-US delay gradient in taste-aversion learning. Learning and Motivation 5:409–423. https://doi.org/10.1016/0023-9690(74)90001-0
- Dong Y, Dong S (2008) Induced thermotolerance and expression of heat shock protein 70 in sea cucumber Apostichopus japonicus. Fish Sci 74:573–578. https://doi.org/10.1111/j.1444-2906.2008.01560.x
- Eggler AL, Gay KA, Mesecar AD (2008) Molecular mechanisms of natural products in chemoprevention: Induction of cytoprotective enzymes by Nrf2. Mol Nutr Food Res. https://doi.org/10.1002/mnfr.200700249
- Fangue NA, Hofmeister M, Schulte PM (2006) Intraspecific variation in thermal tolerance and heat shock protein gene expression in common killifish, Fundulus heteroclitus. J Exp Biol 209:2859–2872. https://doi.org/10.1242/jeb.02260
- El-Gebali S, Mistry J, Bateman A, et al (2019) The Pfam protein families database in 2019. Nucleic Acids Research 47:D427–D432. https://doi.org/10.1093/nar/gky995
- Feder ME, Hofmann GE (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu Rev Physiol 61:243–282. https://doi.org/10.1146/annurev.physiol.61.1.243
- Forsman A (2015) Rethinking phenotypic plasticity and its consequences for individuals, populations, and species. Heredity 115:276–284. https://doi.org/10.1038/hdy.2014.92

- Foster A (1983) On the excitotoxic properties of quinolinic acid, 2,3-piperidine dicarboxylic acids and structurally related compounds. Neuropharmacology 22:1331–1342. https://doi.org/10.1016/0028-3908(83)90221-6
- Foster NL, Lukowiak K, Henry TB (2015) Time-related expression profiles for heat shock protein gene transcripts (HSP40, HSP70) in the central nervous system of Lymnaea stagnalis exposed to thermal stress. Commun Integr Biol 8:. https://doi.org/10.1080/19420889.2015.1040954
- Fruson L, Dalesman S, Lukowiak K (2012) A flavonol present in cocoa [(-)epicatechin] enhances snail memory. Journal of Experimental Biology 215:3566–3576. https://doi.org/10.1242/jeb.070300
- Fujigaki H, Mouri A, Yamamoto Y, et al (2019) Linking phencyclidine intoxication to the tryptophan-kynurenine pathway: Therapeutic implications for schizophrenia. Neurochemistry International 125:1–6. https://doi.org/10.1016/j.neuint.2019.02.001
- Ganong AH, Cotman CW (1986) Kynurenic acid and quinolinic acid act at N-methyl-D-aspartate receptors in the rat hippocampus. J Pharmacol Exp Ther 236:293–299
- Garcia J, Ervin FR, Koelling RA (1966) Learning with prolonged delay of reinforcement. Psychon Sci 5:121–122. https://doi.org/10.3758/BF03328311
- Garcia J, Ervin FR, Yorke CH, Koelling RA (1967) Conditioning with delayed vitamin injections. Science 155:716–718. https://doi.org/10.1126/science.155.3763.716
- Garcia J, Hankins WG, Rusiniak KW (1974) Behavioral regulation of the milieu interne in man and rat. Science 185:824–831. https://doi.org/10.1126/science.185.4154.824
- Garcia J, Kimeldorf DJ, Koelling RA (1955) Conditioned aversion to saccharin resulting from exposure to gamma radiation. Science 122:157–158. https://doi.org/10.1126/science.122.3160.157
- Garrison AM, Parrott JM, Tuñon A, et al (2018) Kynurenine pathway metabolic balance influences microglia activity: Targeting kynurenine monooxygenase to dampen neuroinflammation. Psychoneuroendocrinology 94:1–10. https://doi.org/10.1016/j.psyneuen.2018.04.019
- Geraerts WPM, Smit AB, Li KW, Hordijk PL (1992) The Light Green Cells of Lymnaea: A neuroendocrine model system for stimulus-induced expression of multiple peptide genes in a single cell type. Experientia 48:464–473. https://doi.org/10.1007/BF01928165
- Golia MT, Poggini S, Alboni S, et al (2019) Interplay between inflammation and neural plasticity: Both immune activation and suppression impair LTP and BDNF expression. Brain Behav Immun 81:484–494. https://doi.org/10.1016/j.bbi.2019.07.003
- Guillemin GJ (2012) Quinolinic acid, the inescapable neurotoxin: Quinolinic acid, the inescapable neurotoxin. FEBS Journal 279:1356–1365. https://doi.org/10.1111/j.1742-4658.2012.08485.x
- Haleagrahara N, Siew CJ, Mitra NK, Kumari M (2011) Neuroprotective effect of bioflavonoid quercetin in 6-hydroxydopamine-induced oxidative stress biomarkers in the rat striatum. Neurosci Lett 500:139–143. https://doi.org/10.1016/j.neulet.2011.06.021
- Han Q, Cai T, Tagle DA, Li J (2010) Structure, expression, and function of kynurenine aminotransferases in human and rodent brains. Cell Mol Life Sci 67:353–368. https://doi.org/10.1007/s00018-009-0166-4
- Hoffmann AA, Sørensen JG, Loeschcke V (2003) Adaptation of Drosophila to temperature extremes: bringing together quantitative and molecular approaches. Journal of Thermal Biology 28:175–216. https://doi.org/10.1016/S0306-4565(02)00057-8
- Hosokawa N, Hirayoshi K, Nakai A, et al (1990) Flavonoids inhibit the expression of heat shock proteins. Cell Structure and Function 15:393–401. https://doi.org/10.1247/csf.15.393
- Hosokawa N, Hirayoshi K, Nakai A, et al (1990) Flavonoids inhibit the expression of heat shock proteins. Cell Struct Funct 15:393–401. https://doi.org/10.1247/csf.15.393
- Hughes E, Shymansky T, Swinton E, et al (2017) Strain-specific differences of the effects of stress on memory in Lymnaea. Journal of Experimental Biology 220:891–899. https://doi.org/10.1242/jeb.149161
- Hung C-W, Chen Y-C, Hsieh W-L, et al (2010) Ageing and neurodegenerative diseases. Ageing Research Reviews 9:S36–S46. https://doi.org/10.1016/j.arr.2010.08.006
- Igaz LM, Bekinschtein P, Izquierdo I, Medina JH (2004) One-trial aversive learning induces late changes in hippocampal CaMKIIα, Homer 1a, Syntaxin 1a and ERK2 protein levels. Molecular Brain Research 132:1–12. https://doi.org/10.1016/j.molbrainres.2004.08.016
- Il-Han J, Janes T, Lukowiak K (2010) The role of serotonin in the enhancement of long-term memory resulting from predator detection in Lymnaea. Journal of Experimental Biology 213:3603–3614. https://doi.org/10.1242/jeb.048256
- Ito E, Totani Y, Oike A (2017) Necessity knows no law in a snail. The European Zoological Journal 84:457–464. https://doi.org/10.1080/24750263.2017.1363303

- Itoh A, Komatsuzaki Y, Lukowiak K, Saito M (2021) Epicatechin increases the persistence of long-term memory formed by conditioned taste aversion in Lymnaea. J Exp Biol 224:jeb238055. https://doi.org/10.1242/jeb.238055
- Jaronen M, Quintana FJ (2014) Immunological relevance of the coevolution of IDO1 and AHR. Frontiers in Immunology 5:521
- Jayasena T, Poljak A, Smythe G, et al (2013) The role of polyphenols in the modulation of sirtuins and other pathways involved in Alzheimer's disease. Ageing Research Reviews 12:867–883. https://doi.org/10.1016/j.arr.2013.06.003
- Kandel ER, Dudai Y, Mayford MR (2014) The molecular and systems biology of memory. Cell 157:163–186. https://doi.org/10.1016/j.cell.2014.03.001
- Karnik V, Dalesman S, Lukowiak K (2012) Input from a chemosensory organ, the osphradium, does not mediate aerial respiration in Lymnaea stagnalis. Aquat Biol 15:167–173. https://doi.org/10.3354/ab00416
- Kemenes G, Benjamin PR (1989) Appetitive learning in snails shows characteristics of conditioning in vertebrates. Brain Res 489:163–166. https://doi.org/10.1016/0006-8993(89)90019-x
- Khan MTH, Orhan I, Şenol FS, et al (2009) Cholinesterase inhibitory activities of some flavonoid derivatives and chosen xanthone and their molecular docking studies. Chemico-Biological Interactions 181:383–389. https://doi.org/10.1016/j.cbi.2009.06.024
- Knezevic B, Lukowiak K (2014) The flavonol epicatechin reverses the suppressive effects of a stressor on long-term memory formation. Journal of Experimental Biology 217:4004–4009. https://doi.org/10.1242/jeb.110726
- Koert CE, Spencer GE, van Minnen J, et al (2001) Functional implications of neurotransmitter expression during axonal regeneration: serotonin, but not peptides, auto-regulate axon growth of an identified central neuron. J Neurosci 21:5597–5606
- Kojima S, Nanakamura H, Nagayama S, et al (1997) Enhancement of an inhibitory input to the feeding central pattern generator in Lymnaea stagnalis during conditioned taste-aversion learning. Neuroscience Letters 230:179–182. https://doi.org/10.1016/S0304-3940(97)00507-7
- Kojima S, Sunada H, Mita K, et al (2015) Function of insulin in snail brain in associative learning. J Comp Physiol A 201:969–981. https://doi.org/10.1007/s00359-015-1032-5
- Kojima S, Yamanaka M, Fujito Y, Ito E (1996) Differential Neuroethological Effects of Aversive and Appetitive Reinforcing Stimuli on Associative Learning in Lymnaea stagnalis. Zoological Science 13:803–812. https://doi.org/10.2108/zsj.13.803
- Kumar S, Stecher G, Li M, et al (2018) MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol Biol Evol 35:1547–1549. https://doi.org/10.1093/molbev/msy096
- Kuo ESL, Sanford E (2009) Geographic variation in the upper thermal limits of an intertidal snail: implications for climate envelope models. Marine Ecology Progress Series 388:137–146. https://doi.org/10.3354/meps08102
- Lackie RE, Maciejewski A, Ostapchenko VG, et al (2017) The Hsp70/Hsp90 chaperone machinery in neurodegenerative diseases. Front Neurosci 11:254. https://doi.org/10.3389/fnins.2017.00254
- Lapin IP, Mutovkina LG, Ryzov IV, Mirzaev S (1996) Anxiogenic activity of quinolinic acid and kynurenine in the social interaction test in mice. J Psychopharmacol 10:246–249. https://doi.org/10.1177/026988119601000312
- Letenneur L, Proust-Lima C, Le Gouge A, et al (2007) Flavonoid Intake and Cognitive Decline over a 10-Year Period. American Journal of Epidemiology 165:1364–1371. https://doi.org/10.1093/aje/kwm036
- Lindquist S, Craig EA (1988) The Heat-Shock Proteins. Annu Rev Genet 22:631–677. https://doi.org/10.1146/annurev.ge.22.120188.003215
- Logue AW, Ophir I, Strauss KE (1981) The acquisition of taste aversions in humans. Behaviour Research and Therapy 19:319–333. https://doi.org/10.1016/0005-7967(81)90053-X
- Lovelace MD, Varney B, Sundaram G, et al (2017) Recent evidence for an expanded role of the kynurenine pathway of tryptophan metabolism in neurological diseases. Neuropharmacology 112:373–388. https://doi.org/10.1016/j.neuropharm.2016.03.024
- Lukowiak K, Ringseis E, Spencer G, et al (1996) Operant conditioning of aerial respiratory behaviour in Lymnaea stagnalis. J Exp Biol 199:683–691
- Macready AL, Kennedy OB, Ellis JA, et al (2009) Flavonoids and cognitive function: a review of human randomized controlled trial studies and recommendations for future studies. Genes Nutr 4:227–242. https://doi.org/10.1007/s12263-009-0135-4
- Manach C, Scalbert A, Morand C, et al (2004) Polyphenols: food sources and bioavailability. The American Journal of Clinical Nutrition 79:727–747. https://doi.org/10.1093/ajcn/79.5.727
- Marszalek-Grabska M, Walczak K, Gawel K, et al (2021) Kynurenine emerges from the shadows – Current knowledge on its fate and function. Pharmacology & Therapeutics 225:107845. https://doi.org/10.1016/j.pharmthera.2021.107845

- Mastroiacovo D, Kwik-Uribe C, Grassi D, et al (2015) Cocoa flavanol consumption improves cognitive function, blood pressure control, and metabolic profile in elderly subjects: the Cocoa, Cognition, and Aging (CoCoA) Study—a randomized controlled trial. The American Journal of Clinical Nutrition 101:538–548. https://doi.org/10.3945/ajcn.114.092189
- McDonald SC (1973) Activity patterns of Lymnaea stagnalis (L.) in relation to temperature conditions: a preliminary study. Malacologia 14:395–396
- McKenzie S, Eichenbaum H (2011) Consolidation and reconsolidation: Two lives of memories? Neuron 71:224–233. https://doi.org/10.1016/j.neuron.2011.06.037
- Morimoto RI (1993) Cells in stress: transcriptional activation of heat shock genes. Science 259:1409–1410. https://doi.org/10.1126/science.8451637
- Muneer A (2020) Kynurenine pathway of tryptophan metabolism in neuropsychiatric disorders: pathophysiologic and therapeutic considerations. Clin Psychopharmacol Neurosci 18:507–526. https://doi.org/10.9758/cpn.2020.18.4.507
- Murakami J, Okada R, Fujito Y, et al (2013) Paired pulse ratio analysis of insulin-induced synaptic plasticity in the snail brain. Journal of Experimental Biology 216:1771–1773. https://doi.org/10.1242/jeb.083469
- Nachman M, Ashe JH (1973) Learned taste aversions in rats as a function of dosage, concentration, and route of administration of LiCl. Physiology & Behavior 10:73–78. https://doi.org/10.1016/0031-9384(73)90089-9
- Nader K (2003) Memory traces unbound. Trends in Neurosciences 26:65–72. https://doi.org/10.1016/S0166-2236(02)00042-5
- Nader K, Einarsson EÖ (2010) Memory reconsolidation: an update. Annals of the New York Academy of Sciences 1191:27–41. https://doi.org/10.1111/j.1749-6632.2010.05443.x
- Narum SR, Campbell NR, Meyer KA, et al (2013) Thermal adaptation and acclimation of ectotherms from differing aquatic climates. Molecular Ecology 22:3090–3097. https://doi.org/10.1111/mec.12240
- Nikitin ES, Vavoulis DV, Kemenes I, et al (2008) Persistent sodium current is a nonsynaptic substrate for long-term associative memory. Current Biology 18:1221–1226. https://doi.org/10.1016/j.cub.2008.07.030
- Nishi M, Yoshikawa K, Higashijima J, et al (2018) The impact of indoleamine 2,3-dioxygenase (IDO) expression on stage III gastric cancer. Anticancer Res 38:3387–3392. https://doi.org/10.21873/anticanres.12605
- Orr M, Hittel K, Lukowiak KS, et al (2009) Differences in LTM-forming capability between geographically different strains of Alberta Lymnaea stagnalis are maintained whether they are trained in the lab or in the wild. Journal of Experimental Biology 212:3911–3918. https://doi.org/10.1242/jeb.024281
- Parrott JM, Redus L, Santana-Coelho D, et al (2016) Neurotoxic kynurenine metabolism is increased in the dorsal hippocampus and drives distinct depressive behaviors during inflammation. Transl Psychiatry 6:e918–e918. https://doi.org/10.1038/tp.2016.200
- Parvez K (2005) Boosting intermediate-term into long-term memory. Journal of Experimental Biology 208:1525–1536. https://doi.org/10.1242/jeb.01545
- Parvez K, Moisseev V, Lukowiak K (2006) A context-specific single contingent-reinforcing stimulus boosts intermediate-term memory into long-term memory. European Journal of Neuroscience 24:606–616. https://doi.org/10.1111/j.1460-9568.2006.04952.x
- Parvez K, Moisseev V, Lukowiak K (2006) A context-specific single contingent-reinforcing stimulus boosts intermediate-term memory into long-term memory. European Journal of Neuroscience 24:606–616. https://doi.org/10.1111/j.1460-9568.2006.04952.x
- Pfefferkorn ER (1984) Interferon gamma blocks the growth of Toxoplasma gondii in human fibroblasts by inducing the host cells to degrade tryptophan. Proc Natl Acad Sci U S A 81:908–912. https://doi.org/10.1073/pnas.81.3.908
- Pizarro JM, Haro LS, Barea-Rodriguez EJ (2003) Learning associated increase in heat shock cognate 70 mRNA and protein expression. Neurobiology of Learning and Memory 79:142–151. https://doi.org/10.1016/S1074-7427(02)00008-4
- Porto RR, Dutra FD, Crestani AP, et al (2018) HSP70 facilitates memory consolidation of fear conditioning through MAPK pathway in the Hippocampus. Neuroscience 375:108–118. https://doi.org/10.1016/j.neuroscience.2018.01.028
- Qin L, Wu X, Block ML, et al (2007) Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. Glia 55:453–462. https://doi.org/10.1002/glia.20467
- Ribeiro MJ (2005) Activation of MAPK is necessary for long-term memory consolidation following food-reward conditioning. Learning & Memory 12:538–545. https://doi.org/10.1101/lm.8305
- Ribeiro MJ, Serfozo Z, Papp A, et al (2003) Cyclic AMP response element-binding (CREB)-like proteins in a molluscan brain: cellular localization and learning-induced phosphorylation. Eur J Neurosci 18:1223–1234. https://doi.org/10.1046/j.1460-9568.2003.02856.x

- Rivi V, Benatti C, Colliva C, et al (2020) Lymnaea stagnalis as model for translational neuroscience research: From pond to bench. Neuroscience & Biobehavioral Reviews 108:602–616. https://doi.org/10.1016/j.neubiorev.2019.11.020
- Robak J, Gryglewski RJ (1988) Flavonoids are scavengers of superoxide anions. Biochemical Pharmacology 37:837–841. https://doi.org/10.1016/0006-2952(88)90169-4
- Rosadini CV, Kagan JC (2017) Early innate immune responses to bacterial LPS. Curr Opin Immunol 44:14–19. https://doi.org/10.1016/j.coi.2016.10.005
- Rossi F, Miggiano R, Ferraris DM, Rizzi M (2019) The Synthesis of Kynurenic Acid in Mammals: An Updated Kynurenine Aminotransferase Structural KATalogue. Front Mol Biosci 6:7. https://doi.org/10.3389/fmolb.2019.00007
- Rzóska J (1953) Bait shyness, a study in rat behaviour. The British Journal of Animal Behaviour 1:128–135. https://doi.org/10.1016/S0950-5601(53)80011-0
- Sadamoto H (2010) Learning-dependent gene expression of CREB1 isoforms in the molluscan brain. Front Behav Neurosci 4:. https://doi.org/10.3389/fnbeh.2010.00025
- Sadamoto H, Sato H, Kobayashi S, et al (2004) CREB in the pond snail Lymnaea stagnalis: cloning, gene expression and function in identifiable neurons of the central nervous system. Journal of neurobiology 58:455–66. https://doi.org/10.1002/neu.10296
- Sadamoto H, Takahashi H, Okada T, et al (2012) De novo sequencing and transcriptome analysis of the central nervous system of mollusc Lymnaea stagnalis by deep RNA sequencing. PLoS ONE 7:e42546. https://doi.org/10.1371/journal.pone.0042546
- Salo T, Stamm C, Burdon FJ, et al (2017) Resilience to heat waves in the aquatic snail Lymnaea stagnalis: Additive and interactive effects with micropollutants. Freshwater Biology 62:1831–1846. https://doi.org/10.1111/fwb.12999
- Sangha S (2003) Intermediate and long-term memories of associative learning are differentially affected by transcription versus translation blockers inLymnaea. Journal of Experimental Biology 206:1605–1613. https://doi.org/10.1242/jeb.00301
- Savitz J (2020) The kynurenine pathway: a finger in every pie. Mol Psychiatry 25:131–147. https://doi.org/10.1038/s41380-019-0414-4
- Savvateeva E, Popov A, Kamyshev N, et al (2000) Age-dependent memory loss, synaptic pathology and altered brain plasticity in the Drosophila mutant cardinal accumulating 3-hydroxykynurenine. J Neural Transm (Vienna) 107:581–601. https://doi.org/10.1007/s007020070080
- Schwarcz R, Stone TW (2017) The kynurenine pathway and the brain: Challenges, controversies and promises. Neuropharmacology 112:237–247. https://doi.org/10.1016/j.neuropharm.2016.08.003
- Schwarcz R, Whetsell WO, Mangano RM (1983) Quinolinic acid: an endogenous metabolite that produces axon-sparing lesions in rat brain. Science 219:316–318. https://doi.org/10.1126/science.6849138
- Seligman ME (1970) On the generality of the laws of learning. Psychological Review 77:406–418. https://doi.org/10.1037/h0029790
- Socci V, Tempesta D, Desideri G, et al (2017) Enhancing human cognition with cocoa flavonoids. Front Nutr 4:19. https://doi.org/10.3389/fnut.2017.00019
- Somero GN (2010) The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine 'winners' and 'losers.' Journal of Experimental Biology 213:912–920. https://doi.org/10.1242/jeb.037473
- Song H-M, Mu X-D, Gu D-E, et al (2014) Molecular characteristics of the HSP70 gene and its differential expression in female and male golden apple snails (Pomacea canaliculata) under temperature stimulation. Cell Stress Chaperones 19:579–589. https://doi.org/10.1007/s12192-013-0485-0
- Sørensen JG, Kristensen TN, Loeschcke V (2003) The evolutionary and ecological role of heat shock proteins. Ecology Letters 6:1025–1037. https://doi.org/10.1046/j.1461-0248.2003.00528.x
- Spencer JPE (2007) The interactions of flavonoids within neuronal signalling pathways. Genes Nutr 2:257–273. https://doi.org/10.1007/s12263-007-0056-z
- Squire LR, Genzel L, Wixted JT, Morris RG (2015) Memory consolidation. Cold Spring Harb Perspect Biol 7:. https://doi.org/10.1101/cshperspect.a021766
- Stensmyr MC, Caron SJC (2020) Neuroscience: The secret of sauce Béarnaise syndrome is in the circuit. Current Biology 30:R1413–R1415. https://doi.org/10.1016/j.cub.2020.09.085
- Stevens CF (1994) CREB and memory consolidation. Neuron 13:769–770. https://doi.org/10.1016/0896-6273(94)90244-5
- Storch D, Santelices P, Barria J, et al (2009) Thermal tolerance of crustacean larvae (zoea I) in two different populations of the kelp crab Taliepus dentatus (Milne-Edwards). J Exp Biol 212:1371–1376. https://doi.org/10.1242/jeb.030205

- Storniolo A, Raciti M, Cucina A, et al (2015) Quercetin affects Hsp70/IRE1α mediated protection from death induced by endoplasmic reticulum stress. Oxid Med Cell Longev 2015:. https://doi.org/10.1155/2015/645157
- Sugai R (2006) Taste discrimination in conditioned taste aversion of the pond snail Lymnaea stagnalis. Journal of Experimental Biology 209:826–833. https://doi.org/10.1242/jeb.02069
- Sunada H, Riaz H, Freitas E de, et al (2016) Heat stress enhances LTM formation in Lymnaea: role of HSPs and DNA methylation. Journal of Experimental Biology 219:1337–1345. https://doi.org/10.1242/jeb.134296
- Swinton E, de Freitas E, Swinton C, et al (2018) Green tea and cocoa enhance cognition in Lymnaea. Communicative & Integrative Biology 11:e1434390. https://doi.org/10.1080/19420889.2018.1434390
- Swinton E, Shymansky T, Swinton C, Lukowiak K (2020) Stress before training alters memory retrieval of a non-declarative memory in Lymnaea. Journal of Experimental Biology jeb.223727. https://doi.org/10.1242/jeb.223727
- Tascedda F, Malagoli D, Accorsi A, et al (2015) Molluscs as models for translational medicine. Med Sci Monit Basic Res 21:96–99. https://doi.org/10.12659/MSMBR.894221
- Teskey ML, Lukowiak KS, Riaz H, et al (2012) What's hot: the enhancing effects of thermal stress on long-term memory formation in Lymnaea stagnalis. Journal of Experimental Biology 215:4322–4329. https://doi.org/10.1242/jeb.075960
- Thirtamara-Rajamani K, Li P, Escobar Galvis ML, et al (2017) Is the enzyme ACMSD a novel therapeutic target in Parkinson's Disease? J Parkinsons Dis 7:577–587. https://doi.org/10.3233/JPD-171240
- Thorvaldsdóttir H, Robinson JT, Mesirov JP (2013) Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration, Briefings in Bioinformatics, Volume 14, Issue 2, Pages 178–192, https://doi.org/10.1093/bib/bbs017
- Tronson NC, Taylor JR (2007) Molecular mechanisms of memory reconsolidation. Nat Rev Neurosci 8:262–275. https://doi.org/10.1038/nrn2090
- Tully T (1984) Drosophila Learning: Behavior and biochemistry. Behav Genet 14:527–557. https://doi.org/10.1007/BF01065446
- van der Goot AT, Zhu W, Vázquez-Manrique RP, et al (2012) Delaying aging and the aging-associated decline in protein homeostasis by inhibition of tryptophan degradation. Proc Natl Acad Sci U S A 109:14912–14917. https://doi.org/10.1073/pnas.1203083109
- Vaughn CM (1953) Effects of temperature on hatching and growth of Lymnaea stagnalis. American Midland Naturalist 49:214. https://doi.org/10.2307/2422289
- Vauzour D, Vafeiadou K, Rice-Evans C, et al (2007) Activation of pro-survival Akt and ERK1/2 signalling pathways underlie the anti-apoptotic effects of flavanones in cortical neurons. J Neurochem 103:1355–1367. https://doi.org/10.1111/j.1471-4159.2007.04841.x
- Wagatsuma A, Sadamoto H, Kitahashi T, et al (2005) Determination of the exact copy numbers of particular mRNAs in a single cell by quantitative real-time RT-PCR. Journal of Experimental Biology 208:2389–2398. https://doi.org/10.1242/jeb.01625
- Wang J, Ho L, Zhao W, et al (2008) Grape-derived polyphenolics prevent a oligomerization and attenuate cognitive deterioration in a mouse model of Alzheimer's Disease. Journal of Neuroscience 28:6388–6392. https://doi.org/10.1523/JNEUROSCI.0364-08.2008
- Wang Y, Lawson MA, Dantzer R, Kelley KW (2010) LPS-induced indoleamine 2,3-dioxygenase is regulated in an interferon-gamma-independent manner by a JNK signaling pathway in primary murine microglia. Brain Behav Immun 24:201–209. https://doi.org/10.1016/j.bbi.2009.06.152
- Williams RJ, Spencer JPE, Rice-Evans C (2004) Flavonoids: antioxidants or signalling molecules? Free Radical Biology and Medicine 36:838–849. https://doi.org/10.1016/j.freeradbiomed.2004.01.001
- Wirthgen E, Hoeflich A, Rebl A, Günther J (2018) Kynurenic Acid: the janus-faced role of an immunomodulatory tryptophan metabolite and its link to pathological conditions. Frontiers in Immunology 8:1957. https://doi.org/10.3389/fimmu.2017.01957
- Wu G-M, Barrette M, Boivin G, et al (2011) Temperature influences the handling efficiency of an aphid parasitoid through body size-mediated effects. Environ Entomol 40:737–742. https://doi.org/10.1603/EN11018
- Yadav R, Zammit DJ, Lefrancois L, Vella AT (2006) Effects of LPS-mediated bystander activation in the innate immune system. J Leukoc Biol 80:1251–1261. https://doi.org/10.1189/jlb.0406253
- Yevchak AM, Loeb SJ, Fick DM (2008) Promoting cognitive health and vitality: a review of clinical implications. Geriatric Nursing 29:302–310. https://doi.org/10.1016/j.gerinurse.2007.10.017
- Yin JC, Tully T (1996) CREB and the formation of long-term memory. Current Opinion in Neurobiology 6:264–268. https://doi.org/10.1016/S0959-4388(96)80082-1

Websites and media sources

- http://www.ncbi.nlm.nih.gov/genome
- https://ftp.ncbi.nih.gov/genomes/Biomphalaria_glabrata/protein/
- https://ftp.ncbi.nih.gov/genomes/M_musculus/protein/
- https://ftp.ncbi.nih.gov/genomes/Homo_sapiens/protein/
- http://nadv.herokuapp.com
- https://ftp.ncbi.nih.gov/genomes
- http://www.ddbj.nig.ac.jp
- http://www.broadinstitute.org/igv
- https://www.ncbi.nlm.nih.gov/orffinder
- https://pfam.xfam.org/
- https://www.ncbi.nlm.nih.gov/tools/primer-blast/
- http://geneontology.org
- https://www.genome.jp/kegg/pathway.html
- https://www.genenames.org/
- Figures 3, 34, and 45 have been created using https://biorender.com
- The figure on the front cover has been adapted from Davison and Neiman (2021) https://doi.org/10.1098/rstb.2020.0163

Publications

1. ***A change in taste: the role of MicroRNAs in altering hedonic value***
   Kagan D., Batabyal A., **Rivi V.**, Lukowiak K.
   Journal of experimental biology (2022) – in press

ABSTRACT: The mechanisms associated with neophobia, and anhedonia remain largely unknown. Neuropsychological disorders such as depression and schizophrenia are associated with excessive fear and anhedonia and have been linked to microRNA 137. We hypothesized that microRNAs (miRNAs) in the snail *Lymnaea stagnalis* are important for regulating feeding behaviour through either preventing neophobia or establishing hedonic value. To test these hypotheses, we used an injection of Poly-L-Lysine (PLL) to inhibit miRNA biogenesis and observed its effects on feeding behaviour. We repeated these experiments with pre-exposure to novel stimuli capable of eliciting neophobia to disentangle the processes predicted to regulate feeding behaviour. Next, we exposed snails to food stimuli of high hedonic value after PLL injection to reset their hedonic value for that food. Finally, we consolidated our results with previous research by examining the effect of PLL injection on a one trial appetitive classical conditioning procedure (1TT) to induce long term memory (LTM). We found that miRNAs are likely not required for preventing neophobia. Moreover, we discovered that snails experienced anhedonia in response to inhibition of miRNA biogenesis, resulting in diminished feeding behaviour for food stimuli with a previously high hedonic value. Snails showed diminished feeding behaviour for multiple food stimuli of high hedonic value post 1TT with PLL injection. This finding suggested that PLL causes anhedonia rather than an impairment of LTM formation following the 1TT procedure. This is the first evidence suggesting that inhibiting the biogenesis of miRNAs contributes to anhedonia in *Lymnaea*.

2. ***Nature versus nurture in heat stress induced learning between inbred and outbred populations of Lymnaea stagnalis***

Rivi V., Batabyal A., Benatti C., Blom, JMC., Lukowiak K.

Journal of thermal biology (2022) - doi.org/10.1016/j.jtherbio.2021.103170.

ABSTRACT: Changing environmental conditions often lead to microevolution of traits that are adaptive under the current selection pressure. Currently, one of the major selection pressures is the rise in temperatures globally that has a severe impact on the behavioral ecology of animals. However, the role of thermal stress on neuronal plasticity and memory formation is not well understood. Thermal tolerance and sensitivity to heat stress show variation across populations of the same species experiencing different thermal regimes. We used two populations of the pond snail *Lymnaea* stagnalis: one lab-bred W-snails and the other wild Delta snails to test heat shock induced learning and memory formation for the Garcia effect learning paradigm. In Garcia effect, a single pairing of a heat stressor (30°C for 1h) with a novel taste results in a taste-specific negative hedonic shift lasting 24h as long-term memory (LTM) in lab bred W-snails. In this study, we used a repeated heat stress procedure to test for increased or decreased sensitivity to the heat before testing for the Garcia effect. We found that lab-bred W-snails show increased sensitivity to heat stress after repeated heat exposure for 7days, leading to enhanced LTM for Garcia effect with only 15min of heat exposure instead of standard 1h. Surprisingly, the freshly collected wild snails do not show Garcia effect. Additionally, F1 generation of wild snails raised and maintained under laboratory conditions still retain their heat stress tolerance similar to their parents and do not show a Garcia effect under standard learning paradigm or even after repeated heat stressor. Thus, we found a differential effect of heat stress on memory formation in wild and lab bred snails. Most interestingly we also show that local environmental (temperature) conditions for one generation is not enough to alter thermal sensitivity in a wild population of *Lymnaea*.

3. ***Quercetin enhances long-term memory formation if encountered at different times in the learning, memory formation, and memory recall continuum*.**

Rivi V., Batabyal A., Benatti C., Tascedda F., Blom, JMC., Lukowiak K.

Journal of comparative physiology A (2021) - doi: 10.1007/s00359-021-01522-1.

ABSTRACT: A major extrinsic factor influencing memory and neuro-cognitive performances across *taxa* is diet. Studies from vertebrates have shown the effects of a flavonoid rich diet on cognitive performance, but the mechanism underlying this action is still poorly understood. A common and abundant flavonoid present in numerous food substances is quercetin (Q). The present study provides the first support for Q-modulated enhancement of cognitive function in an invertebrate model, the pond snail *Lymnaea* stagnalis, after an operant conditioning procedure. We found that when snails were exposed to Q 3h before or after a single 0.5 h training session, which typically results in memory lasting ~ 3 h, they formed a long-term memory (LTM) lasting for at least 24 h. Additionally, we assessed the effects of the combined presentation of a single reinforcing stimulus (at 24h post-training or 24h before training) and Q-exposure on both LTM formation and reconsolidation. Thus, when applied within 3h of critical periods of memory, Q regulates four different phases: (1) acquisition (i.e., a learning event), (2) consolidation processes after acquisition, (3) memory recall, and (4) memory reconsolidation. In all these phases Q-exposure enhanced LTM persistence.

4. ***Long term memory of configural learning is enhanced via CREB upregulation by the flavonoid Quercetin in Lymnaea stagnalis***

Batabyal A*., **Rivi V.*,** Benatti C., Blom JMC., Lukowiak K. * These authors should be considered joint first authors.

Journal of experimental biology (2021) - doi: 10.1242/jeb.242761.

ABSTRACT: Animals respond to acute stressors by modifying their behaviour and physiology. The pond snail *Lymnaea* stagnalis exhibits configural learning (CL), a form of higher order associative learning. In CL snails develop a landscape of fear when they experience a predatory cue along with a taste of food. This experience results in a suppression of the food response; but the memory only persists for 3 h. *Lymnaea* has also been found to upregulate heat shock proteins (HSPs) as a result of acute heat stress, which leads to the enhancement of memory formation. A plant flavonoid quercetin blocks the upregulation of HSPs when experienced prior to heat stress. Here, we used this blocking mechanism to test the hypothesis that HSP upregulation plays a critical role in CL. Snails experienced quercetin prior to CL training and surprisingly instead of blocking memory formation it enhanced the memory such that it now persisted for at least 24 h. Quercetin exposure either prior to or after CL enhanced long-term memory (LTM) up to 48 h. We quantified mRNA levels of the transcription factor CREB1 in the *Lymnaea* central nervous system and found LymCREB1 to be upregulated following quercetin exposure. The enhanced LTM phenotype in *Lymnaea* was most pronounced when quercetin was experienced during the consolidation phase. Additionally, quercetin exposure during the memory reconsolidation phase also led to memory enhancement. Thus, we found no support of our original hypothesis but found that quercetin exposure upregulated LymCREB1 leading to LTM formation for CL.

5. ***The temperature-sensitivity of memory formation and persistence is altered by cold acclimation in a pond snail.***

Fernell M., **Rivi V.**, Batabyal A., Lukowiak K.

Journal of experimental biology (2021) - doi:10.1242/jeb.242513

ABSTRACT: There are reports on the inability of inbred, laboratory-reared *Lymnaea* stagnalis to perform feeding and aerial respiration in the cold. It has also been suggested that laboratory-bred snails have an inability to perform aerial respiration in winter months in the laboratory. Here, we used an inbred, laboratory-reared strain of *Lymnaea* (the S-strain) to demonstrate that the snails are capable of performing those behaviours in a cold (4°C) environment after a 2 day acclimation period. In addition, the inbred snails were able to perform aerial respiration during winter months at room temperature (20°C) in the laboratory. The persistence of long-term memory (LTM) was extended for at least 4 weeks by placing S-strain snails into a 4°C environment following training. Typically, the cold block (CB) procedure (1 h at 4°C) immediately after a training session blocks LTM formation in the S-strain but not in a freshly collected strain. Four weeks at 4°C transformed the S-strain phenotype into one resisting the CB procedure. Thus, with a 4-week cold spell snails gain a resistance to the CB procedure, and that would explain why freshly collected snails are resistant to the procedure. However, we found that F1 progeny of a freshly collected strain reared in the laboratory were resistant to the CB procedure. This suggests that an unknown selection resulted in the S-strain being susceptible to the CB procedure.

6. ***To eat or not to eat: A Garcia effect in pond snails (Lymnaea stagnalis).***

Rivi V., Batabyal A., Juego K., Kakadiya M., Benatti C., Blom JMC., Lukowiak K.

Journal of comparative physiology A - doi: 10.1007/s00359-021-01491-5.

ABSTRACT: Taste aversion learning is universal. In animals, a single presentation of a novel food substance followed hours later by visceral illness causes animals to avoid that taste. This is known as bait-shyness or the Garcia effect. Humans demonstrate this by avoiding a certain food following the development of nausea after ingesting that food ('Sauce Bearnaise effect'). Here, we show that the pond snail *Lymnaea* stagnalis is capable of the Garcia effect. A single 'pairing' of a novel taste, a carrot slurry followed hours later by a heat shock stressor (HS) is sufficient to suppress feeding response elicited by carrot for at least 24 h. Other food tastes are not suppressed. If snails had previously been exposed to carrot as their food source, the Garcia-like effect does not occur when carrot is 'paired' with the HS. The HS up-regulates two heat shock proteins (HSPs), HSP70 and HSP40. Blocking the up-regulation of the HSPs by a flavonoid, quercetin, before the heat shock, prevented the Garcia effect in the snails. Finally, we found that snails exhibit Garcia effect following a period of food deprivation but the long-term memory (LTM) phenotype can be observed only if the animals are tested in a food satiated state.

7. ***What can we teach Lymnaea and what can Lymnaea teach us?***

Rivi V., Benatti C., Lukowiak K., Colliva C., Alboni S., Tascedda F., Blom JMC.

Biological Reviews - (2021). doi: 10.1111/brv.12716

ABSTRACT: This review describes the advantages of adopting a molluscan complementary model, the freshwater snail *Lymnaea* stagnalis, to study the neural basis of learning and memory in appetitive and avoidance classical conditioning; as well as operant conditioning of its aerial respiratory and escape behaviour. We firstly explored 'what we can teach *Lymnaea*' by discussing a variety of sensitive, solid, easily reproducible and simple behavioural tests that have been used to uncover the memory abilities of this model system. Answering this question will allow us to open new frontiers in neuroscience and behavioural research to enhance our understanding of how the nervous system mediates learning and memory. In fact, from a translational perspective, *Lymnaea* and its nervous system can help to understand the neural transformation pathways from behavioural output to sensory coding in more complex systems like the mammalian brain. Moving on to the second question: 'what can *Lymnaea* teach us?', it is now known that *Lymnaea* shares important associative learning characteristics with vertebrates, including stimulus generalization, generalization of extinction and discriminative learning, opening the possibility to use snails as animal models for neuroscience translational research.

8. ***Lymnaea stagnalis as model for translational neuroscience research: From pond to bench.***

Rivi V., Benatti C., Radighieri G., Colliva C., Brunello N., Tascedda F., Blom, JCM.

Neuroscience and Biobehavioural reviews (2020) - doi: 10.1016/j.neubiorev.2019.11.020.

ABSTRACT: The purpose of this review is to illustrate how a reductionistic, but sophisticated, approach based on the use of a simple model system such as the pond snail *Lymnaea* stagnalis (*Lymnaea*), might be useful to address fundamental questions in learning and memory. *Lymnaea*, as a model, provides an interesting platform to investigate the dialog between the synapse and the nucleus and vice versa during memory and learning. More importantly, the "molecular actors" of the memory dialogue are well-conserved both across phylogenetic groups and learning paradigms, involving single- or multi-trials, aversion or reward, operant or classical conditioning. At the same time, this model could help to study how, where and when the memory dialog is impaired in stressful conditions and during aging and neurodegeneration in humans and thus offers new insights and targets in order to develop innovative therapies and technology for the treatment of a range of neurological and neurodegenerative disorders.

9. ***Redefining operant conditioning of escape behaviour in Lymnaea stagnalis.***

Benatti C., **Rivi V.**, Radighieri G., Colliva C., Tascedda F., Blom JMC.

Invertebrate survival journal - doi:10.25431/1824-307X/isj.v0i0.129-137.

ABSTRACT: The escape behaviour is one of the many behavioural responses that can be operantly conditioned in a stimulus-dependent manner in both vertebrates and invertebrates. By exposing the pond snail *Lymnaea stagnalis* repeatedly to a negative reinforcement its natural tendency to explore its surroundings can be operantly conditioned in both adult and aged snails. When adult snails were trained with 100 mM of KCl their number of escapes was significantly decreased and the latency to first escape was significantly increased. Our behavioural protocol allowed us to investigate memory acquisition, consolidation, and retrieval in pre- and post-training sessions over different days. From the 3rd day of training the learned response was strengthened: the number of the escapes in the post-test session remained significantly reduced even when animals were presented with distilled water. Moreover, adult snails exposed to the negative reinforcement for at least 4 days started to escape significantly less than the control group also in the pre-test session. This effect became more pronounced in the following days and was accompanied by a significant increase in the latency to first escape at the beginning of the pre-test on day 6 and 7. Aged snails, instead, showed selective deficiencies when operantly conditioned: memory retention appeared only after 7 days, while memory retrieval could not be induced. This redefined paradigm can help unravelling a variety of sophisticated cognitive phenomena in *Lymnaea* and could be employed also to study the basis of memory impairment occurring during neuro-aging.

Other papers published during my PhD- not *Lymnaea*-related

10. ***Mild to severe neurological manifestations of COVID-19: cases reports.***

Melegari G., **Rivi, V.**, Zelent G., Nasillo V., De Santis E., Melegari A., Bevilacqua C., Zoli M., Meletti S., Barbieri A.

International journal of environmental research and public health - doi: 10.3390/ijerph18073673

ABSTRACT: The main focus of Coronavirus disease 2019 (COVID-19) infection is pulmonary complications through virus-related neurological manifestations, ranging from mild to severe, such as encephalitis, cerebral thrombosis, neurocognitive (dementia-like) syndrome, and delirium. The hospital screening procedures for quickly recognizing neurological manifestations of COVID-19 are often complicated by other coexisting symptoms and can be obscured by the deep sedation procedures required for critically ill patients. Here, we present two different case-reports of COVID-19 patients, describing neurological complications, diagnostic imaging such as olfactory bulb damage (a mild and unclear underestimated complication) and a severe and sudden thrombotic stroke complicated with hemorrhage with a low-level cytokine storm and respiratory symptom resolution. We discuss the possible mechanisms of virus entrance, together with the causes of COVID-19-related encephalitis, olfactory bulb damage, ischemic stroke, and intracranial hemorrhage.

11. ***How to humanise the COVID-19 intensive care units.***

Rivi V., Melegari G., Blom JMC.

BMJ Evidence-Based Medicine (2021) - doi:10.1136/bmjebm-2020-111513.

ABSTRACT: The COVID-19 is altering the way patients and families endure illness and death. To mitigate the spread of the virus, patient isolation and visitor restrictions in hospitals have been implemented at a scale never seen before. These measures could make both patients and relatives vulnerable to different degrees of stress disorders as well as depression and anxiety. Because these symptoms will likely continue even after the pandemic has subsided, virtual and/or on-site psychological support should be proposed promptly to patients and their families during the hospitalisation and after discharge from the hospital. What to do? How to act? Here, we proposed some solutions for humanising ICUs during this challenging period.

12. ***Mind the mother when considering breastfeeding.***

Rivi V., Petrilli G., Blom JMC.

Frontiers – Global women's health (2020) - doi:10.3389/fgwh.2020.00003

ABSTRACT: Depression and anxiety disorders represent the most common obstetric complications during pregnancy and the first-year post-partum, reducing the mother's ability to effectively perceive, decipher, and respond to their infant needs. Maternal mental health and breastfeeding have a tight but mixed relationship. Given this and considering that breastfeeding recommendations are a critical and complex topic in public health discussions, our aim is to highlight various aspects surrounding breastfeeding with the purpose to propose to stably include screening for mental health in programs tailored to the mother.

Paper published before my PhD- not Lymnaea-related

13. *Genomic and cytogenetic localization of the carotenoid genes in the aphid genome.*

Mandrioli M., **Rivi V.**, Nardelli A., Manicardi GC.

Cytogenetic and Genome Research (2016)

ABSTRACT: Data published in the scientific literature suggests a possible link between chromosomal rearrangements involving autosomes 1 and 3 and the presence of red morphs in the peach-potato aphid *Myzus persicae* (Sulzer). In order to begin a study of this relationship, we analysed the genomic and chromosomal location of genes involved in carotenoid biosynthesis in *M. persicae* and the pea aphid, *Acyrthosiphon pisum* (Harris), since carotenoids are the basis of the colour in many aphid species. Genomic analysis identified a DNA sequence containing carotenoid genes in synteny between the 2 species. According to the results obtained using in situ PCR, carotenoid genes were located in a subterminal portion of autosome 1 in both species. The same localization has also been observed in the onion aphid *Neotoxoptera formosana* Takahashi that, as *M. persicae* and *A. pisum*, belongs to the tribe *Macrosiphini*, thereby suggesting a synteny of this chromosomal region in aphids. In situ PCR experiments performed on *M. persicae* asexual lineages bearing heterozygous translocations involving autosomes 1 and 3 revealed that carotenoid genes were located within chromosomal portions involved in recurrent rearrangements. We also verified by bioinformatics analyses the presence of fragile sites that could explain these recurrent rearrangements in *M. persicae*.