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Caenorhabditis elegans: Learning Outcomes in Conditioned Inhibition Protocols are Consistent with Alpha Conditioning

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Conditioned inhibition (CI) refers to the learned property of a stimulus to predict the absence of an unconditioned stimulus (US). While excitatory forms of conditioning, like alpha conditioning, appear early in metazoan evolution, evidence of true CI in invertebrates remains scant. In the present study, we employed a negative contingency CI training design with an approach/withdrawal test to identify excitatory components involved in CI-like protocols in *Caenorhabditis elegans*. By using a joint-presentation procedure and alternating stimulus application sites across blocks, we minimised non-associative sensory effects. A control group was added to further consider context-dependent effects. Our results point to learning effects being consistent with excitatory (alpha) conditioning, as trained behavior was facilitated but did not depend on the conditioned stimulus. This underlines the widespread significance of alpha conditioning in invertebrate learning and its contribution to CI-like processes.

Keywords: alpha conditioning, associative learning, *Caenorhabditis elegans*, conditioned inhibition, non-associative learning

線虫（*Caenorhabditis elegans*）: 条件性抑制プロトコルにおける学習結果はアルファ条件付けと一致する

条件性抑制（CI）とは、無条件刺激（US）の不在を予測するという、刺激の学習された特性のことである。アルファ条件付けのような興奮性の条件付け様式は、後生動物の進化の初期段階で見られるが、無脊椎動物における真の CI の証拠は未だにほとんど見られない。本研究では、線虫（*Caenorhabditis elegans*）における CI 様プロトコルに関与した興奮性コンポーネントを特定するために、接近/回避テストによる負の随伴性 CI 訓練デザインを用いた。共同提示手続きを用い、さらに刺激の適用部位をブロックごとに交互に変えることで、非連合性の感覚効果の影響を最小化した。さらに文脈依存効果を考慮した対照群を追加した。結果として、訓練した動作は促進されたが、条件刺激には依存しておらず、興奮性の条件付け（アルファ条件付け）と一致した学習効果を示した。このことは、無脊椎動物の学習におけるアルファ条件付けの広範な意義と CI 様プロセスへの貢献を浮き彫りにしている。

キーワード：アルファ条件付け、連合学習、*Caenorhabditis elegans*、条件性抑制、非連合学習

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Caenorhabditis elegans: Resultados del aprendizaje en protocolos de inhibición condicionada son consistentes con el condicionamiento alfa

La inhibición condicionada (IC) se refiere a la propiedad aprendida de un estímulo para predecir la ausencia de un estímulo incondicional (EI). Aunque las formas excitatorias del condicionamiento, como el condicionamiento alfa, parecen haber surgido tempranamente en la evolución de los metazoos, la evidencia de una verdadera IC en invertebrados sigue siendo limitada. En el presente estudio se utilizó un diseño de entrenamiento de IC basado en contingencia negativa, junto con una prueba de aproximación/evitación, con el objetivo de identificar los componentes excitatorios implicados en protocolos similares a la IC en *Caenorhabditis elegans*. Mediante el uso de un procedimiento de presentación conjunta de estímulos y la alternancia de los sitios de aplicación de los estímulos entre bloques experimentales, se minimizaron los efectos sensoriales no asociativos. Adicionalmente, se incorporó un grupo control para considerar de manera más rigurosa los posibles efectos dependientes del contexto. Los resultados obtenidos sugieren que los efectos observados son consistentes con procesos de aprendizaje de carácter excitatorio, específicamente con el condicionamiento alfa, dado que la conducta entrenada fue facilitada, pero no dependió del estímulo condicionado. Estos hallazgos resaltan la importancia generalizada del condicionamiento alfa en el aprendizaje de los invertebrados y su contribución a los procesos que se asemejan a la inhibición condicionada.

Palabras clave: Condicionamiento alfa, aprendizaje asociativo, *Caenorhabditis elegans*, inhibición condicionada, aprendizaje no asociativo

Conditioned inhibition (CI) refers to a learned process in which a stimulus predicts the absence of an unconditioned stimulus (US), resulting in the omission or reduction of the response that would normally be elicited by that US (Savastano et al., 1999). Unlike conditioned suppression, CI is not based on the presence of an aversive US during training, but on the encoding of a contingency in which the US is omitted. Whereas excitatory conditioning and non-associative learning appeared early in metazoan evolution, CI has traditionally been thought to be restricted to vertebrates (Moore, 2004). Beyond these basic learning processes, the evolution of more integrated behavioral representations—supported by hierarchically organised inhibitory circuits—has also been proposed as a prerequisite for CI (Anokhin, 2016). Such circuits govern integrated behavioral responses or sequences via central pattern generators (CPGs; Papini, 2021).

In *Caenorhabditis elegans*, a locomotor CPG integrates input from multiple sensory modalities (Rakowski et al., 2013). Sensory neurons at anterior and posterior regions include receptors for chemical and mechanical stimuli and project directly to command interneurons that coordinate motor neurons in the ventral nerve cord (Cho et al., 2020; Hilliard et al., 2002; Stirman et al., 2011). Activation in anterior sensory neurons excites interneurons responsible for backward movement while inhibiting those mediating forward movement, whereas posterior stimulation produces the opposite pattern (e.g., Hilliard et al., 2002).

These inhibitory stimulations are mediated both by lateral connections among command interneurons (Cho et al., 2020; Hilliard et al., 2002; Stirman et al., 2011) and by second-layer interneurons, such as ring interneuron M (RIM), which partially orchestrate mutual inhibition within this network (Piggott et al., 2011; Stetak et al., 2009). While non-associative learning in *C. elegans* is closely linked to sensory neuron adaptation, associative learning has been connected to plasticity within this CPG-interneuron circuitry.

In excitatory conditioning, changes in command interneurons and in their direct and RIM-mediated synapses play a central role (Amano & Maruyama, 2011; Nishijima & Maruyama, 2017; Piggott et al., 2011; Rahmani & Chew, 2021; Stetak et al., 2009). Although *C. elegans* exhibits several forms of non-associative and associative learning (e.g., Rahmani & Chew, 2021; Roman et al., 2023), the distinction between both mechanisms can be difficult to establish (Nishijima & Maruyama, 2017). For example, sensory sensitisation can explain some learning outcomes commonly attributed to aversive associative learning (Abramson & Buckbee, 1995), and many forms of excitatory associative learning may reflect alpha conditioning—that is, the strengthening of pre-existing neural pathways—sometimes mimicking long-time or conditioned sensitisation (hence also called pseudo-conditioning; Moore, 2004). Alpha conditioning is considered an evolutionary precursor to instrumental conditioning and has been defined as a form of associative learning where a stimulus eliciting a response (US) is paired to a non-neutral stimulus, and the conditioned response

derives from an existing innate connection (Abramson, 1994).

Evidence for CI in invertebrates is scant (Chandra et al., 2010; Moore, 2004), with honeybees (*Apis mellifera*) and snails (*Helix aspersa*) representing notable exceptions (Abramson, 1986; Abramson & Bitterman, 1986; Acebes et al., 2012; Menzel et al., 1984). While these animals have also been shown to learn via conditioned suppression (Abramson et al., 2009), this seems to have certain limitations to stimulus-appropriate responses (Smith et al., 1991). Notably, processes resembling CI in invertebrates often intertwine with non-associative mechanisms or excitatory conditioning, complicating their interpretation (Frequet, 1997; Roman et al., 2023). Latent inhibition, which can mimic CI under negative contingency protocols (Savastano et al., 1999), has been linked to context-specific mechanisms in invertebrates (Muñiz-Moreno & Loy, 2022) and specifically to context-dependent habituation in *C. elegans* (Chandra et al., 2010). Unlike CI, context conditioning effects in *C. elegans* have been well established (Dissegna et al., 2021; Lau et al., 2013; Rankin, 2000). Issues to precisely classify learning processes in invertebrates are well known and stem not only from theoretical inconsistencies, but also from the need to incorporate these categorical questions into empirical research (Abramson & Wells, 2018).

The aim of the present study was to test whether excitatory conditioning processes, like alpha conditioning, independently from non-associative learning processes, contribute to the behavioral outcomes in CI-like protocols in *C. elegans.* We employed a negative contingency procedure in which a CS predicted the absence of an aversive US. Approach and withdrawal responses to the US were measured under joint CS-US presentation before and after the training (*cf*. Savastano et al., 1999). This allowed us to quantify learning as a change in the US-elicited behavior relative to pre-training levels. We opted for these design choices rather than classical CI tests (i.e., summation and retardation) because our aim was to highlight the contribution of alpha conditioning processes within CI-like protocols, rather than to establish CI itself. Accordingly, we predicted that behavioral changes would reflect the trained response pattern rather than an inhibitory effect of the CS.

Method

Ethical Approval

We inquired the process for approval from the ethical committee of the University of Girona. Because this study only worked with invertebrates, an exemption from requiring ethical approval was granted.

Sample

The experiment was conducted in the Laboratory of Experimental Sciences of the Faculty of Education and Psychology at the University of Girona. Wild-type *Caenorhabditis elegans* were tested four days after hatching to control for developmental effects (cf. Amano & Maruyama, 2011). We raised the animals on standard agar-based nematode growth medium seeded with *Escherichia coli* (OP 50) as a food source and maintained at 19 °C.

Experimental Design

We implemented a mixed-model design and randomly assigned subjects to an experimental group (n = 12) or a control group (n = 12). The experiment was structured into three phases: pre-test (t_0), learning phase, and post-test (t_1, t_2).

We removed each subject individually from the *E. coli*-based (OP 50) nematode growth medium and transferred it with a worm pick (Picodna, flexible 0.2 mm platinum wire, flattened tip) to a separate Ø 35 mm Petri dish containing 5 mm of water. Pre-test observations confirmed that subjects moved freely under these conditions and responded reliably to the stimuli.

We administered two classes of stimuli: chemical and mechanosensory ones. We applied a drop-assay protocol for the stimulation of the chemical receptors (*cf*. Hilliard et al., 2002) and a modified eyelash method for mechanosensory stimulation using a Ø 0.05 mm artificial fibre (Chen & Chalfie, 2014; Stirman et al., 2011). This approach is similar to the von Frey hair algesiometry, where the characteristics of the mechanical touch are partially determined by the physical properties (i.e., stiffness, length) of the fibre that is used for the stimulation (Malfait et al., 2013). Following these principles, we selected parameters for fibres used to deliver the mechanical stimuli in pre-test evaluations of the animals' escape response. In order to avoid an increase of the sodium dodecyl sulphate (SDS) concentration in the dish over trials, we removed the subjects with a pipette and washed out the chemical solution by covering the pipette tip with a 30 µm nylon mesh, which retained the animals while allowing flow-through (*cf*. Amano & Maruyama, 2011) after each trial. Previous studies applied 0.1% SDS as chemical (repellent) stimulus in *C. elegans* (Cho et al., 2020; Hilliard et al., 2002). Our pre-tests showed that varying the SDS concentration by ± 0.05% altered the reaction strength towards the stimulus. Because mechanical force can vary substantially across experimenters (Nekimken et al., 2017), a single experimenter performed all tactile stimulations after calibrating individual force levels for light and hard touch according to the animals' behavioral responses.

Before the learning phase, we measured each subject's baseline response to the four possible combinations of stimulus modality and intensity: light touch with 0.05% SDS, hard touch with 0.05% SDS, light touch with 0.15% SDS, and hard touch with 0.15% SDS. We systematically varied the stimulus intensities in the baseline trials to account for potential differences in sensitivity towards the sensory modalities. Modality-site mappings were counterbalanced across subjects. In each of these pre-test trials, we delivered the tactile and chemical stimuli simultaneously from opposite body sites. We randomised trial order and introduced a ten-minute inter-trial interval (ITI) between single measurements. We classified behavioral responses as forward or backward movement. The classification of the response was grounded on wave propagation (forward movement: head-to-tail; backward movement: tail-to-head) and a displacement threshold of half a body length. Ambiguous observations and non-locomotor behaviors, like body bending, were classified separately and later discarded from further analysis.

In the training phase, the experimental group received ten CS- trials. Hereby, we counterbalanced the CS sensory modality and application site across the individuals, and stimulus intensity over sessions. This was done to compensate for potentially pre-existing biases in the processing of either modality or site. For every individual, we inverted the mapping of the CS relative to pre-test and post-test phases. In the training phase, the control group was allowed to habituate to the context and was given the same procedure with regards to their handling, except that they did not receive a CS-. This allowed for the control of temporal, contextual and procedural confounds (e.g., mechanical stimulation during transfers, temporary food restriction and absence of conspecifics).

The post-tests followed the same logic as the pre-test. We simultaneously administered CS and US to both sites, using the same stimulus combinations and the same modality-location mapping as in the pre-test. We conducted two post-tests to evaluate learning results: immediately after the learning period (t_1), to evaluate short-term memory, and three hours later (t_2), to assess longer-lasting memory effects (*cf*. Amano & Maruyama, 2011).

Table 1

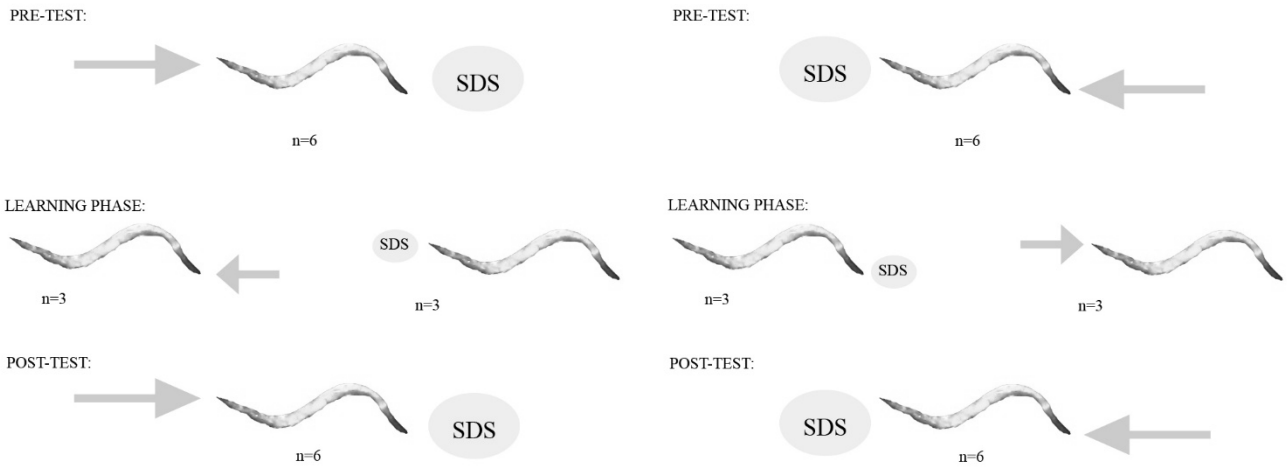
Stimulus Combinations and Counterbalancing for the Experimental Group

Subgroup	Experimental Phase		
	Pre-Measure	Training	Post-Tests
Subgroup I (***n*** = 3)	Front: Chemical Stimulus Back: Tactile Stimulus	CS-: Tactile (frontal application)	Front: Chemical Stimulus Back: Tactile Stimulus
Subgroup II (***n*** = 3)	Front: Tactile Stimulus Back: Chemical Stimulus	CS-: Tactile (posterior application)	Front: Tactile Stimulus Back: Chemical Stimulus
Subgroup III (***n*** = 3)	Front: Chemical Stimulus Back: Tactile Stimulus	CS-: Chemical (posterior application)	Front: Chemical Stimulus Back: Tactile Stimulus
Subgroup IV (***n*** = 3)	Front: Tactile Stimulus Back: Chemical Stimulus	CS-: Chemical (frontal application)	Front: Tactile Stimulus Back: Chemical Stimulus

Note. CS-modality and site were counterbalanced across individuals; stimulus intensity was counterbalanced across trials.

Figure 1

Experimental Design for the Experimental Group



Note. C. elegans with head facing right in the illustrations; "SDS" = sodium dodecyl sulphate; Arrows indicate the application of the mechanosensory stimulus. The illustrations of pre-test and post-test apply equally for the control group.

Statistical Analyses

We ran a Generalized Linear Mixed Model (GLMM) with a binomial distribution, using the *glmmTMB* (Brooks et al., 2017), *performance* (Lüdecke et al., 2019), *emmeans* (Lenth, 2025) and *DHARMa* packages (Hartig, 2016) in R (R Core Team, 2023). Following the logic of the approach/withdrawal test, we classified behavioral responses as one of two different outcomes. We expected behavioral changes between pre- and post-tests based on the hypothesized alpha conditioning processes when the initial response differed from the trained response. In contrast, we did not expect changes in coincident cases and in the control group. Thus we were able to model a binary response variable indicating whether individuals exhibited (1) or did not exhibit (0) a change in movement direction from pre-test to post-test within the same trial. In light of the experimental protocol, the use of a binary outcome measure was deemed the simplest adequate representation of the observations. Because we obtained two post-test measurements per trial (i.e., t_1 and t_2), we extracted two data points per trial. Excluding cases where the movement direction was ambiguous in either phase, our final dataset included 151 valid observations (107 no-expected changes, 44 expected changes).

In the full model, we included as test predictors the two-way interaction between condition (i.e., t_1 and t_2) and whether a change in movement direction was expected (i.e., yes or no). The full model also included the main terms of the interaction, sensory modality of the stimuli used in the learning phase as a control. We treated trial identity nested within subject identity as random intercepts to account for repeated measures. As we counterbalanced application site (front vs. tail) and stimulus intensity (high vs. low) across groups, we did not include these variables as predictors in the model.

We compared the full and null models using likelihood ratio tests (Dobson & Barnett, 2018) with $\alpha = 0.05$. When the full model outperformed the null model, we used the drop1 function to assess the significance of individual predictors. For significant interactions, we conducted Tukey-adjusted post-hoc comparisons using the *emmeans* package (Lenth, 2025). We checked model assumptions with the *DHARMa* package (Hartig, 2016), including residual diagnostics and overdispersion. We used the *performance* package (Lüdecke et al., 2019) to check for multicollinearity, which was low (maximum variance inflation factors across models = 1.34).

Results

Our analysis showed that the full model was significantly better than the null model (GLMM, $\chi^2 = 50.47$, $df = 3$, $p < .001$). In particular, we found a significant effect of the two-way interaction between condition (i.e., t_1 and t_2) and whether a change in movement direction was expected ($p = .004$). Post-hoc tests showed that subjects were more likely to change their movement direction when such a change was expected (i.e., when the trained reaction was opposite to the reaction performed in the pre-test), as compared to when it was not, but this effect was stronger in t_1 than in t_2 measurements (i.e., it was stronger immediately after the

learning phase than after 3 hours; p = .026). The control variable, sensory modality of the CS, did not significantly affect the likelihood of the directional change. In summary, our results showed that animals increasingly reproduced the escape response in the same orientation as during training, with a slight decline over time. The escape response in *C. elegans* was characterized as a rapid movement away from a stimulus, involving either reversal of movement or forward locomotion depending on the stimulus location.

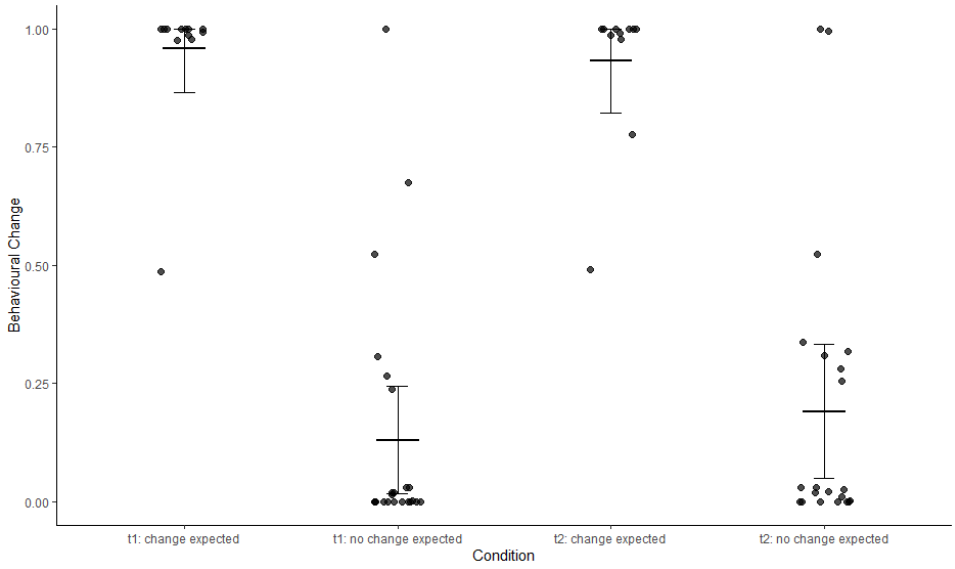
Table 2

Summary with the Results of the Generalized Linear Mixed Models; Significant Predictors are marked with an Asterisk, Reference Categories are in Parentheses, and Controls in Italics

	Estimate	95% CI	Std. Error	Pr(>|z|)
(Intercept)	-10.75	[-15.51,-5.99]	2.43	-
Expected Change in Direction * Measurement point (t_2)	-10.58	[-19.15 -2.01]	4.37	.016 *
Expected Change in Direction	28.82	[15.79 41.84]	6.64	
Measurement Point (t_2)	1.81	[-1.18, 4.80]	1.53	
Modality of stimuli in learning phase (Chemical)	-0.10	[-6.42, 6.22]	3.22	.975
Modality of stimuli in learning phase (Mechanical)	-0.20	[-6.93, 6.52]	3.43	.953

Figure 2

Probability of Behavioral Changes Across the Different Conditions



Note. Black circles represent average values for each individual and condition. Thick lines represent the mean of the individual values for each condition, and the ends of the whiskers represent the 95% confidence intervals, as assessed from the raw data.

Discussion

In this study, we applied a CI-like negative contingency training protocol. Before and after training, we measured the ratio of approach versus withdrawal from the US when it was presented jointly with the CS. We compared the outcomes between one pre-test and two post-tests in both an experimental group and a control group.

Our results indicate that, in the applied protocol, changes in movement direction after the learning phase *C. elegans* can be predicted based on the behavioral implication of the conditioned stimulus (i.e., the site of application, regardless of the CS itself). This pattern of results is consistent with alpha conditioning, in which presentation of a stimulus during training strengthens the same response to a different stimulus. Our experiment minimized the impact of non-associative sensory effects and context-dependent learning commonly occurring in CI (especially negative contingency) protocols mentioned in the literature (Abramson & Bitterman, 1986; Savastano et al., 1999) through a stimulus-switching and context-control group design. Furthermore, the simultaneous presentation of CS and US speaks against true CI as a base for the observed outcomes. This supports our hypothesis of alpha conditioning processes as a behavioral component in CI-like protocols in *C. elegans*. Our results are therefore in line with previous findings on learning in *C. elegans,* which support the broader hypothesis that alpha conditioning is widespread in metazoans. On the other hand, evidence of true CI in invertebrates remains scarce (Chandra et al., 2010; Menzel et al., 1984; Moore, 2004). Contrary to this idea, reliable evidence has been described for the presence of CI processes in honeybees (Abramson & Aquino, 2002; Chandra et al., 2010; Giurfa & Sandoz, 2012). The division of labour in honeybees has been related to learning abilities (e.g., latent inhibition; Ben-Shahar et al., 2000) and is highly subjected to epigenetic control (Herb et al., 2012; Kucharski et al., 2008). Foraging behavior, social organisation (Kurismaa, 2018), and learning processes including conditioned (Mahoney et al., 2024) and latent inhibition (Chandra et al., 2000) are closely influenced by epigenetic mechanisms. These differences in cognitive processes have been reported to relate to developmental changes brain regions associated with sensory integration and hierarchical control over behavioral responses (Fahrbach et al., 1998). Genetic fixation of developmental pathways is a key driver of evolutionary novelty (Hall, 1999; Kurismaa, 2018) and is often mediated through changes in developmental timing. Differences in CI between populations of honeybees have been associated with (rapid) environmental changes being accompanied by changes in developmental timing (Abramson & Aquino, 2002). Development of brain regions responsible for sensory integration and hierarchical behavioral control is considered a key factor in the evolution of CI and other executive functions in honeybees (Farris, 2013; Menzel, 2012), and similar hierarchical elaboration characterises vertebrate brain evolution (Farris, 2013; Kaas, 2017).

Even though the locomotor CPG of *C. elegans* demonstrates that its nervous system can support sensory and behavioral integration—including external inhibition—the present findings underline the importance of relatively simple excitatory mechanisms (alpha conditioning) in CI-like protocols. Ultimately, the distinction between excitatory mechanisms and true CI likely reflects both qualitative and quantitative differences in neural architecture. Whereas *C. elegans*' nervous system is comprised of exactly 302 neurons, a honeybee brain contains roughly one million. The evolution of CI therefore seems to involve not only increased number of neurons, but also the emergence of additional layers of hierarchical processing (Anokhin, 2016).

Limitations

Our study has some technical limitations. First, the use of an automated stimulus-delivery system would increase the methodological precision. Specifically, adopting a microfluid platform similar to Cho et al. (2020) could greatly improve standardisation and reliability of stimulus application. While a common practice (*cf*. Chen & Chalfie, 2014; Stirman et al., 2011), the manual administration of mechanosensory stimuli has been argued to have certain drawbacks with regards to its precision (Nekimken et al., 2017). Automation of the mechanosensory stimulus would allow for a finer tuning of the stimulus parameters and the inquiry of

more nuanced hypotheses. Furthermore, automation would also allow greater throughput and sample sizes. In general, sample sizes of single-animal assays (e.g., Kuhara & Mori, 2006; Murakami et al., 2005; Nekimken et al., 2017; Youssef et al., 2021) are considerably smaller compared to massed-trials. Automated systems therefore represent a promising means to increase the sample sizes in single-animal experiments in future work. An increased sample size would provide the opportunity to include additional experimental conditions and controls (such as an equivalent exposure control for unspecific motivational effects), in order to further expand the understanding of factors implied in the observed learning processes.

Additionally, future studies would benefit from incorporating analyses of neuronal activity, such as calcium imaging (e.g., Cho et al., 2020), to identify physiological correlates of the observed behavioral changes. As a model organism, *C. elegans* provides a series of advantages and opportunities for neuroscientific research. We acknowledge that integrating behavioral and neural measurements further strengthens the understanding of mechanisms underlying learning processes in invertebrates.

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Availability of Data and Materials

R-Codes for the statistical analysis, data and additional visual materials are publicly available via Open Science Framework (https://osf.io/jevw4/?view_only=105a97418f6a49f6948d11b4b4e377fb).

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