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Trace conditioning: insights from invertebrate models on bridging the temporal gap

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Trace conditioning is the ability to form associations between a conditioned stimulus (CS) and an unconditioned stimulus (US) separated in time. This fundamental cognitive process allows organisms to predict outcomes, avoid danger and exploit resources even when predictive stimuli are temporally discontinuous. This process has been thought to depend on complex neural structures such as the mammalian hippocampus, marking it as exclusive to vertebrates. However, emerging evidence from honeybees, fruit flies and molluscs challenges this notion, showing that trace conditioning can occur in invertebrates with relatively simple nervous systems. We highlight some current findings on trace conditioning across invertebrate species and explore promising directions for future research. We propose a two-tier mechanistic model that integrates stimulus salience and attentional processes into trace learning. We highlight the great pond snail, *Lymnaea stagnalis*, as a model for utilizing this framework. Our framework is based on rapid, stimulus-specific encoding of the conditioned stimulus and attention-based memory. By varying CS–US intervals, stimulus salience and distractor presence, we propose a paradigm to explicitly test how sensory inputs are transformed into persistent, attention-based memory during trace learning. This work lays a foundation for a broader, mechanistically informed framework for learning and memory across the animal kingdom.

1. Introduction

At the beginning of the twentieth century, associative learning emerged as the primary mechanism of learning, where some studies focused on the association between two stimuli and some on associations between a stimulus and a response. In classical conditioning, for example, organisms learn to associate two stimuli—the conditioned and unconditioned stimuli (CS and US) (table 1)—and contiguity in the two stimuli has been thought of as an essential component for this kind of learning, wherein most studies test delay conditioning where CS–US overlap is found [1]. Similarly, in operant conditioning, behaviours are reinforced more effectively when rewards or punishments follow closely in time [2]. However, notable exceptions challenge this rule of contiguity in stimuli. One such case is the Garcia effect (table 1) [3], a special form of conditioned taste aversion (CTA), in which an animal learns to avoid a novel taste after a single pairing with illness, even when several hours separate ingestion from the onset of malaise [4,5] (figure 1). This ecologically adaptive response demonstrates that, under certain conditions, the brain can bridge substantial temporal gaps.

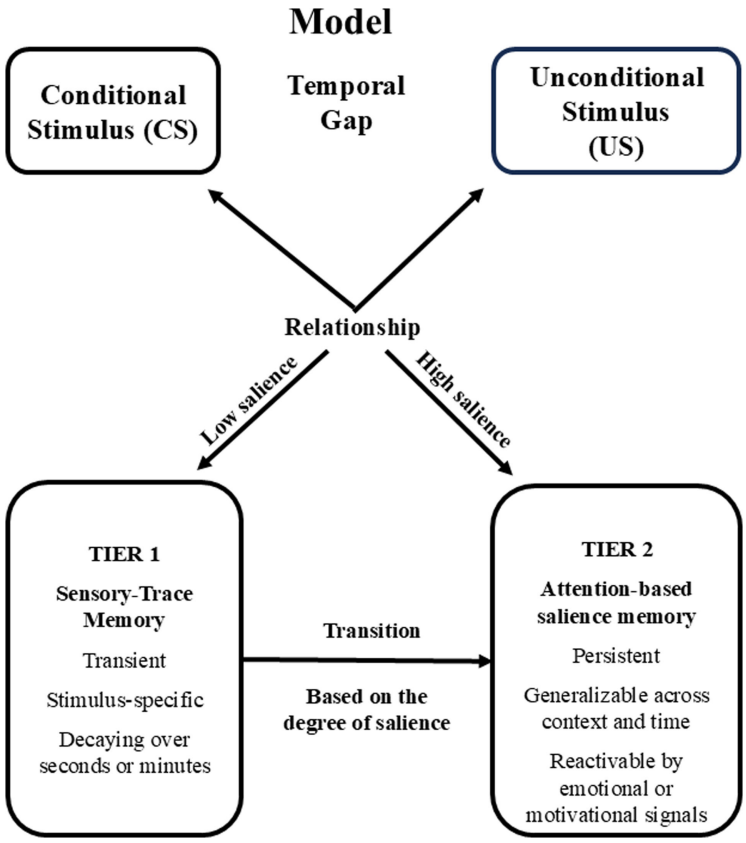

Figure 1. A two-tiered model of memory for testing trace conditioning under salience manipulation. The model outlines two distinct memory systems engaged during trace conditioning, depending on the salience of the conditioned stimulus (CS) and unconditioned stimulus (US). Low-salience CS–US pairings activate sensory-trace memory—a transient, stimulus-specific system that decays within seconds or minutes. In contrast, high-salience pairings engage attention-based salience memory, which is a more persistent, generalizable system reinforced by emotional or motivational signals, enabling longer retention and reactivation.

Table 1. Glossary of key terms.

term	definition	example/note
trace conditioning	learning where the CS and US are separated by a time gap	e.g. a tone followed by a shock after several seconds of the offset of the tone
CS (conditioned stimulus)	initially, a neutral stimulus that gains significance through pairing with a US	e.g. a sound or a novel taste
US (unconditioned stimulus)	a stimulus that naturally elicits a response	e.g. food, pain or illness
partial trace memory	CS–US association is formed without precise knowledge of the interval	organism learns 'CS predicts US', but timing is approximate
full trace memory	CS–US association is formed with precise timing of the US	organism learns 'CS predicts US in exactly 10 s'
salience	the behavioural or evolutionary significance of a stimulus that attracts attention	e.g. a highly bitter taste versus a mildly bitter taste, where highly bitter is more salient if organisms associate bitterness with an internal state
attention	an internal process that enhances the processing of salient stimuli and supports memory across time gaps	necessary for maintaining CS traces in trace conditioning
sensory-trace memory	short-lived, modality-specific memory formed immediately after the CS	maintains a fleeting representation of the stimulus
attention-based salience memory	longer-lasting memory reinforced by attention and motivational relevance	highly salient stimuli (food, pain) are remembered longer
Garcia effect	conditioned taste aversion in which an organism learns to avoid a taste associated with illness, even after a long delay. It requires a novel food/taste	e.g. rats avoid a novel flavour hours after becoming sick

Trace conditioning (table 1) provides another notable exception. Here, a CS is presented and then removed before the US appears, creating a temporal gap between the two events [6–8]. Traditionally, the ability to associate such temporally separated stimuli was seen as a characteristic of higher cognition reliant on structures like the hippocampus and prefrontal cortex [9–12].

However, recent studies in invertebrates show that trace learning can also develop in simpler nervous systems [7,13,14]. In this review, we highlight representative cases that best illustrate the phenomena discussed.

The fruit fly *Drosophila melanogaster* can associate visual and olfactory cues with rewards or punishments across seconds-long delays [13,15,16], the honeybee *Apis mellifera* also shows trace learning for similar appetitive olfactory learning using visual and olfactory cues [14,17,18], while the moth *Manduca sexta* shows how neural representations are associated with olfactory conditioning [19]. Even the 302-neuron nematode *Caenorhabditis elegans* can associate cues during aversive conditioning separated over a time gap of seconds [20,21]. Finally, the pond snail *Lymnaea stagnalis* can sustain CTA across CS–US intervals of minutes to hours [22–24]. Together, these findings suggest that bridging temporal gaps does not require complex neural architectures but instead relies on evolutionarily conserved mechanisms [7,24].

From an evolutionary perspective, associative learning across a temporal gap can enable organisms to predict outcomes, avoid threats and exploit resources even when the predictive cause and effect are separated in time. We do observe such trace memory maintenance, but with a diversity of results across systems. This raises a central question: what mechanisms allow a stimulus to be maintained across time, and why do some associations persist robustly while others decay?

One way to approach this question is to compare how CS representations are maintained and how the effectiveness of this process depends on stimulus salience (table 1) and attention (table 1). These are distinct features: stimulus salience is intrinsic to the stimulus or its ecological relevance, while attention relates to the organism's internal state during resource allocation [21,25–27]. To understand how salience and attention influence temporal bridging, we need to consider the theoretical frameworks that offer complementary insights into how learning unfolds over time.

According to the Rescorla–Wagner model for associative learning, the strength of the association depends on the extent to which CS predicts US (prediction error) and the salience of the CS and the US [28]. Highly salient cues (such as for pain, food, etc.), whether in the form of CS or US, would invoke attentional processes that drive stronger associations [29–31]. While highly successful in accounting for many conditioning phenomena, this model does not explicitly specify how stimulus representations persist or evolve over time once the CS has ended. In contrast, attentional models of learning like Mackintosh's model and the Pearce–Hall model focus on how the associability of a CS changes across experience, wherein attention is allocated to cues that are reliable and surprising [31,32]. Thus, attention enhances the processing of salient stimuli, allowing CS representations to persist across temporal gaps—a key feature of trace conditioning [7,33]. A complementary perspective is provided by Wagner's standard operating procedure or sometimes opponent processes (SOP) model, which explains associative learning in terms of time-dependent stimulus representations rather than a single static memory trace [34]. In SOP, each stimulus is represented by a set of elements that transition through three activation states: an inactive state, a primary active state when the stimulus is present and a secondary, decaying activation state following stimulus offset, before returning to inactivity, and associative learning depends on the temporal overlap of these states for CS and US. This provides a mechanistic framework to understand how a CS bridges the temporal gap to the US. Taken together, these perspectives highlight complementary aspects of associative learning: how learning occurs, which cues are prioritized for learning, and how stimulus representations might persist across time [35].

A direct behavioural consequence of how CS representations are maintained across time is the temporal resolution with which an association is expressed. Depending on the resolution of the memory, trace conditioning can result in full trace conditioning (table 1), where the precise timing of the CS–US association is present and the organism can anticipate the arrival of the US at the exact time point post-CS presentation or partial trace conditioning (table 1), where the timing of the CS–US association is not present or displayed by the organism but only the association is present as a memory trace [12]. These differences can underscore how attention and salience shape the temporal resolution of learning, influencing the way CS representation occurs and is maintained. Experimental processes such as repeated training can extend trace intervals, reflecting the plasticity of attentional and neuromodulatory systems [14,15]. These distinctions form the foundation of our proposed two-tier model of trace memory, which integrates rapid sensory-trace processes (table 1) (Tier 1) with slower, attention-driven maintenance (table 1) (Tier 2) to account for variability across species and experimental conditions (figure 1). Tier 1 corresponds to rapid sensory encoding broadly analogous to the primary activation state described in SOP, whereas Tier 2 captures processes that maintain or reinstate CS representations over longer delays, functionally consistent with extended secondary activation under conditions of high salience and attentional engagement. Evidence from invertebrate models illustrates how these processes may be implemented in biological systems with relatively simple central nervous systems. Recent work on honeybees highlights how attentional processes are required to bridge the CS–US gap in trace conditioning, as distractors were found to impair trace learning, signifying the role of attention or awareness involved in the process [35]. Experiments with different CS and US varying in their salience, along with the use of distractors, are therefore necessary to test the roles of salience and attention in trace conditioning. A single model capable of testing these processes would be ideal. We aim to address this in our current study by providing a unified experimental framework to examine this in the great pond snail, *L. stagnalis*.

Building on this framework, we organize this article around three interrelated questions:

(1) What molecular and neural mechanisms sustain a CS across a temporal gap (possible mechanism for Tier 1 of our model)?
(2) How do salience and attention contribute to active maintenance during the interval (possible mechanism for Tier 2 of our model)?
(3) How can invertebrate models be used to understand mechanisms of trace conditioning using the two-tier model framework?

By framing trace conditioning within this comparative perspective, we aim to connect mechanistic insights, behavioural phenomena and evolutionary principles to better understand how even simple nervous systems bridge time in associative learning (table 2).

2. Molecular and neural mechanisms supporting temporal bridging (Tier 1)

Tier 1 sensory-trace memory reflects the initial, quickly fading encoding of the CS. It captures modality-specific features (i.e. olfactory, tactile, visual) and provides the immediate neural representation needed for the organism to recognize and respond to the stimulus. Across invertebrate taxa, several conserved mechanisms support this process, making these systems ideal for studying how organisms bridge temporal gaps in associative learning [7,24] (table 2).

Tier 1 sensory-trace memory relies on fast neurotransmitters and short-timescale modulatory systems that generate and stabilize initial CS representations. Acetylcholine and glutamatergic signalling through ionotropic and NMDA-like receptors produce modality-specific CS traces and stabilize the CS representations over the interstimulus interval [7,57,58]. In *C. elegans*, GLR-1 (AMPA-type) and NMR-1 (NMDA-type) receptors in interneurons, such as RIM and AWC circuits, mediate short- and long-term trace-like memories, interacting with CREB (CRH-1) pathways to associate sensory cues with delayed food or aversive outcomes [55,59]. These mechanisms closely parallel cholinergic pathways in vertebrate hippocampus-dependent trace learning [60–62], highlighting deep conservation of glutamatergic mechanisms. Cholinergic circuits in insects further modulate the excitability of learning related circuits and contribute to arousal-like states and attentional gating that enhances stimulus salience during learning [63]. This occurs by selectively amplifying CS inputs while suppressing background noise, a function analogous to cortical cholinergic circuits in vertebrates [64,65]. These processes form the immediate substrate for CS recognition and short-term maintenance across the temporal gap.

Neuromodulators then refine and temporally tune Tier 1 sensory traces. In *Drosophila*, dopaminergic neurons modulate mushroom body circuits during the trace interval [16], allowing learning at longer CS–US delays [66]. Dopamine signals both prediction error, the discrepancy between expected and actual outcomes, and the expected timing of the US, bridging the interval and adjusting synaptic weights in a temporally precise manner [7,67,68]. In bees, octopamine contributes to reward signalling and enhances attention to salient CS cues [41,42,69], paralleling vertebrate noradrenaline and dopaminergic reward circuits [70,71]. These effects can be understood within the Rescorla–Wagner framework, in which the strength of the CS–US association increases proportionally to prediction error [72]. That is, when the CS–US interval closely matches the learned expectation, prediction error is small and conditioned response (CR) timing is more accurate. Conversely, deviations from the expected interval generate prediction errors that serve to update synaptic weights and improve temporal accuracy [67].

Serotonin (5-HT) further refines temporal precision by regulating the coincidence time window of CS–US associations and can either shorten or prolong the activity of the neurons across the time gap during trace conditioning [45]. Receptor-specific serotonergic signalling modulates both the probability of CR acquisition and the temporal precision of those responses [45]. Local 5-HT release adjusts neuronal excitability, synaptic plasticity and aligns neuronal firing towards motivationally relevant and reward-predicting stimuli that are expected under highly salient CS–US associations [73]. In *Drosophila* and *Lymnaea*, 5-HT interacts with calcium-dependent signalling and the cAMP–PKA–CREB cascade to stabilize CS representations and supports memory formation [74–76]. This coordination maintains the strength and timing of the CS trace, effectively constraining the window during which associative plasticity can occur ensuring that the organism can generate precisely timed CR. Along with these neurotransmitters and modulators, some neuropeptides emerge as potential candidates that bridge the Tier 1 with the Tier 2 attention-based processes, linking internal state to trace persistence. Neuropeptides such as sNPF in *Drosophila* and honeybees [51,77,78] and FMRFamide in *Lymnaea* [53,54] act on slower timescales, modulating motivation, arousal and excitability to bias networks towards salient stimuli and stabilize ongoing activity. Cholinergic circuits also contribute to attentional gating by selectively amplifying CS inputs and suppressing distractors [79,80]. These systems converge on intracellular pathways, including cAMP–PKA–CREB and calcium-dependent signalling, integrating rapid sensory-trace input with longer-term plasticity [81,82]. By doing so, they reinforce the CS representation beyond the initial Tier 1 timescale and prepare it for attentional enhancement during Tier 2 maintenance.

By orchestrating excitability, attention and internal state, these mechanisms collectively maintain CS representations across the interstimulus interval, enabling organisms to accurately detect, evaluate and respond to the US. Thus, fast neurotransmitters and short-term modulators—including acetylcholine, glutamate, dopamine, octopamine and serotonin—form the core Tier 1 substrate, whereas slower modulators and neuropeptides interface with Tier 2 to tag salience, adjust temporal windows and reinforce trace persistence. The striking parallels between invertebrate and vertebrate systems underscore that the molecular and cellular logic of trace conditioning is deeply conserved [83].

3. Interaction of salience and attention for uncovering trace memory (Tier 2)

While sensory-trace memory (Tier 1) provides the initial encoding of the CS, it is insufficient to sustain associations across extended CS–US intervals. Tier 2—attention-based salience memory—operates more slowly but is essential for persistence. This dependence on sustained attention makes it uniquely vulnerable to interference (figure 1). Distractors—whether environmental noises or irrelevant cues—readily disrupt trace conditioning [8,13,33,84,85]. In *Drosophila*, Grover *et al.* [13] showed that brief air puffs or virtually induced auditory stimuli delivered as distractors during the trace interval impaired learning by degrading both CS- and US-related persistent activity at behavioural and neural levels. Similarly, Clark *et al.* [33] showed how trace

Table 2. Summary of molecular systems involved in trace conditioning across invertebrate models. To clarify, the table shows whether each role is backed by empirical evidence or remains hypothetical/extrapolated. These entries highlight selected examples and are not meant to be a complete survey of all models or taxa.

molecular actor	model	role in trace conditioning	mechanistic notes	status	key references
dopamine	*D. melanogaster*, *A. mellifera*, *M. sexta*	modulates mushroom body circuits during trace intervals; role in motivation necessary for learning with longer CS–US gaps	acts via cAMP–PKA–CREB pathways; encodes prediction error	**empirical** in flies and bees; **hypothetical** extension to moths	[13,16,19,36–40]
octopamine	*A. mellifera*	reinforces appetitive learning; modulates reward salience	analogous to vertebrate noradrenaline, it strengthens the persistence of the CS trace	**empirical** in bees; **hypothetical** in other insects/molluscs	[41,42]
serotonin (5-HT)	*L. stagnalis*, *D. melanogaster*, *A. mellifera*	modulates taste aversion learning; regulates timing precision and extension of CS trace	receptor activity modulates synaptic plasticity, excitability, CREB cascade	**empirical** in snails, flies and bees; likely conserved across taxa	[22,35,43–45]
cholinergic system (ACh)	*D. melanogaster*	mediates initial CS encoding; functions in appetitive learning; facilitates plasticity	nicotinic receptors involved in attention-like processing; interacts with DA and 5-HT	**empirical** in flies; **hypothetical** in molluscs/insects broadly	[46–48]
sNPF (short neuropeptide F)	*D. melanogaster*, *A. mellifera*	modulates motivational states; enhances trace robustness under high salience	homologue of vertebrate NPY; regulates feeding and arousal	**empirical** in flies and bees	[49–52]
FMRFamide and related peptides	*L. stagnalis*	prolong neural activity; can gate learning circuits by controlling excitability	operate on slower timescales; connect internal state to persistence	**empirical** in snails	[53,54]
glutamate (iGluRs, NMDA-like)	*C. elegans*	critical for associating sensory cues with delayed outcomes	GLR-1 (AMPA) and NMR-1 (NMDA) receptors in RIM/AWC circuits; CREB-dependent	**empirical** in worms; parallels vertebrate NMDA-based trace learning	[20,55,56]

learning is strongly dependent on expectancy and declarative awareness, making it vulnerable to distractions as also shown in the *Drosophila* study. These findings highlight the reliance of invertebrate trace conditioning on attentional maintenance, paralleling mechanisms described in mammals.

The attentional load in trace conditioning is likely to depend on the properties of both CS and US, as well as whether information is processed within a single modality or across multiple modalities. Salient or novel cues capture attention strongly and persist longer, whereas weak or familiar cues fade rapidly [86]. How attentional systems interact with salience across single- and multimodal contexts remains poorly understood. In all cases, attentional networks must sustain the fading CS, filter competing inputs and align it with the expected US. These demands are likely to intensify in cross-modal conditioning, where multiple sensory streams—each engaging distinct neural substrates—must be coordinated simultaneously to preserve stimulus traces across the temporal gap.

Neurobiological studies in invertebrates shed light on how modality-specific circuits address the challenge of temporal bridging. In *Drosophila* visual trace conditioning, central complex circuits maintain the CS: ellipsoid-body ring neurons (R2 and R4m) extend CS-related activity into the gap, while dopaminergic PPM3 neurons project US-related activity forward. Both processes are hindered by distractors [13,87]. Conversely, olfactory trace conditioning relies on mushroom bodies, where dopaminergic reinforcement can help stabilize fading odour traces, since dopaminergic neuronal output signals about the dynamics of salience [70,88]. This divergence prompts important questions about how attentional systems adapt when multiple modalities are involved. A salient CS may resist distraction by strongly capturing attention, but its enhanced representation might also increase vulnerability by over-engaging attentional resources. Planned experiments in *Lymnaea* will evaluate whether distractors have different effects on salient versus non-salient CSs, aiding in disentangling these possibilities.

Learning dynamics further emphasize the flexibility of attentional systems. Repeated training can extend the effective trace interval at the behavioural and neuronal level [14,15,88,89]. Grover *et al.* [13] demonstrated corresponding plasticity in *Drosophila* at the neural level: CS-responsive neurons showed progressively stronger oscillatory persistence, while US-responsive neurons gradually came to encode the CS, consistent with the substitution principle. Repeated presentations of the CS

can engage attentional systems, as the recurrence of the same CS–US pairing reinforces its importance and strengthens the perceived cause-and-effect relationship. These bridging mechanisms, however, remain fragile under distraction, highlighting the attentional basis of trace conditioning at the neural level. Some species precisely time CRs to anticipated US onset, while others do not, suggesting distinctions between full and partial trace conditioning. The correspondence between behavioural and neural temporal dynamics remains unresolved in such cases. Neural activity may exhibit temporal alignment between CR and US onset without manifesting in overt behaviour. Experiments manipulating CS–US salience and timing in *Lymnaea* could shed light on this gap, clarifying how attention interacts with overt behaviour and neural processes to jointly sustain full versus partial trace conditioning. Interestingly, partial trace CR might result from two different processes. Firstly, if only the sensory trace memory (Tier 1) is engaged without attentional network involvement, this can result in partial trace conditioning. However, when training involves a highly salient US, attentional networks may be engaged and the organism may encode the CS–US interval, yet still fail to display full trace conditioning. In such cases, presentation of the CS during testing triggers an immediate response that showcases partial trace memory phenotype, as the organism anticipates a strongly rewarding or aversive US and thus does not wait for its expected onset. This is explicitly included in our experimental testing framework to understand whether such a phenomenon exists.

Together, Tiers 1 and 2 enable organisms to sustain associations over extended CS–US intervals. Current invertebrate data show occasional mismatches between the timing of CRs and the expected US [8,14]. However, a recent study by Paoli *et al.* [90] shows a clear demonstration of the CR at the expected US arrival timing in their trace conditioning protocol modelled by mushroom body output neuron activity and the proboscis extension reflex that are common indicators of learning. Within our proposed two-tier framework, the match between anticipated US and CR timings can be understood through partial versus full trace conditioning, depending on the salience of the CS/US and the involvement of Tier 2 attentional systems. Overall, the interaction between Tier 1 and Tier 2 creates a mechanistic link from molecular and cellular processes to behavioural outcomes. It suggests how attentional networks, neuromodulators and sensory traces work together to allow invertebrates to shift from partial to full trace conditioning and also how it maintains CS representations across intervals ranging from seconds to minutes.

4. Experimental testing in invertebrates: validating the model

Among the invertebrate models presented in the previous sections, *Lymnaea* offers a uniquely tractable system for experimentally validating the two-tier model of trace conditioning. While direct evidence that *Lymnaea* acquires canonical trace conditioning under controlled conditions is limited, snails are capable of long-delay CTA [22,91]. The combination of *Lymnaea*'s neural circuit tractability and its conserved neuromodulatory pathways provides a unique opportunity to dissect Tier 1 and Tier 2 [92–96]. In particular, serotonergic and neuropeptide systems in *Lymnaea* can prolong CS traces over minutes to hours, integrating motivational state to stabilize memory thereby linking the sensory persistence (Tier 1) with modulatory control of motivation and salience for consolidation (Tier 2) [97,98]. These examples reveal a conserved principle: Tier 1 provides rapid encoding and stimulus specificity, whereas Tier 2 recruits higher-order networks to maintain and organize memory across time.

CTA learning is well suited for testing trace conditioning. Unlike standard trace paradigms, which operate across a seconds-to-minutes gap through attentional bridging of transient sensory traces [12], CTA engages neuromodulatory and motivational systems that maintain associations over much longer intervals [4,5,99]. However, the robustness of CTA to long CS–US delay may also reflect the absence of competitive cues during trace learning. But still CTA is not an exception to the two-tier framework but an expansion of it: canonical trace conditioning examines the transition from Tier 1 sensory-trace memory to Tier 2 attention-based salience mechanisms within seconds to minutes, while CTA demonstrates how similar mechanisms function over extended periods via neuromodulatory amplification. Therefore, the two-tier model offers a unified framework that connects short-interval trace conditioning with longer-lasting associative processes, with differences mainly in the timescale and neuromodulatory resources involved [93,100].

To induce CTA in *Lymnaea*, the CS is a chemical cue, typically sucrose applied to the lips. The US is a noxious stimulus, such as potassium chloride (KCl), which naturally suppresses feeding, and the CR is the suppression of rhythmic feeding movements ('rasps') in response to the CS alone [22,101]. The KCl concentration used in previous studies (10 mM) can be considered a standard KCl concentration, which we also propose for our experimental framework. The prolonged feeding suppression in *Lymnaea* following CS–US intervals ranging from seconds to minutes provides a window for exploring the maintenance of sensory-trace memory (Tier 1) and its reinforcement by attention-mediated salience memory (Tier 2). We also incorporate distractor stimuli during CS–US interval to directly test CTA under cue competition to assess whether the long delay learning during CTA is due to active attentional maintenance rather than due to the absence of competing cues.

(a) Experimental framework of the two-tier model in *Lymnaea stagnalis*

Manipulating motivational value (e.g. CS salience: standard sucrose versus novel carrot reward and US salience: high or low concentration of KCl) allows testing of how salience modulates attentional recruitment to stabilize CS traces. High-salience CS is predicted to induce stronger distraction resistance and faster memory acquisition and retention, along with the ability to associate CS–US across an increased temporal gap (inter-stimulus interval, ISI) when compared to a low-salience CS (figure 2). High-salience pretraining should engage Tier 2, whereas low-salience stimuli may remain confined to Tier 1, yielding short-lived CRs. Controlled distractors (brief water-flow stimuli during the trace interval) allow direct testing of attention-mediated Tier 2 protection of the CS trace. Control experiments with only distractors and CS paired with distractors showcase whether

the response is altered by the distractor-only condition (figure 2). Graded effects of distractors across salience conditions would provide evidence for interaction between sensory traces and attentional reinforcement.

(b) Trace interval and temporal precision

The CR in *Lymnaea* can be quantified in terms of latency, magnitude and probability relative to the expected US, providing direct readouts of partial versus full trace conditioning. Partial trace conditioning is indicated when the organism responds outside the expected US window, suggesting that the association is learned but the timing is not encoded. In contrast, full trace conditioning occurs when responses are temporally aligned with the expected US, reflecting precise temporal learning. To test for partial versus full trace conditioning, manipulating the salience of the US can be a possible approach, where a high-salience US results in partial trace conditioning, as the aversion response is strong and the animal immediately responds with a withdrawal of feeding when anticipating a high-salience US. However, with a low-salience US and repeated training, a full trace CR might emerge wherein animals would forego the feeding response at the anticipated time of arrival of the US based on pre-training ISIs (figure 3). We test three ISIs spanning established CTA performance: 15 s (canonical), 1–3 min (intermediate) and 5 min (upper bound), all with 15 min inter-trial intervals with a low and high concentration of US (KCl). The concentrations need to be determined based on initial testing. This brackets the window within which *Lymnaea* CTA still forms and where timing/distraction effects can be revealed.

(c) Pharmacological and naturalistic manipulations

Pharmacological interventions using dopamine or octopamine can enhance salience but may non-specifically affect motivation, arousal or other neuromodulatory pathways. To disentangle these effects, naturalistic variations in CS/US salience—such as adjusting sucrose concentration or giving foods that differ in salience or value—can complement pharmacological approaches. Shifts in the internal state of the organism such as starvation or stress can also modulate motivation and arousal. This dual strategy strengthens interpretability and provides a mechanistically grounded test of how Tier 1 and Tier 2 interact to sustain temporal bridging. Along with behavioural tests for memory, electrophysiology measurements from known neurons involved in learning and attention/reward pathways, as well as molecular signatures for Tier 1 versus Tier 2, can be quantified. However, for the current study, we have limited our methodology to primarily address behavioural measures without pharmacological manipulations.

(d) Training and test schedule for experimental framework

- *Pre-test (baseline feeding)*: present CS (sucrose or carrot) for 15 s; count bites for 1 min (exclude snails with <10 bites min^{-1}).
- *CTA training*: 10 CS–US pairings (15 s CS; gap per assigned ISI; 15 s US); inter-trial interval = 15 min; training performed in the morning; experimenter blind to group.
- *Immediate post-test*: CS alone; bites min^{-1} for 1 min.
- *Retention and extinction*: CS-only tests at 24 h and 7 d; additional CS-only trials (extinction series) to assess robustness. (*Lymnaea* CTA typically yields durable long-term memory and is resistant to extinction.)

(e) Experimental design

2 (CS salience: high versus low) × 2 (distractor: no versus yes) × 3 (ISI: 15 s, 1–3 min, 5 min) between-subjects; repeated measures over test time (pre, immediate post, 24 h, 7 d). *A priori* contrasts compare (i) distractor effects within each ISI and salience, (ii) high versus low salience at long ISIs, and (iii) forward versus backward controls.

(f) Controls

- Backward conditioning (US–CS) at matched ISIs. This group is a comparative control rather than a non-associative baseline.
- Naive (handled, no CS/US).
- Distractor control where the distractor cue is paired with the CS without US.

These directly address timing specificity and pseudoconditioning.

(g) Partial versus full trace conditioning: operational criteria

Beyond overall suppression, we score the latency of the first feeding suppression relative to CS onset and the learned ISI:

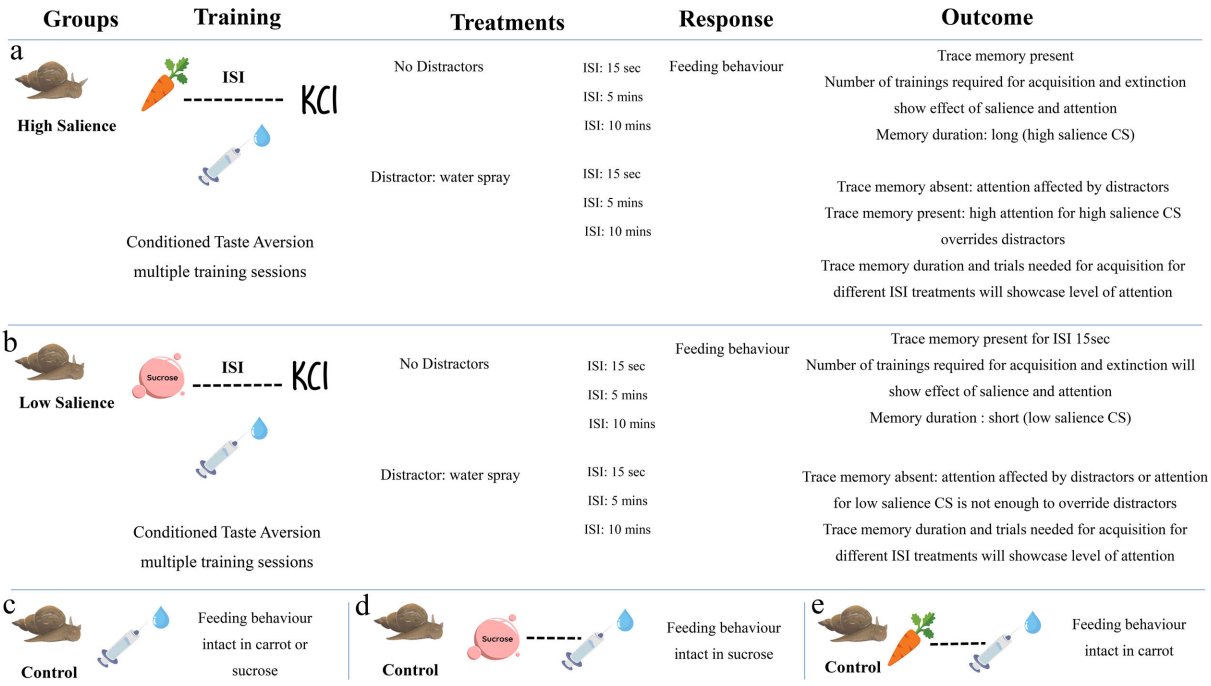

Figure 2. Experimental testing. This illustration shows how to test the two-tier model using *Lymnaea stagnalis*. (a) This group receives high-salience pretraining with carrot as CS and is expected to maintain the CS–US association even in the presence of distractors, suggesting the engagement of attention-based memory. (b) This group, with low-salience pretraining with sucrose CS, is expected to lose the association under distraction, indicating reliance on short-lived sensory traces. (c–e) Control groups are included to rule out pseudoconditioning and effects related to specific methodological protocols. ISI: inter-stimulus interval.

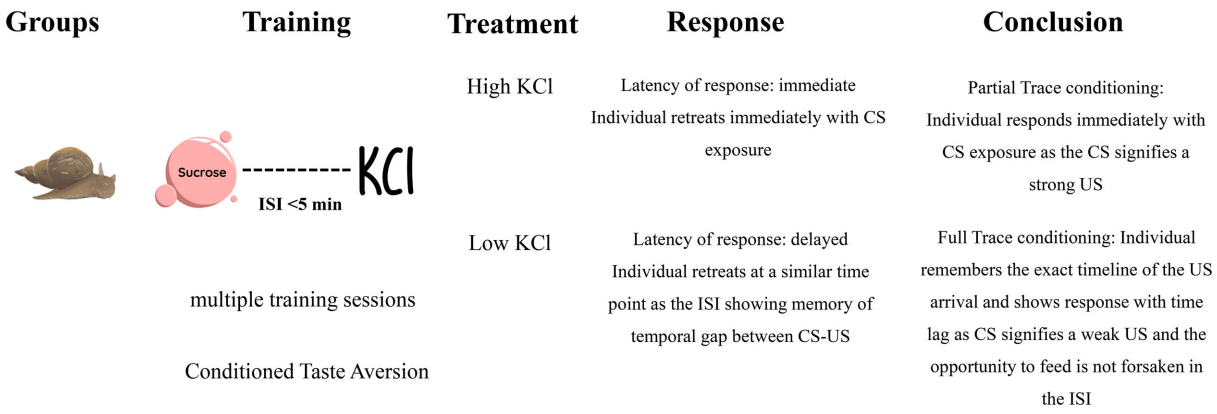

Figure 3. Partial versus full trace conditioning protocol. This illustration shows how to test for partial versus full trace conditioning using *Lymnaea stagnalis*. The US (KCl) is manipulated to be low or high salience, giving rise to responses where either the inter-stimulus interval is remembered by the organism when responding to CS only memory test, showing a delayed feeding withdrawal corresponding to the timing of the US arrival, or no memory of the temporal gap is present, and feeding withdrawal is immediate following exposure to CS. ISI: inter-stimulus interval.

— *Partial trace*: suppression begins immediately upon CS presentation (CS predicts 'aversive US is coming', but timing is not encoded).
— *Full trace*: suppression onsets near the expected US time (learned timing: the organism remembers when 'aversive US will come', so it continues feeding till that time).

(h) Outcome measures

— *Primary*: bites min^{-1} (pre versus post/retention).
— *Secondary*: latency-to-suppression (ms resolution from CS onset), distraction cost (Δ in suppression with versus without distractor) and extinction rate (trials-to-criterion).

(i) Predicted patterns and interpretation

Salience × *ISI*: At 15 s, both salience levels acquire CTA; at ≥1–3 min, high salience maintains suppression and shows higher distraction-resilience; at 5 min, performance declines and forward/backward begin to converge.

Distractor effects: Greater loss of suppression under distractor for low salience and longer ISIs; minimal effect at 15 s with high salience.

Timing readout: Latency clustering around the trained ISI (versus immediate) distinguishes full from partial trace conditioning.

5. Conclusions

In summary, our two-tier model of trace conditioning offers a mechanistic framework linking sensory-trace memory (Tier 1) and attention-based salience memory (Tier 2) to molecular, neural and behavioural processes that can be tested in different taxa, from invertebrates to mammals. By showing how transient sensory representations can be stabilized and temporally extended through neuromodulatory and attentional mechanisms, this model generates clear, testable predictions regarding the conditions under which partial versus full trace conditioning emerges. Experiments in *Lymnaea*—manipulating CS–US salience, trace interval duration and resistance to distractors—have the potential to reveal the minimal neural and molecular components required to bridge temporal gaps in associative learning. That said, our framework does not explicitly address how motivational states dynamically interact with attentional gating in real time. However, the model has the provision to test such conditions. We also do not yet specify the precise circuit architectures or neuromodulatory signatures that enable transitions between Tier 1 and Tier 2. Critical next experiments should therefore probe whether similar two-tier dynamics are observed across diverse invertebrate taxa, and whether manipulating internal states such as hunger, arousal or stress systematically alters trace learning. Comparative work combining behavioural assays with pharmacological interventions and circuit-level recordings will be essential to determine whether the mechanisms proposed here represent a conserved evolutionary pathway or multiple convergent strategies. Finally, this work highlights trace conditioning as a general cognitive capability rather than a vertebrate-exclusive cognitive phenomenon. Invertebrate models reveal how attentional and neuromodulatory systems sustain memory traces and bring forth some of the conserved evolutionary underpinnings of memory formation. By embracing invertebrate systems, we gain not only a deeper understanding of trace conditioning but also fundamental insights into how even the simplest nervous systems adapt across time, offering lessons that resonate broadly across species.

Ethics. This work did not require ethical approval from a human subject or animal welfare committee.

Data accessibility. This article has no additional data.

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. V.R.: conceptualization, investigation, methodology, visualization, writing—original draft; A.P.: conceptualization, investigation, methodology, visualization, writing—original draft; K.L.: conceptualization, writing—review and editing; A.B.: conceptualization, investigation, methodology, supervision, visualization, writing—original draft.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

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