Extracellular vesicles aid in the transfer of long-term associative memory between *Caenorhabditis elegans*

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Abstract:

Memory formation is necessary for the survival of animals across phyla. Here, we elucidate the mechanism underlying the formation of long-term associative memory (LTAM) formed by treating *Caenorhabditis elegans* with a volatile chemoattractant and heat. Previous work has shown that training animals with a paradigm involving heat and isoamyl alcohol (IAA) simultaneously, causes *C. elegans* to lose their attraction to IAA. In this study, we elaborate on the mechanism behind this LTAM formation and suggest that during training with heat and IAA, *C. elegans* release extracellular vesicles (EVs) that upon being taken up by the same trained animals or their untrained counterparts causes the organism to lose attraction to IAA. Our data suggests that the vesicles are highly specific to the training paradigms used and differ with differing cues. Finally, we show that this mechanism of transfer of LTAM appears to be conserved between *C. elegans* and *C. briggsae* allowing for both intra and interspecies transfer of memory.

Introduction

Native preferences and innate behaviours are essential for the survival of living organisms. Many innate behaviours represent the hardwired neural circuitry put in place during the development of an animal (Bateson & Mameli, 2007; Tierney, 1986). *Caenorhabditis elegans* has been widely used to study behavioural plasticity because of its ability to learn and modify its innate behaviour (reviewed in (Ardiel & Rankin, 2010)). With a nervous system consisting of 302 neurons, these animals show a variety of robust and adaptable behaviours like chemotaxis and thermotaxis (reviewed in (Ardiel & Rankin, 2010; Hart, 2006)). These behaviours allow *C. elegans* to move towards food sources, avoid toxic bacteria, and find mates, all of which rely heavily on chemosensation by the animal and innate preferences for certain odours ((Ballestriero *et al*, 2016) and reviewed in (Ferkey *et al*, 2021)). For example, under well fed naïve condition, *C. elegans* are attracted to odorants like isoamyl alcohol (IAA), which can be sensed by the AWC neuron (reviewed in (Bargmann, 2006)). However, this odour to behaviour relationship can be modified if the animal is exposed to periods of starvation along with IAA (Pereira & van der Kooy, 2012). Similarly, neutral concentrations of butanone can be turned attractive when repetitively paired with food. These modified behaviours can persist for up to forty hours post training (Kauffman *et al*, 2011). The above examples indicate the formation of associative memory, that is brought about as a result of the animal's interaction with two simultaneous cues.

Associative memory in *C. elegans* can be classified as short-term or long-term associative memory, depending on how long it can last. Previous studies have shown that pairing cues for a single conditioning period (mass training) forms short-term associative memory (STAM) in *C. elegans*, that last for around 2 hours. However, multiple conditioning along with periods of rest in between forms long-term associative memory (LTAM), which can last from 20 to 40 hours (Kauffman *et al.*, 2011). LTAM formation in *C. elegans* has largely been examined by pairing the presence/absence of food (unconditioned stimulus) with a variety of cues (Amano & Maruyama, 2011; Kauffman *et al.*, 2011; Nishijima & Maruyama, 2017). We had shown in a previous study that changes in innate behaviours using the chemoattractive odorant IAA and an aversive heat stimulus leads to loss of attraction to IAA, that lasts more than twenty

hours after training, hence giving rise to LTAM formation (Dahiya *et al*, 2019). Here, we propose a novel mechanism for LTAM formation and show that the effect of training *C. elegans* with a chemoattractant and heat may lead to the release of training cue specific extracellular vesicles (EVs). Further, these EVs may then be taken up by the same or by untrained counterparts that in turn allow naïve animals to gain LTAM, resulting in impaired attraction to the chemoattractant.

Caenorhabditis elegans can release EVs environmentally through sensory ciliary neurons, which can act as a mode of communication. Previous studies have shown the involvement of EVs in inter-animal communication in the context of mating ((Wang *et al*, 2014) and reviewed in (Wang *et al*, 2024a)). There are multiple ciliated EV releasing neurons (EVNs) in *C. elegans.* 21 of these are male-specific neurons in the head and tail, 6 are inner labial type 2 (IL2) neurons which are present in both males and hermaphrodites (O'Hagan *et al*, 2017). It has been previously reported that CIL-7, a myristoylated protein regulates EV biogenesis and is known to be an EV cargo. The kinesin-3 protein, KLP-6, is required for the release of EVs into the environment. Both these genes, *cil-7* and *klp-6*, are expressed in the IL2 neurons of hermaphrodite *C. elegans* (Kahn-Kirby & Bargmann, 2006; Maguire *et al*, 2015; Morsci & Barr, 2011; Peden & Barr, 2005; Wang *et al*, 2021). Recent work has also shown that multiple ciliated sensory neurons pack and export ciliary membranes and excess ciliary proteins through EVs (Lobo *et al*, 2025; Razzauti & Laurent, 2021). Here we report that EVs facilitate the transfer of LTAM from trained animals to both naïve and memory-defective *C. elegans*. The cAMP response element binding protein (CREB) has been shown to be involved in memory formation across phyla, with mutants in *creb1* shown to be defective in long-term memory formation in multiple organisms ((Dash *et al*, 1990) and reviewed in (Flavell & Greenberg, 2008; Silva *et al*, 1998)). We show that *creb1/crh-1* mutant animals that show no LTAM formation in this IAA and heat-based paradigm (Dahiya *et al*., 2019), show LTAM from exposure to the extrinsic factors (likely EVs) released by trained wild-type (WT) animals.

These data incentivised us to perform LC-MS experiments to identify the chemicals that may be sent out by trained *C. elegans*. Using the LC-MS data, we go on to show that a

113 cocktail of three chemicals sent out by *C. elegans* during the training process, likely
114 through EVs, allows for the animals to show no attraction specifically to IAA
115 recapitulating associative memory without a training paradigm.

116 This work adds to the growing body of literature describing the role of EVs in multiple
117 processes including cell death, neurodegenerative diseases and therapeutics
118 (Mohamed *et al*, 2025; Wang *et al*, 2025; Wiersema *et al*, 2024; Wright *et al*, 2025).

119 Lastly, we report that LTAM can be transferred across species of *Caenorhabditis*, where
120 *C. elegans* and *C. briggsae* can transfer LTAM from trained animals of one species to
121 naïve animals of the other species. Like most previous studies involving LTAM formation
122 in *C. elegans* and across phyla, we had speculated that the mechanism of LTAM
123 formation as a result of IAA and heat training, was intrinsic to the organism (Dahiya *et*
124 *al.*, 2019). Contrary to this hypothesis, our findings in this study indicate that LTAM can
125 be transferred from one *C. elegans* to another through environmentally released
126 extracellular vesicles (EVs), thus challenging the conventional notion of LTAM being
127 intrinsic to an organism.

128

129

130 **Materials and Methods**

131 **Strain maintenance and transgene generation**

132 All strains were cultured on nematode growth medium (NGM) plates, inoculated with
133 OP50 *E. coli* bacteria, and maintained under standard conditions at 22°C, following
134 established protocols outlined in previous literature (Brenner, 1974). The N2 Bristol strain
135 served as the standard wild-type (WT) control for comparative analyses across all
136 experimental procedures. A synchronous population of young adult *C. elegans* was
137 obtained using previously established protocols (Porta-de-la-Riva *et al*, 2012). The list
138 of strains used in this study is tabulated in Supplementary Table 1 (S1) and primers used
139 for genotyping the strains are tabulated in Supplementary Table 2 (S2). The *klp-6* rescue
140 construct P*klp-6*::GFP::KLP-6 (gift from Maureen Barr, details in (Wang *et al.*, 2014))

141 was microinjected in the *klp-6* mutant *C. elegans* to prepare transgenic lines as
142 previously described (C. Mello and Fire 1995; C. C. Mello et al. 1991).

143

144 **Assay to study long-term associative memory in *C. elegans***

145 All assays were performed as previously described in (Dahiya *et al.*, 2019) with minor
146 modifications. Briefly, the training paradigm involves simultaneous exposure of *C.*
147 *elegans* or *C. briggsae* young adults to two cues; heat and a chemoattractant (isoamyl
148 alcohol (IAA)/ Diacetyl (depending on the experiment)), for two minutes followed by ten
149 minutes of rest period at 22°C. This cycle is repeated five times, and the animals are
150 then kept at 22°C for 20 hours (illustrated in Figure 1A). This leads to the formation of
151 long-term associative memory (LTAM), seen in the form of reduced attraction towards
152 the chemoattractant, which can last for as long as 24 hours. LTAM is quantified at 20-24
153 hours from the time of training, using a readout called the chemotaxis index (CI), where
154 CI= Displacement along IAA, heptanone or diacetyl (depending on the odorant used)
155 gradient/Total distance traversed. The concentrations of the odorants used are as
156 previously published (Dahiya *et al.*, 2019; Zhang *et al*, 2016). CI is calculated by
157 analysing the videos of the *C. elegans* undergoing chemotaxis with respect to the
158 chemoattractant. The videos were analysed using FIJI, and an associated plugin,
159 Trackmate (Ershov *et al*, 2022; Schindelin *et al*, 2012). Each plot indicates a set of 3-6
160 replicates with all conditions/genotypes in the graph performed at the same time over
161 multiple days. Each replicate was performed using 8-10 *C. elegans*. A detailed
162 experimental protocol can be found in the Supplementary section.

163

164 **Exchange assays**

165 Naïve animals were transferred to an empty plate, followed by trained animals being
166 transferred to the plate that previously had naïve *C. elegans.* The final step involved
167 transferring the naïve animals to the plate that had had trained *C. elegans* (illustrated in
168 Figure 2A). This exchange protocol was completed within 30 minutes from the end of
169 training. After exchange, *C. elegans* were kept in a 22°C incubator and LTAM was tested
170 using the chemotaxis assay 20-24 hours after training. The exchange assays were

171 performed between *C. elegans* and *C. briggsae* in a similar manner with naïve *C.*
172 *elegans* transferred to plates that had contained trained *C. briggsae* and naïve *C.*
173 *briggsae* transferred to plates that had had trained *C. elegans*. All plates used for
174 training and transfer were 60 mm in diameter.

175

176 **Imaging experiments**

177 Freshly trained young adult hermaphrodite *C. elegans* were used for imaging as
178 previously described with minor modifications (Tikiyani *et al*, 2018). Briefly, *C. elegans*
179 were mounted on 1% agarose pads with 34 mg/ml 2,3-Butanedione Monoxime (BDM)
180 solution in M9 medium. Images were acquired as Z-stacks on a Zeiss Apotome
181 microscope using the 63X oil objective within four hours of the training paradigm. The
182 order of imaging of trained and control animals was changed for each of the three
183 replicates. For processing and analysis of images, FIJI (Schindelin *et al.*, 2012) was
184 used to quantify fluorescence intensity of cell body and number of puncta in each
185 condition by selecting specific regions of interest. A threshold was set for analysing
186 particles of each *C. elegans* to separately quantify cell body intensity and punctal
187 number across the neuronal processes. The summation of mean grey values using
188 "Analyze Particles" from each cell body per worm and the punctal number along each
189 neuronal process was counted using "Analyze Particles". Both cell body intensity and
190 punctal numbers were plotted as independent graphs. Each replicate had 4-7 *C.*
191 *elegans* that were imaged per condition over three replicates and each dot on the plots
192 indicates a single animal.

193

194 **Liquid Chromatography-Mass Spectrometry (LC-MS)**

195 The protocol used was modified from a previous study (Nikonorova *et al*, 2022). Sample
196 collection involved four 60 mm plates with approximately 70 *C. elegans*/plate for each
197 sample. Freshly trained WT animals were washed with M9 buffer and collected as one
198 sample. This was repeated for every group of *C. elegans*; naïve, trained, only IAA and
199 only heat. Next differential centrifugation was performed. Firstly, the *C. elegans*
200 suspension was centrifuged at 3,000 g for 15 minutes at 15°C to pellet the animals and

bacteria. This supernatant was transferred to fresh tubes and then centrifuged at 10,000 g for 30 minutes at 4°C. This step was repeated thrice. Lastly, the collected supernatant was sent to the IISc LC-MS facility for identification of extrinsic factor(s). Samples were similarly collected from the EV release defective mutants as naïve and trained animals as well as empty plates with and without *E. coli*. Each sample was processed in triplicate over multiple days.

Chemical supplementation assays

The surface of unseeded NGM plates were supplemented with 300 µl of 100 mM imazapyr (Sigma Aldrich, cat. no. 37877), 2-methoxy 5-methyl aniline (2M5M, Sigma Aldrich, cat. no. 103284) or sodium salt of glycochenodeoxycholic acid (SGCDC, SRL, cat. no. 97971). For the fourth condition, all three chemicals were supplemented together (100 µl each of 100 mM of imazapyr, 2M5M and SGCDC). These plates were freshly seeded with OP50 *E. coli* bacteria after the chemical solutions had dried. Finally, a synchronized young adult population of naïve *C. elegans* was transferred (30-35 animals) to each plate. Chemotaxis to IAA and diacetyl was tested at 3.5 hours and 6 hours from the time-point of supplementation. These concentrations and the time for assays were chosen after standardization procedures involving multiple concentrations of the chemicals and multiple time-points at which the assays were performed.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 8. Outliers were identified using Grubbs method ($\alpha = 0.05$) for all plots. For all plots, the mean values were compared using one-way ANOVA with Dunnett's correction. The level of significance was set as $p \leq 0.05$. The statistical analyses used for each plot is indicated in the figure legends.

Results

Long-term Associative memory in *C. elegans* requires extrinsic factor(s) released by the animals

We have previously published a training paradigm to study long-term associative memory (LTAM) in wild-type (WT) *C. elegans* using isoamyl alcohol (IAA) and heat ((Dahiya *et al.*, 2019) and illustrated in Figure 1A). Using this paradigm, we replicated the data showing that unlike naïve animals, their trained counterparts showed a significantly lower chemotaxis index (CI) to IAA (Figure 1B, tracks indicated in Figure S1A). To investigate the underlying molecular mechanism of LTAM formation we planned to perform RNA sequencing experiments using naïve and trained WT animals.

In order to perform this experiment optimally, adult *C. elegans* had to be synchronized (without eggs and L1 animals) at 20 hours post training for RNA extraction ((Brenner, 1974) and illustrated in Figure S1B)). To remove eggs and L1s from the population, the *C. elegans* were separated from eggs twice (at 12 and 8 hours) during the twenty-hour resting stage after training and a similar protocol was followed for naïve animals (illustrated in Figure S1B). We initially gently washed the *C. elegans* and eggs with M9 to remove the *C. elegans* from the eggs on the plate at both time points. However, upon testing the animals for LTAM after the washes, we found the animals no longer showed LTAM (Figure S1C). This prompted us to pick animals at the standardized 12-hour time point during the 20-hour resting period to circumvent the effects of washing. Again, we found that the animals removed from the plates at the 12-hour time-point no longer showed LTAM (Figure 1F). This prompted us to hypothesize that trained *C. elegans* may be releasing factor(s) onto the plate that could allow for LTAM formation. In order to test the time-point at which the animals may be releasing unknown factor(s) we tested the animals by picking them from the plate within a few minutes of training (t_0), 6 hours after training (t_6), 12 hours after training (t_{12}), and keeping some animals in the original plate (illustrated in Figure 1C). We found that only the animals on the original plate showed LTAM formation (Figure 1B), while all other animals lost LTAM associated with heat and IAA (Figures 1D-F).

Our experiments indicated that removal of *C. elegans* to new plates immediately after training led to a loss of LTAM allowing us to conclude that *C. elegans* may be releasing unknown factor(s) during training or immediately after training. We then decided to test if these factor(s) could impart LTAM to naïve animals.

Naïve *C. elegans* on trained plates gain LTAM

To investigate if the factor(s) released by *C. elegans* during training were indeed required for LTAM formation, we decided to expose naïve *C. elegans* to the factor(s) released by the animals during training. For this, we performed an exchange assay, where naïve animals were transferred to plates that previously had trained animals, and vice versa (illustrated in Figure 2A). This exchange was performed within 30 minutes of training. Intriguingly, we observed that naïve *C. elegans* that were transferred to plates that had had trained animals showed LTAM, similar to what we observed in trained control animals (Figure 2B, tracks indicated in Figure S2A). Consistent with our previous results, trained animals that were transferred to plates that had had naïve *C. elegans*, showed a loss of LTAM (Figure 2B, tracks indicated in Figure S2A). This led us to conclude that these factor(s) released by *C. elegans* during training are sufficient to induce LTAM in naïve animals.

Next, to make sure that the factor(s) released on the plate were not produced by bacteria on the plate as the animals are well fed with OP50 *E. coli* throughout their training and resting phases, we performed the same experiments with heat-killed *E. coli.* The *E. coli* were heat-killed as previously described (Qi *et al*, 2017). We observed the same results as previously seen with live *E. coli* (Figure 2C). To confirm the associative nature of the transferred LTAM, we tested WT *C. elegans* with heat and IAA in an unpaired assay. In order to perform this experiment, we exposed naïve *C. elegans* to heat and IAA one after the other as illustrated in Figure S2B and found that in neither case could the animals form LTAM. Further, the plates that had had trained *C. elegans* did not allow for naïve *C. elegans* placed on them to gain LTAM (Figures S2C and D).

287 Our data so far indicates that the associative training paradigm allows *C. elegans* to
288 release factor(s) onto its surroundings that may be taken up by naïve animals which then
289 go on to show LTAM, hence allowing for the horizontal transfer of LTAM across animals.
290 Next, we wanted to understand the specificity of these released factor(s).

291

292 **The training cue lends specificity to factor(s) released by trained *C. elegans***

293 Our results so far indicate horizontal transfer of LTAM in naïve animals when transferred
294 to a plate that had previously contained trained animals. We wanted to understand the
295 specificity of the released factor(s). Different odours can be sensed by largely distinct
296 neurons in *C. elegans* (reviewed in (Chou et al, 1996)). IAA is known to be sensed by the
297 AWC neurons, that can also sense heptanone ((Colbert & Bargmann, 1995; Zhang *et al.*,
298 2016) and illustrated in Figure S3). In order to test the specificity of the secreted factor(s)
299 we took animals trained using the IAA and heat paradigm, along with naïve animals
300 transferred to plates that had contained trained *C. elegans* and tested their
301 chemoattraction towards heptanone. We observed that these *C. elegans* showed no
302 LTAM formation with respect to heptanone (Figure 3A). These results indicate a specificity
303 of the released factor(s) that allow for LTAM formation only with respect to the odour used
304 for training.

305 We next decided to test LTAM formation with respect to another chemoattractive odour
306 sensed by a different neuron. Diacetyl is an odour that is sensed by the AWA neuron
307 ((Sengupta *et al*, 1996; Sengupta *et al*, 1994) and illustrated in Figure S3). We trained
308 animals with heat and IAA and found that trained animals and naïve animals transferred
309 to plates that had contained trained animals did not show any defects in chemotaxis with
310 respect to diacetyl (Figure 3B). Our previous work has shown that the heat and
311 chemoattractant based training paradigm may be used with diacetyl and heat in a process
312 similar to that performed with IAA and heat (Dahiya *et al.*, 2019). We next trained the
313 animals with heat and diacetyl and found that they showed LTAM towards diacetyl (Figure
314 3C). Further, naïve animals placed on a plate that had had trained *C. elegans* showed
315 LTAM towards diacetyl (Figure 3C). This experiment indicates that a release of factor(s)
316 occurs independent of whether the odour is IAA or diacetyl; the nature of these factor(s),

317 however, appears to be extremely specific to the odorant used during training. Finally, we
318 trained animals with diacetyl and tested them with IAA and found no LTAM formation
319 towards IAA (Figures 3D). These data together indicate a high degree of specificity of the
320 factor(s) released for allowing LTAM formation only with respect to the cue present in the
321 training paradigm.

322 We next wanted to study the role of these factor(s) in animals that are known to be
323 defective in memory formation.

324

325 **LTAM defective *C. elegans* show LTAM from exposure to plates that had contained**
326 **trained WT animals**

327 A plethora of literature has implicated CREB1 (cAMP Response Element Binding protein
328 1) in long-term memory formation across phyla ((Bartsch *et al*, 1998; Timbers & Rankin,
329 2011; Yin *et al*, 1995; Yin *et al*, 1994) and reviewed in (Flavell & Greenberg, 2008;
330 Johannessen *et al*, 2004; Silva *et al.*, 1998; Yin & Tully, 1996)). We have shown that the
331 mutants of the ortholog of mammalian *creb1*, *crh-1* in *C. elegans,* show LTAM defects in
332 the heat and IAA paired assay (Dahiya *et al.*, 2019). We decided to test if *crh-1* mutants
333 allowed for LTAM formation when transferred to a plate that had contained trained WT
334 animals (illustrated in Figure 4A). Our data indicates that *crh-1* mutants that basally show
335 no LTAM, show LTAM when transferred to a plate which had had WT trained animals. We
336 also found that WT trained animals transferred to a plate that had contained trained *crh-1*
337 mutant *C. elegans* do not show LTAM (Figure 4B and tracks indicated in Figure S4). These
338 data allowed us to conclude that *crh-1* mutants may have defects in the synthesis or
339 release of the factor(s) that allow for LTAM formation but appear to be able to uptake these
340 factor(s) to show LTAM.

341 We next wanted to gain insight into the mechanism of secretion of the factor(s) during
342 training of *C. elegans*.

343

344 **Extracellular vesicles are required for LTAM formation**

A well-studied mechanism of social behaviour in *C. elegans* is through secreted small molecule signalling by ascarosides (reviewed in (Yang *et al*, 2023)). Pre-exposure to different ascarosides can change the chemotaxis of *C. elegans* to different odours (Wu *et al*, 2023). We decided to test for LTAM formation in the *daf-22* mutant *C. elegans* that are defective in ascaroside biosynthesis (Golden & Riddle, 1985). We found that *daf-22* mutants showed no defects in LTAM formation (Figure 5A). These data indicate that the secreted factor(s) may not be *daf-22* dependent. Apart from ascarosides, sensory signalling allowing for behaviours like mate sensing through chemoattraction requires extracellular vesicle (EV) release from *C. elegans* (reviewed in (Wang *et al.*, 2024a)). We next tested mutants in EV biogenesis (*cil-7*) and release (*klp-6*) for LTAM formation (Wang *et al.*, 2021). We found that both mutants showed no LTAM formation (Figure 5B and C). In order to further confirm the role of EVs in the process of LTAM, we attempted to rescue the loss of LTAM phenotype in *klp-6* mutants by expressing KLP-6 specifically in the IL2 neurons where KLP-6 has been shown to be expressed in hermaphrodites (Peden & Barr, 2005; Wang *et al*, 2024b). We observed that expressing KLP-6 in the mutant *klp-6* animals rescued the loss of LTAM phenotype where both trained *C. elegans* as well as naïve animals transferred to plates that had contained trained animals now showed LTAM formation (Figure 5D). We next decided to test if *klp-6* mutants allowed for LTAM formation when transferred to a plate that had trained WT animals. We found that trained *klp-6* mutants transferred to a plates which had had trained WT animals showed LTAM, while trained WT animals transferred to plates that had contained trained *klp-6* mutant *C. elegans* did not show LTAM (Figure 5E and tracks indicated in Figure S5). These data allow us to conclude that EV release may be the basis for LTAM formation in a chemoattractant and heat-based associative training paradigm.

We next investigated changes in EV expression in the nervous system upon training *C. elegans* with heat and IAA.

Changes in EV biogenesis marker expression upon heat and IAA training

Previous work has elegantly shown that an EV cargo, CIL-7, is released from the IL2 neurons into the external region around *C. elegans* (Wang *et al.*, 2024b). We tested the

375 same line for changes in fluorescence expression in the IL2 neurons and found that the
376 *cil-7 (my61[cil-7::mNG])I; him-5(e1490)* line showed increased fluorescence in the IL2 cell
377 bodies and an increase in fluorescent puncta along the IL2 neuronal processes in trained
378 WT animals when compared to the control *C. elegans* that were either naïve or trained
379 with only IAA or only heat (Figures 6A and B). With these data indicating the involvement
380 of EVs in LTAM transfer. We next decided to perform a Liquid Chromatography-Mass
381 Spectrometry (LC-MS) to study the differences in the released products upon heat and
382 IAA training when compared to control animals.
383

384 **A mixture of three chemicals mimics memory formation in *C. elegans***

385 LC-MS was performed using WT *C. elegans* as well as *klp-6* mutant animals. Multiple LC-
386 MS experiments were performed, each in triplicate as indicated in Figure 7A. This
387 experiment allowed us to find 9 consistent hits that were only present in the heat and IAA
388 trained *C. elegans* plates (Figure 7A and S6). These data suggest that multiple ions are
389 differentially released onto the plates upon heat and IAA training. These ions may allow
390 for LTAM formation in *C. elegans*. Based on the availability of chemicals, we
391 supplemented the plates of naïve animals with three chemicals from our candidate list –
392 imazapyr, 2M5M and SGCDC. Each molecule was supplemented on NGM plates onto
393 which naïve animals were placed. To understand their cumulative role, we also
394 supplemented all three chemicals together.

395 we observed that imazapyr or SGCDC alone did not alter the chemotaxis towards IAA in
396 treated *C. elegans*, whereas 2M5M supplementation led to the loss of attraction to IAA,
397 seen as a lower chemotaxis index when compared to naïve animals This loss of attraction
398 to IAA phenotype was more pronounced in *C. elegans* treated with all three molecules
399 (Figure 7B, and track images in S7A). This effect was seen at the 3.5 hours' time-point
400 from supplementation and is lost by 6 hours after supplementation (Figure 7C), indicating
401 that these molecules allow for memory formation, although not LTAM formation. This could
402 be because of the absence of the other six molecules from our candidate list, along with
403 other cargo including nucleic acids that are usually known to be carried by EVs released
404 into the environment (Nikonorova *et al.*, 2022). We also checked the chemotaxis of naïve
405 and animals trained with IAA and heat towards Diacetyl at the 3.5- and 6-hour time-points.

At both time points we observed no loss of attraction towards Diacetyl (Figures 7D and E). As controls we performed the training and testing protocols with WT *C. elegans* where testing was performed at 3.5 and 6 hours after training. The aversion to IAA was consistently seen at both time points (Figures S7B and C). These results establish our findings that specific factors released in response to training, could lead to specific loss of attraction towards the odorant used in the training paradigm.

Finally, we wanted to test if LTAM formation is conserved across *Caenorhabditis* species. In order to perform this experiment, we investigated LTAM formation in *Caenorhabditis briggsae.*

***Caenorhabditis* show interspecies transfer of LTAM**

We first tested the *C. briggsae* strain for LTAM formation to heat and IAA and found that *C. briggsae* were surprisingly not attracted to IAA even in naïve conditions (Figure S8A and B). This precluded us from studying LTAM formation in *C. briggsae* using the IAA and heat based associative paradigm. However, since *C. briggsae* naïve animals behaved like *C. elegans* trained animals (Figure S8B), we went on to transfer *C. briggsae* naïve animals to a plate that had contained *C. elegans* trained animals and found that there was no change in *C. briggsae* chemotaxis index. We also transferred trained *C. elegans* to a plate that had contained naïve *C. briggsae* (exchange assay) and found that these animals behaved like naïve *C. elegans* (Figure S8B). These data could not allow us to make any conclusions other than the fact that *C. briggsae* was not attracted to IAA in the naïve condition unlike *C. elegans*.

We next tested if *C. briggsae* was attracted to diacetyl. We found that *C. briggsae* was both attracted to and formed LTAM with heat and diacetyl (Figure 8A). We went on to transfer *C. briggsae* naïve animals to a plate that had contained *C. briggsae* trained animals and vice versa. We found that *C. briggsae* naïve animals acquired LTAM from the plate that had contained trained *C. briggsae*, and surprisingly the trained *C. briggsae* did not lose LTAM from being on a plate that had had naïve *C. briggsae* (Figure 8B). These data indicate that a similar mechanism may allow for LTAM transfer in *C. briggsae* as is seen in *C. elegans*, however the LTAM appears to be more stable in *C. briggsae* when

compared to *C. elegans*. Finally, we tested if interspecies transfer of LTAM could occur. For this experiment we transferred naïve *C. briggsae* to a plate that had contained trained *C. elegans* and transferred naïve *C. elegans* to a plate that had contained trained *C. briggsae* and found that both sets of animals showed LTAM (Figure 8C). We also transferred trained *C. elegans* to a plate that had contained naïve *C. briggsae* and unsurprisingly found no LTAM formation (Figure 8C).

Together, our data indicates the role of extracellular vesicles in LTAM formation and its horizontal transfer in *C. elegans* along with its possible role in this phenomenon across species.

Discussion

In this study we report three novel findings that have broad implications. First, *C. elegans* form long-term associative memory (LTAM) through environmentally released extracellular vesicles (EVs) from the IL2 neurons when the animals undergo training with heat and a chemoattractant. Further, these EVs appear to be cue specific and allow for LTAM formation only with respect to the cue used in the initial training paradigm. Second, LTAM can be transferred to naïve as well as memory-defective mutant animals possibly through EVs. Third, there appears to be conservation of this behaviour across *Caenorhabditis* species with EVs from *C. elegans* being able to elicit LTAM formation in *C. briggsae* and vice versa. These findings are illustrated in Figure 9. Below, we discuss the significance of these findings.

Novel mechanism for horizontal transfer of LTAM

Previous studies have shown that a form of non-associative memory can be transferred from one *Aplysia* to another by injecting RNA extracted from the trained animal into the naïve counterpart (Bedecarrats *et al*, 2018). More recent studies have shown that learned avoidance to pathogenic bacteria can be transferred from trained to naïve *C. elegans* through the *Cer1* retrotransposon (Moore *et al*, 2021). However, there appear to be no studies showing the organic transfer of LTAM from one organism to another without active communication between the animals. In this study, we show that *C. elegans* when trained

using an IAA and heat-based associative learning paradigm may release extrinsic factor(s). Removal of trained animals from these plates results in loss of LTAM, indicating that these factor(s) are responsible for the process of LTAM formation. Our results also show that these factor(s) are likely released during training. Naïve animals exposed to the factor(s) released by trained animals also show LTAM formation, similar to that seen in the original trained animals. This work uncovers a novel mechanism for the horizontal transfer of LTAM through secreted EVs from one *Caenorhabditis elegans* to another, irrespective of whether the animals have been trained with the associative learning paradigm. Further, our work suggests that EVs released due to training appear to carry extremely specific cargo with respect to the odorant cue used during training. The cargo may dynamically change with changing training cues allowing for cue specific LTAM formation.

LTAM formation in memory defective animals

Apart from allowing for LTAM formation in naïve animals, we show that the factor(s) (EVs) secreted by wild-type (WT) *C. elegans* also show LTAM in memory defective *creb1/crh-1* and EV biogenesis/cargo defective animals using multiple previously described mutants (Dahiya *et al.*, 2019; Maguire *et al.*, 2015; Wang *et al.*, 2021; Wang *et al.*, 2024b). These animals gain LTAM to the specific isoamyl alcohol (IAA) cue by being placed on a plate that had had WT animals trained with heat and IAA. This intriguing finding may allow for a better understanding of the factor(s) released and the mechanism of EV uptake/action that appear to be intact in these mutants that show defects in LTAM formation. Future work may allow for a better understanding of the involvement of CREB/CRH-1 in the observed LTAM formation and transfer phenomenon.

A chemical cocktail that allows *C. elegans* to behave like it has been trained with heat and IAA

We found that a cocktail of Imazapyr, 2-methoxy 5-methylaniline (2M5M) and Glycochenodeoxycholic acid (SGCDC) elicits a loss of attraction specifically to IAA, albeit for a short time-span of upto 3.5 hours. How these chemicals may act throws open interesting future experiments to understand memory and chemosensation in *Caenorhabditis*. Imazapyr is known to be a non-selective herbicide which controls

496 vegetation, especially hard-to-control perennial grass by inhibiting the acetolactate
497 synthase (ALS) enzyme. It can disrupt protein synthesis and interfere with the synthesis
498 of DNA and cell growth (Dersch *et al*, 2016). ALS is the first enzyme in the pathway for
499 the synthesis of branched-chain amino acids (Dezfulian *et al*, 2017). 2M5M is an organic
500 compound that is largely used in the production of dyes and pigments of inks and paints.
501 It is also used as a catalyst in reactions like nitration of aromatic compounds.
502 (https://chem-iso.com/18630). Finally, SGCDC, usually found as a sodium salt is a bile
503 salt found in the liver, generated from chenodeoxycholic acid and glycine. It has been
504 shown to inhibit autophagosome formation lysosomal function leading to apoptosis of
505 human hepatocyte cells (Lan *et al*, 2020). However, to our knowledge, none of these
506 compounds have been studied in the context of *C. elegans*. It would be interesting to test
507 more of the compounds found in our LC-MS experiments to try and get a better
508 understanding of what *C. elegans* release during heat and IAA training.

509 **LTAM transfer between *Caenorhabditis briggsae* and *Caenorhabditis elegans***

510 We show that *C. briggsae*, a relative of *C. elegans*, with a divergence of tens of millions
511 of years (Butler *et al*, 1981) also show LTAM formation through released factor(s) that are
512 likely to be EV dependent. We found that *C. briggsae* do not appear to be attracted to IAA
513 in a manner similar to *C. elegans*, likely due to the high divergence in the olfactory
514 pathways of these animals (Jovelin *et al*, 2009). However, both animals showed attraction
515 towards diacetyl. We show that LTAM can transfer from trained *C. briggsae* to naïve *C.*
516 *elegans* and trained *C. elegans* to naïve *C. briggsae*, indicating a conserved mechanism
517 of LTAM transfer across *Caenorhabditis,* likely through EVs.

518 The one interesting difference we found was that unlike *C. elegans*, *C. briggsae* does not
519 seem to lose LTAM when removed from the plates that they were trained on. This could
520 be because of two possibilities; *C. briggsae* release the extrinsic factor(s) continuously for
521 a period of time, long enough for LTAM consolidation, or these factor(s) (EVs) are stable
522 and functional for longer periods of time in *C. briggsae.* Further studies may allow for
523 parsing out these differences between the two species.

Our work leads to multiple outstanding questions that may allow for a better understanding of the process of LTAM transfer across organisms. We briefly discuss some of these unanswered questions below.

Neuronal circuitry allowing for EV release by IL2 neurons for LTAM formation

Studies have shown that CIL-7, a myristoylated protein regulates EV biogenesis along with being an EV cargo and the kinesin-3 protein KLP-6 is required for the release of EVs. Both these genes, *cil-7* and *klp-6,* are expressed in the IL2 neurons in *C. elegans* hermaphrodites (Kahn-Kirby & Bargmann, 2006; Maguire *et al.*, 2015; Morsci & Barr, 2011; Peden & Barr, 2005; Wang *et al.*, 2021). Here we report that *cil-7* and *klp-6* mutant animals are LTAM defective, uncovering a previously unreported role of the IL2 neurons in LTAM formation.

This raises important questions in understanding the neuronal circuitry involved. Different neurons are responsible for sensing different odorants in *C. elegans*. Our experiments indicate that animals trained with IAA (sensed by the AWC neurons) and heat versus diacetyl (sensed by the AWA neurons) and heat release EVs with different cargo causing cue-specific LTAM formation. Understanding the neuronal circuitry between chemosensory neurons and IL2 neurons may allow for a better understanding of the specificity of LTAM formation.

Dynamic EV cargo for cue-specific LTAM

C. elegans show LTAM formation and transfer, specific to the odorant cue used during training. For examining the extrinsic factor(s) released by trained animals, we performed LC-MS to identify candidate molecules present exclusively on the plates of trained animals (Figure 6B). One of these molecules 4-methylaminobutanal, might be of potential interest. IAA is chemically 3-methylbutanol, and a biological pathway for the conversion of 3-methylbutanol to 4-methylaminobutanal may exist. The steps would include oxidation of 3-methylbutanol to 3-methylbutanal through an alcohol dehydrogenase (Illikoud *et al*, 2018), followed by a transamination step producing 3-methylbutylamine (Encyclopedia of Sensors and Biosensors, 2023). This intermediate could potentially result in 4-methylaminobutanal through additional reactions. We have shown that a cocktail of three

553 of these LC-MS hits allows for C. elegans to lose attraction to IAA, how these and other
554 molecules function would allow for more insight into what EV cargoes may consist of as
555 well allow for a better understanding of memory towards IAA. Since we see specificity of
556 LTAM formation to the odorant used in the training paradigm, it is plausible that different
557 odorants may allow for release of cue-specific cargo. This in turn may give rise to the
558 specificity of the observed LTAM phenomenon.

559 **Uptake of EV cargo for LTAM transfer and a role for RNAs in this process**

560 Transfer of LTAM from one *C. elegans* to another entails factor(s) (EVs) released by
561 trained animals, and its uptake by naïve animals. This uptake could occur through a
562 variety of mechanisms. Is the cargo released through EVs retained within the EV structure
563 after release into the environment? Are the individual components of vesicles dispersed
564 onto the plates? Further, does the uptake mechanism involve only neurons/glia or could
565 other tissues like cuticles/hypodermis be involved? Understanding these aspects of the
566 uptake mechanism may shed more light on the process of LTAM transfer across
567 *Caenorhabditis* species.

568 RNA has been implicated in memory in multiple model organisms. The role of different
569 types of non-coding RNAs like micro-RNAs (miRNAs) and Piwi-interacting RNAs
570 (piRNAs) have been explored and studied in the context of long-term non-associative
571 memory in *Aplysia* (Fiumara *et al*, 2015; Rajasethupathy *et al*, 2012; Rajasethupathy *et*
572 *al*, 2009). Further, it has been shown in *Aplysia* that memory formed from long-term
573 sensitization (non-associative) can be transferred from trained to naïve animals
574 (Bedecarrats *et al.*, 2018). More recently, multiple studies in have defined the role of non-
575 coding RNAs in transgenerational memory as well (reviewed in (Miska & Rechavi, 2021)).
576 Studies have also shown that small non-coding RNAs expressed in *Pseudomonas* strains
577 allow for the transgenerational inheritance of avoidance memory in *C. elegans* (Kaletsky
578 *et al*, 2020; Legue *et al*, 2022; Sengupta *et al*, 2024; Seto *et al*, 2024). Additionally, Moore
579 et al, 2021 has shown that this avoidance memory can also be horizontally transferred
580 from one animal to another (trained to naïve *C. elegans*) through virus-like particles
581 encoded by the *Cer1* retrotransposon (Moore *et al.*, 2021). Multiple studies have shown
582 that extracellular vesicles from *C. elegans* carry proteins, non-coding RNAs, miRNAs, lipid

nanodomains, enzymes and other chemicals (Nikonorova *et al.*, 2022; Russell *et al*, 2020). Collectively, these findings suggest the involvement of RNA in learning and memory and may allow for the hypothesis that non-coding RNAs or miRNAs could be involved in LTAM uptake from trained to naïve animals through EVs.

In summary, we report a novel mechanism of LTAM transfer through environmentally released EVs. This, to the best of our knowledge, is the first report of organic transfer of long-term associative memory between organisms and across species.

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Conflict of Interest:

Authors declare no conflict of interests.

643 **Figure legends:**

644

645 **Figure 1: Long-term associative memory (LTAM) in *C. elegans* requires the release**
646 **of extrinsic factor(s) by trained animals**

647 (A) Illustration of the long-term associative memory (LTAM) training paradigm used in
648 this study. (B) Violin plots showing chemotaxis index (CI) in *C. elegans*. The presence of
649 LTAM is indicated as a reduction in the chemotaxis index (CI) of trained animals. (C)
650 Illustration of the time-point assay to test the time of release of factor(s) required for
651 LTAM formation. (D, E, F) Violin plots showing CI of *C. elegans* that were removed at
652 time-points t_0 (D), t_6 (E) and t_{12} (F) hours respectively from plates on which the animals
653 were trained. Multiple comparisons were performed using one-way ANOVA and p-values
654 were adjusted using Dunnett's correction, "***" indicates p=0.0002 and "ns" indicates not
655 significant.

656

657 **Figure 2: LTAM is gained by naïve *C. elegans* from trained plates**

658 (A) Schematic of the exchange assay to expose naïve *C. elegans* to factor(s) released
659 by freshly trained animals. The numbers along the arrows indicate the sequence of
660 moving *C. elegans* across multiple plates. (B) Violin plots showing CI in naïve *C.*
661 *elegans* when exposed to factor(s) released by trained animals. (C) Violin plots showing
662 CI in *C. elegans* trained on plates with heat-killed bacteria. Multiple comparisons were
663 performed using one-way ANOVA and p-values were adjusted using Dunnett's
664 correction, "****" indicates p< 0.0001 and "ns" indicates not significant.

665

666 **Figure 3: The factor(s) released during training are cue-specific**

667 (A, B) Violin plots showing CI of animals trained using IAA and tested with heptanone (A)
668 and diacetyl (B). Animals trained using IAA show no decrease in CI towards heptanone
669 or diacetyl indicating no LTAM towards a chemoattractant not used during training. (C)
670 Violin plots indicating diacetyl specific CI in diacetyl and heat trained *C. elegans* and
671 naïve animals exposed to factor(s) released by trained animals. (D) Violin plots showing
672 CI of animals trained with diacetyl and tested with IAA. Multiple comparisons were
673 performed using one-way ANOVA and p-values were adjusted using Dunnett's
674 correction, "****" indicates p< 0.0001 and "ns" indicates not significant.

675

676 **Figure 4: LTAM can be gained by memory defective *crh-1* mutant *C. elegans* from**
677 **trained plates that contained wild-type animals**

(A) Schematic representation of exchange assay between wild-type (WT) and *crh-1* mutant *C. elegans.* The numbers along the arrows indicate the sequence of moving *C. elegans* across multiple plates. (B) Violin plots indicating CI in *crh-1* mutant animals (naïve and trained) when exposed to factor(s) released by trained WT animals. Multiple comparisons were performed using one-way ANOVA and p-values were adjusted using Dunnett's correction and Sidak's correction (for within group comparisons), "**" indicates $p < 0.01$, "***" indicates $p < 0.001$ and "ns" indicates not significant. Note that in the *crh-1* trained violin plot, only 16 animals are plotted across 3 replicates, although 8 *C. elegans* were taken per replicate. This is because of multiple tracks that overlapped during analyses and could not be separated out.

Figure 5: Role of extracellular vesicles released from IL2 neurons in LTAM formation and transfer

(A) Violin plots indicating CI in *daf-22* mutant animals defective in ascaroside biosynthesis. (B) Violin plots indicating CI in *klp-6* mutant animals defective in extracellular vesicle release. These animals show no LTAM formation. (C) Violin plots indicating CI in *cil-7* mutant animals defective in extracellular vesicle biosynthesis. These animals also show no LTAM formation. (D) Violin plots indicating CI in the *klp-6* rescuing strain. (E) Violin plots indicating CI in *klp-6* mutant animals when exposed to factor(s) released by WT animals, no LTAM was observed in WT animals exposed to plates that had had trained *klp-6* mutants. Multiple comparisons for all plots were performed using one-way ANOVA and p-values were adjusted using Dunnett's correction, "**" indicates $p < 0.01$, "****" indicates $p < 0.0001$ and "ns" indicates not significant.

Figure 6: Increase in EVs in trained *C. elegans*

(A) Representative fluorescence imaging of the IL2 neurons using the *cil-7*::mNG marker line in naïve, trained, IAA only and heat only conditions. (B) Violin plots showing total fluorescence intensity of cell bodies of IL2 neurons (above) and the number of puncta across the IL2 processes (below). The trained animals show increased cell body fluorescence and increased punctal number along the IL2 processes when compared to the control *C. elegans*. White arrowheads indicate cell bodies and yellow arrowheads indicate puncta in all images. Multiple comparisons were performed using one-way ANOVA and p-values were adjusted using Dunnett's correction, "***" indicates $p < 0.001$, "****" indicates $p < 0.0001$ and "ns" indicates not significant.

Figure 7: A cocktail of molecules released during training from C. elegans is sufficient to induce memory formation in *C. elegans*

715 (A) LC-MS data analysed from eight animal groups processed as three replicates per
716 group; WT trained and controls (4 groups), *klp-6* naïve and trained *C. elegans* (2 groups)
717 and empty plates containing NGM alone or NGM+OP50 *E. coli* (2 groups). The venn
718 diagrams were created using the Venny 2.1 software
719 (https://bioinfogp.cnb.csic.es/tools/venny/). The list of molecules present exclusively on
720 WT trained plates in every replicate is indicated at in the bottom panel of 7A. (B) Violin
721 plots indicating CI towards IAA in naïve *C. elegans* placed on NGM plates with OP50
722 containing nothing (naïve), Imazapyr, 2M5M, SGCDC or all 3 chemicals. This
723 experiment was performed at 3.5 hours after placing young adult animals on the plate.
724 (C) Violin plots indicating CI towards IAA in naïve *C. elegans* placed on NGM plates with
725 OP50 containing nothing (naïve), Imazapyr, 2M5M, SGCDC or all 3 chemicals. This
726 experiment was performed at 6 hours after placing young adult animals on the plate. (D)
727 Violin plots indicating CI towards diacetyl in naïve *C. elegans* placed on NGM plates with
728 OP50 containing nothing (naïve), Imazapyr, 2M5M, SGCDC or all 3 chemicals. This
729 experiment was performed at 3.5 hours after placing young adult animals on the plate.
730 (E) Violin plots indicating CI towards diacetyl in naïve *C. elegans* placed on NGM plates
731 with OP50 containing nothing (naïve), Imazapyr, 2M5M, SGCDC or all 3 chemicals. This
732 experiment was performed at 6 hours after placing young adult animals on the plate.
733 Multiple comparisons were performed using one-way ANOVA and p-values were
734 adjusted using Dunnett's correction and Sidak's correction (for within group
735 comparisons), "*" indicates $p < 0.05$, "**" indicates $p < 0.01$, "****" indicates $p < 0.0001$ and
736 "ns" indicates not significant.

737

738 **Figure 8: LTAM formation and transfer is conserved across *Caenorhabditis***
739 **species**

740 (A) Violin plots indicating CI in *C. briggsae* trained with the Diacetyl and heat associative
741 learning paradigm. (B) Violin plots indicating CI in *C. briggsae* that underwent exchange
742 from trained plates. This plot indicates conservation of the LTAM transfer phenomenon
743 from naïve to trained animals in *C. briggsae*. Further, no LTAM loss is seen in trained *C.*
744 *briggsae* when removed from trained plates, unlike in *C. elegans*. (C) Violin plots
745 indicating CI after transferring *C. briggsae* to plates that had contained trained *C.*
746 *elegans* and transferring *C. elegans* to plates that had contained trained *C. briggsae*.
747 This plot indicates interspecies transfer of LTAM from *C. elegans* to *C. briggsae* and vice
748 versa. Multiple comparisons were performed using one-way ANOVA and p-values were
749 adjusted using Dunnett's correction and Sidak's correction (for within group
750 comparisons), "*" indicates $p < 0.05$, "***" indicates $p < 0.001$, "****" indicates $p < 0.0001$
751 and "ns" indicates not significant.

752

Figure 9: Model

Illustration of the proposed mechanistic model showing that the release of EVs from IL2 in trained *C. elegans* allows for LTAM formation in naïve animals, memory defective *crh-1* mutant *C. elegans* and EV biogenesis defective *klp-6* mutant *C. elegans*. Further, this transfer of LTAM is conserved across *Caenorhabditis*, with LTAM formation occurring in naïve *C. briggsae* from EVs of trained *C. elegans* and vice versa. The numbers indicate the order of processes including EV release followed by uptake.

References:

Amano H, Maruyama IN (2011) Aversive olfactory learning and associative long-term memory in Caenorhabditis elegans. *Learn Mem* 18: 654-665

Ardiel EL, Rankin CH (2010) An elegant mind: learning and memory in Caenorhabditis elegans. *Learn Mem* 17: 191-201

Ballestriero F, Nappi J, Zampi G, Bazzicalupo P, Di Schiavi E, Egan S (2016) Caenorhabditis elegans employs innate and learned aversion in response to bacterial toxic metabolites tambjamine and violacein. *Sci Rep* 6: 29284

Bargmann CI (2006) Chemosensation in C. elegans. *WormBook*: 1-29

Bartsch D, Casadio A, Karl KA, Serodio P, Kandel ER (1998) CREB1 encodes a nuclear activator, a repressor, and a cytoplasmic modulator that form a regulatory unit critical for long-term facilitation. *Cell* 95: 211-223

Bateson P, Mameli M (2007) The innate and the acquired: useful clusters or a residual distinction from folk biology? *Dev Psychobiol* 49: 818-831

Bedecarrats A, Chen S, Pearce K, Cai D, Glanzman DL (2018) RNA from Trained Aplysia Can Induce an Epigenetic Engram for Long-Term Sensitization in Untrained Aplysia. *eNeuro* 5

Brenner S (1974) The genetics of Caenorhabditis elegans. *Genetics* 77: 71-94

Butler MH, Wall SM, Luehrsen KR, Fox GE, Hecht RM (1981) Molecular relationships between closely related strains and species of nematodes. *J Mol Evol* 18: 18-23

Chou JH, Troemel ER, Sengupta P, Colbert HA, Tong L, Tobin DM, Roayaie K, Crump JG, Dwyer ND, Bargmann CI (1996) Olfactory recognition and discrimination in Caenorhabditis elegans. *Cold Spring Harb Symp Quant Biol* 61: 157-164

Colbert HA, Bargmann CI (1995) Odorant-specific adaptation pathways generate olfactory plasticity in C. elegans. *Neuron* 14: 803-812

Dahiya Y, Rose S, Thapliyal S, Bhardwaj S, Prasad M, Babu K (2019) Differential Regulation of Innate and Learned Behavior by Creb1/Crh-1 in Caenorhabditis elegans. *J Neurosci* 39: 7934-7946

Dash PK, Hochner B, Kandel ER (1990) Injection of the cAMP-responsive element into the nucleus of Aplysia sensory neurons blocks long-term facilitation. *Nature* 345: 718-721

Dersch LM, Beckers V, Rasch D, Melzer G, Bolten C, Kiep K, Becker H, Blasing OE, Fuchs R, Ehrhardt T *et al* (2016) Novel Approach for High-Throughput Metabolic Screening of Whole Plants by Stable Isotopes. *Plant Physiol* 171: 25-41

Dezfulian MH, Foreman C, Jalili E, Pal M, Dhaliwal RK, Roberto DK, Imre KM, Kohalmi SE, Crosby WL (2017) Acetolactate synthase regulatory subunits play divergent and overlapping roles in branched-chain amino acid synthesis and Arabidopsis development. *BMC Plant Biol* 17: 71

Ershov D, Phan MS, Pylvanainen JW, Rigaud SU, Le Blanc L, Charles-Orszag A, Conway JRW, Laine RF, Roy NH, Bonazzi D *et al* (2022) TrackMate 7: integrating state-of-the-art segmentation algorithms into tracking pipelines. *Nat Methods* 19: 829-832

Ferkey DM, Sengupta P, L'Etoile ND (2021) Chemosensory signal transduction in Caenorhabditis elegans. *Genetics* 217

Fiumara F, Rajasethupathy P, Antonov I, Kosmidis S, Sossin WS, Kandel ER (2015) MicroRNA-22 Gates Long-Term Heterosynaptic Plasticity in Aplysia through Presynaptic Regulation of CPEB and Downstream Targets. *Cell Rep* 11: 1866-1875

Flavell SW, Greenberg ME (2008) Signaling mechanisms linking neuronal activity to gene expression and plasticity of the nervous system. *Annu Rev Neurosci* 31: 563-590

Golden JW, Riddle DL (1985) A gene affecting production of the Caenorhabditis elegans dauer-inducing pheromone. *Mol Gen Genet* 198: 534-536

Hart AC (2006) Behavior. *WormBook*

Illikoud N, Klopp C, Roulet A, Bouchez O, Marsaud N, Jaffres E, Zagorec M (2018) One complete and three draft genome sequences of four Brochothrix thermosphacta strains, CD 337, TAP 175, BSAS1 3 and EBP 3070. *Stand Genomic Sci* 13: 22

Johannessen M, Delghandi MP, Moens U (2004) What turns CREB on? *Cell Signal* 16: 1211-1227

Jovelin R, Dunham JP, Sung FS, Phillips PC (2009) High nucleotide divergence in developmental regulatory genes contrasts with the structural elements of olfactory pathways in caenorhabditis. *Genetics* 181: 1387-1397

Kahn-Kirby AH, Bargmann CI (2006) TRP channels in C. elegans. *Annu Rev Physiol* 68: 719-736

Kaletsky R, Moore RS, Vrla GD, Parsons LR, Gitai Z, Murphy CT (2020) C. elegans interprets bacterial non-coding RNAs to learn pathogenic avoidance. *Nature* 586: 445-451

Kauffman A, Parsons L, Stein G, Wills A, Kaletsky R, Murphy C (2011) C. elegans positive butanone learning, short-term, and long-term associative memory assays. *J Vis Exp*

Lan W, Chen Z, Chen Y, Tan M, Chen Y, Chen J, Chi X, Chen Y (2020) Glycochenodeoxycholic acid impairs transcription factor E3 -dependent autophagy-lysosome machinery by disrupting reactive oxygen species homeostasis in L02 cells. *Toxicol Lett* 331: 11-21

Legue M, Caneo M, Aguila B, Pollak B, Calixto A (2022) Interspecies effectors of a transgenerational memory of bacterial infection in Caenorhabditis elegans. *iScience* 25: 104627

Lobo T, Haasnoot GH, Nawrocka A, Bruggeman CW, Razzauti A, Peterman EJG, Laurent P (2025) Sensory stimuli and cilium trafficking defects trigger the release of ciliary extracellular vesicles from multiple ciliary locations. *bioRxiv*: 2025.2006.2006.658075

Maguire JE, Silva M, Nguyen KC, Hellen E, Kern AD, Hall DH, Barr MM (2015) Myristoylated CIL-7 regulates ciliary extracellular vesicle biogenesis. *Mol Biol Cell* 26: 2823-2832

Miska EA, Rechavi O (2021) Can brain activity transmit transgenerationally? *Curr Top Dev Biol* 144: 1-18

Mohamed AH, Abaza T, Youssef YA, Rady M, Fahmy SA, Kamel R, Hamdi N, Efthimiado E, Braoudaki M, Youness RA (2025) Extracellular vesicles: from intracellular trafficking molecules to fully fortified delivery vehicles for cancer therapeutics. *Nanoscale Adv* 7: 934-962

Moore RS, Kaletsky R, Lesnik C, Cota V, Blackman E, Parsons LR, Gitai Z, Murphy CT (2021) The role of the Cer1 transposon in horizontal transfer of transgenerational memory. *Cell* 184: 4697-4712 e4618

Morsci NS, Barr MM (2011) Kinesin-3 KLP-6 regulates intraflagellar transport in male-specific cilia of Caenorhabditis elegans. *Curr Biol* 21: 1239-1244

Nikonorova IA, Wang J, Cope AL, Tilton PE, Power KM, Walsh JD, Akella JS, Krauchunas AR, Shah P, Barr MM (2022) Isolation, profiling, and tracking of extracellular vesicle cargo in Caenorhabditis elegans. *Curr Biol* 32: 1924-1936 e1926

Nishijima S, Maruyama IN (2017) Appetitive Olfactory Learning and Long-Term Associative Memory in Caenorhabditis elegans. *Front Behav Neurosci* 11: 80

O'Hagan R, Silva M, Nguyen KCQ, Zhang W, Bellotti S, Ramadan YH, Hall DH, Barr MM (2017) Glutamylation Regulates Transport, Specializes Function, and Sculpts the Structure of Cilia. *Curr Biol* 27: 3430-3441 e3436

Peden EM, Barr MM (2005) The KLP-6 kinesin is required for male mating behaviors and polycystin localization in Caenorhabditis elegans. *Curr Biol* 15: 394-404

Pereira S, van der Kooy D (2012) Two forms of learning following training to a single odorant in Caenorhabditis elegans AWC neurons. *J Neurosci* 32: 9035-9044

Porta-de-la-Riva M, Fontrodona L, Villanueva A, Ceron J (2012) Basic Caenorhabditis elegans methods: synchronization and observation. *J Vis Exp*: e4019

Qi B, Kniazeva M, Han M (2017) A vitamin-B2-sensing mechanism that regulates gut protease activity to impact animal's food behavior and growth. *Elife* 6

Rajasethupathy P, Antonov I, Sheridan R, Frey S, Sander C, Tuschl T, Kandel ER (2012) A role for neuronal piRNAs in the epigenetic control of memory-related synaptic plasticity. *Cell* 149: 693-707

Rajasethupathy P, Fiumara F, Sheridan R, Betel D, Puthanveettil SV, Russo JJ, Sander C, Tuschl T, Kandel E (2009) Characterization of small RNAs in Aplysia reveals a role for miR-124 in constraining synaptic plasticity through CREB. *Neuron* 63: 803-817

Razzauti A, Laurent P (2021) Ectocytosis prevents accumulation of ciliary cargo in C. elegans sensory neurons. *Elife* 10

Russell JC, Kim TK, Noori A, Merrihew GE, Robbins JE, Golubeva A, Wang K, MacCoss MJ, Kaeberlein M (2020) Composition of Caenorhabditis elegans extracellular vesicles suggests roles in metabolism, immunity, and aging. *Geroscience* 42: 1133-1145

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B *et al* (2012) Fiji: an open-source platform for biological-image analysis. *Nat Methods* 9: 676-682

Sengupta P, Chou JH, Bargmann CI (1996) odr-10 encodes a seven transmembrane domain olfactory receptor required for responses to the odorant diacetyl. *Cell* 84: 899-909

Sengupta P, Colbert HA, Bargmann CI (1994) The C. elegans gene odr-7 encodes an olfactory-specific member of the nuclear receptor superfamily. *Cell* 79: 971-980

Sengupta T, St Ange J, Kaletsky R, Moore RS, Seto RJ, Marogi J, Myhrvold C, Gitai Z, Murphy CT (2024) A natural bacterial pathogen of C. elegans uses a small RNA to induce transgenerational inheritance of learned avoidance. *PLoS Genet* 20: e1011178

Seto R, Brown R, Kaletsky R, Parsons LR, Moore RS, Murphy CT (2024) Pseudomonas fluorescens 15 small RNA Pfs1 mediates transgenerational epigenetic inheritance of pathogen avoidance in C. elegans through the Ephrin receptor VAB-1. *bioRxiv*

Silva AJ, Kogan JH, Frankland PW, Kida S (1998) CREB and memory. *Annu Rev Neurosci* 21: 127-148

Tierney AJ (1986) The evolution of learned and innate behavior: Contributions from genetics and neurobiology to a theory of behavioral evolution. *Anim Learn Behav* 14: 339-348

Tikiyani V, Li L, Sharma P, Liu H, Hu Z, Babu K (2018) Wnt Secretion Is Regulated by the Tetraspan Protein HIC-1 through Its Interaction with Neurabin/NAB-1. *Cell Rep* 25: 1856-1871 e1856

Timbers TA, Rankin CH (2011) Tap withdrawal circuit interneurons require CREB for long-term habituation in Caenorhabditis elegans. *Behav Neurosci* 125: 560-566

Wang J, Barr MM, Wehman AM (2024a) Extracellular vesicles. *Genetics* 227

Wang J, Nikonorova IA, Silva M, Walsh JD, Tilton PE, Gu A, Akella JS, Barr MM (2021) Sensory cilia act as a specialized venue for regulated extracellular vesicle biogenesis and signaling. *Curr Biol* 31: 3943-3951 e3943

Wang J, Saul J, Nikonorova IA, Cruz CN, Power KM, Nguyen KC, Hall DH, Barr MM (2024b) Ciliary intrinsic mechanisms regulate dynamic ciliary extracellular vesicle release from sensory neurons. *Curr Biol* 34: 2756-2763 e2752

Wang J, Silva M, Haas LA, Morsci NS, Nguyen KC, Hall DH, Barr MM (2014) C. elegans ciliated sensory neurons release extracellular vesicles that function in animal communication. *Curr Biol* 24: 519-525

Wang J, Wang X, Luo H, Xie Y, Cao H, Mao L, Liu T, Yue Y, Qian H (2025) Extracellular vesicles in Helicobacter pylori-mediated diseases: mechanisms and therapeutic potential. *Cell Commun Signal* 23: 79

Wiersema AF, Rennenberg A, Smith G, Varderidou-Minasian S, Pasterkamp RJ (2024) Shared and distinct changes in the molecular cargo of extracellular vesicles in different neurodegenerative diseases. *Cell Mol Life Sci* 81: 479

940 Wright SS, Kumari P, Fraile-Agreda V, Wang C, Shivcharan S, Kappelhoff S, Margheritis EG,
941 Matz A, Vasudevan SO, Rubio I *et al* (2025) Transplantation of gasdermin pores by extracellular
942 vesicles propagates pyroptosis to bystander cells. *Cell* 188: 280-291 e217
943 Wu T, Ge M, Wu M, Duan F, Liang J, Chen M, Gracida X, Liu H, Yang W, Dar AR *et al* (2023)
944 Pathogenic bacteria modulate pheromone response to promote mating. *Nature* 613: 324-331
945 Yang B, Wang J, Zheng X, Wang X (2023) Nematode Pheromones: Structures and Functions.
946 *Molecules* 28
947 Yin JC, Del Vecchio M, Zhou H, Tully T (1995) CREB as a memory modulator: induced
948 expression of a dCREB2 activator isoform enhances long-term memory in Drosophila. *Cell* 81:
949 107-115
950 Yin JC, Tully T (1996) CREB and the formation of long-term memory. *Curr Opin Neurobiol* 6:
951 264-268
952 Yin JC, Wallach JS, Del Vecchio M, Wilder EL, Zhou H, Quinn WG, Tully T (1994) Induction of a
953 dominant negative CREB transgene specifically blocks long-term memory in Drosophila. *Cell* 79:
954 49-58
955 Zhang C, Zhao N, Chen Y, Zhang D, Yan J, Zou W, Zhang K, Huang X (2016) The Signaling
956 Pathway of Caenorhabditis elegans Mediates Chemotaxis Response to the Attractant 2-
957 Heptanone in a Trojan Horse-like Pathogenesis. *J Biol Chem* 291: 23618-23627

958

Figure 1

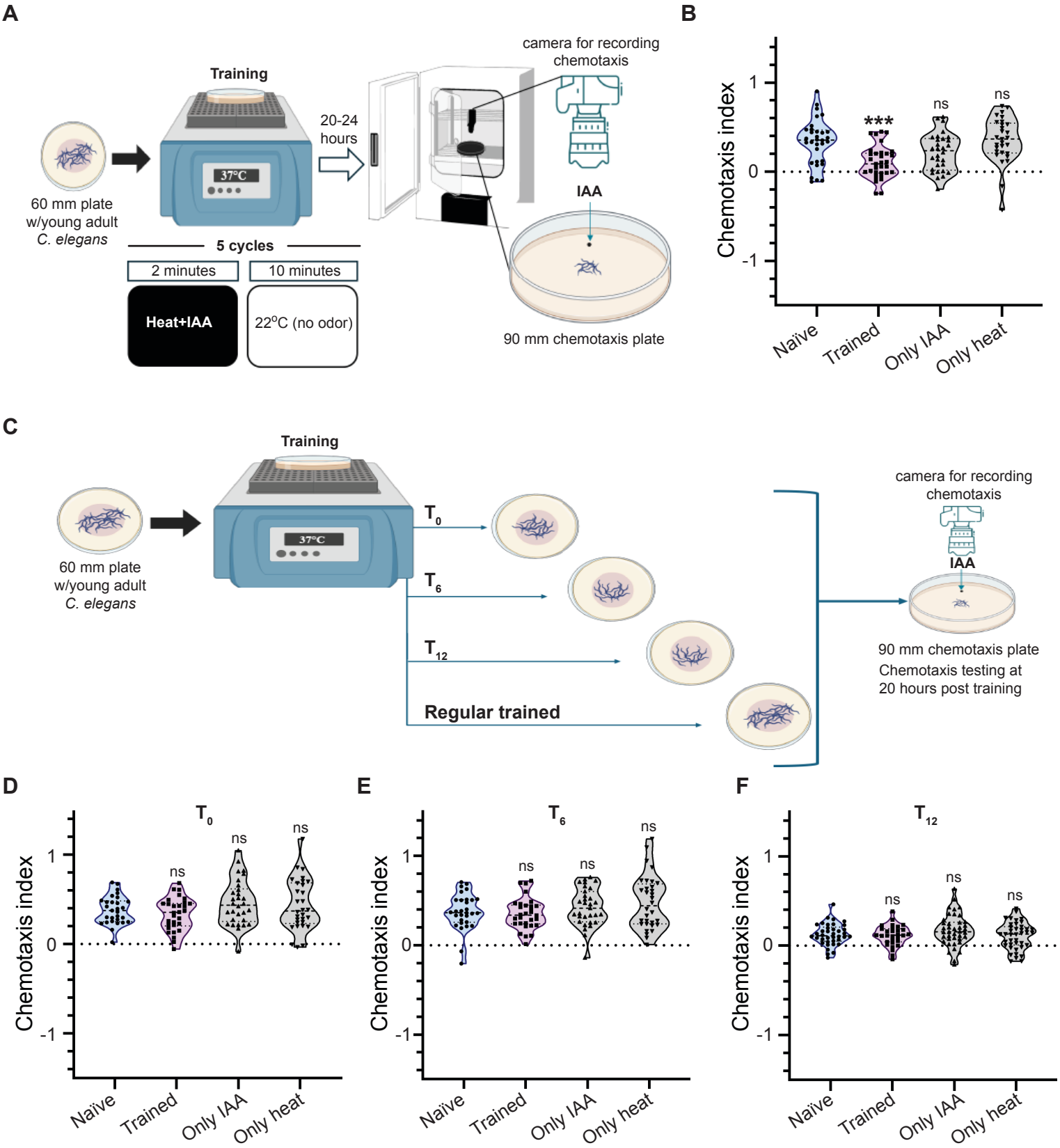
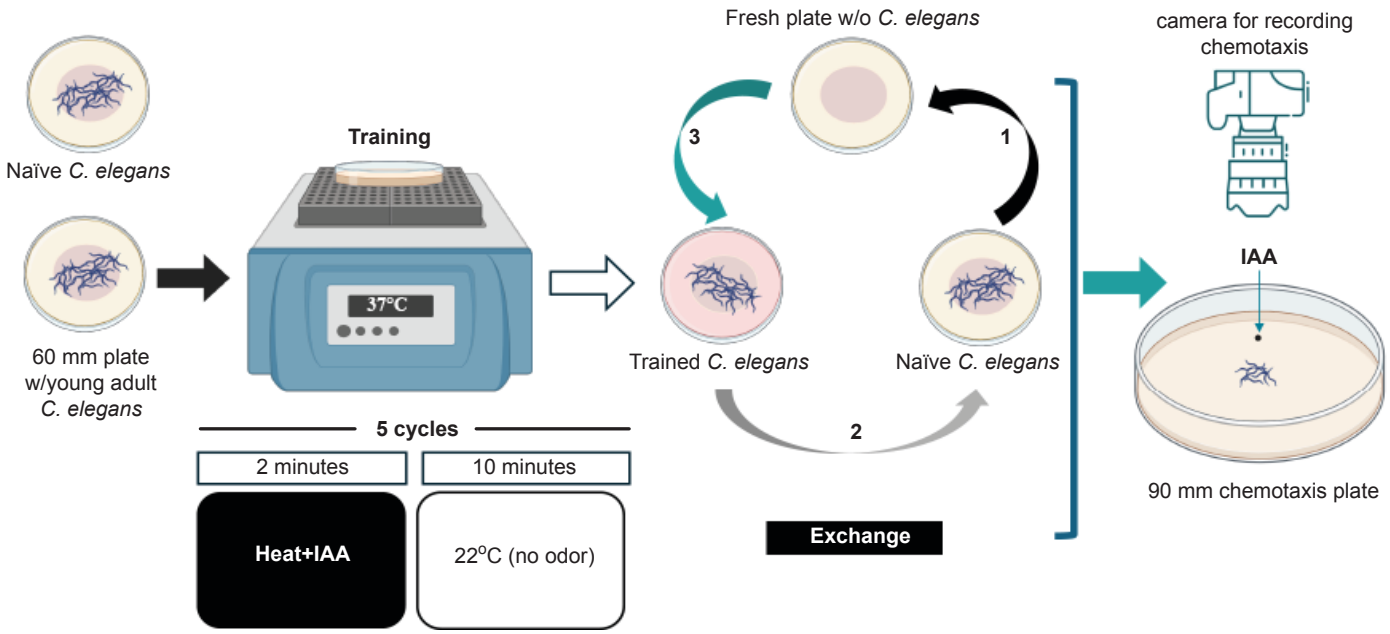
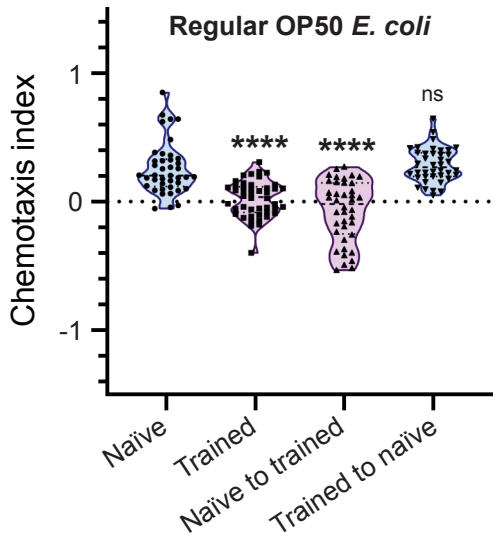

A
Training
37°C
60 mm plate
w/young adult
C. elegans
20-24
hours
camera for recording
chemotaxis
IAA
90 mm chemotaxis plate
5 cycles
2 minutes
10 minutes
Heat+IAA
22°C (no odor)
B
Chemotaxis index
1
0
-1
***
ns
ns
Naïve
Trained
Only IAA
Only heat
C
Training
37°C
60 mm plate
w/young adult
C. elegans
T0
T6
T12
Regular trained
camera for recording
chemotaxis
IAA
90 mm chemotaxis plate
Chemotaxis testing at
20 hours post training
D
T0
E
T6
F
T12
Chemotaxis index
ns
Naïve
Trained
Only IAA
Only heat

Figure 2

A


Naïve C. elegans
60 mm plate w/young adult C. elegans
Training
37°C
5 cycles
2 minutes
10 minutes
Heat+IAA
22°C (no odor)
Fresh plate w/o C. elegans
3
1
Trained C. elegans
Naïve C. elegans
2
Exchange
camera for recording chemotaxis
IAA
90 mm chemotaxis plate

B


Regular OP50 E. coli
Chemotaxis index
1
0
-1
****
****
ns
Naïve
Trained
Naïve to trained
Trained to naïve

C

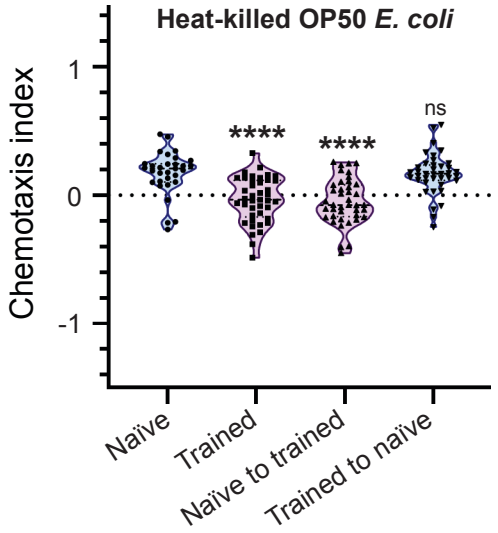

Heat-killed OP50 E. coli
Chemotaxis index
1
0
-1
****
****
ns
Naïve
Trained
Naïve to trained
Trained to naïve

Figure 3

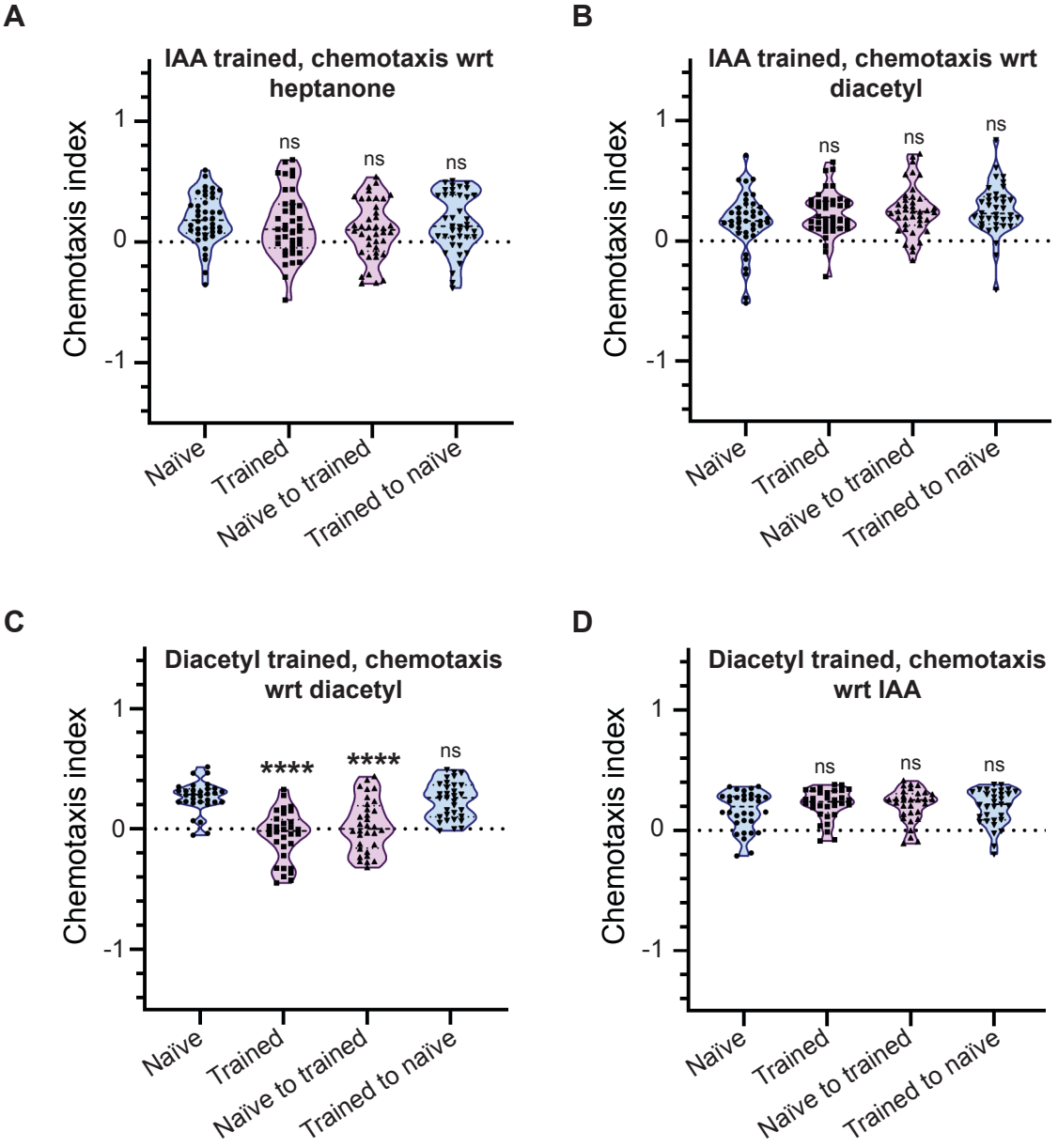
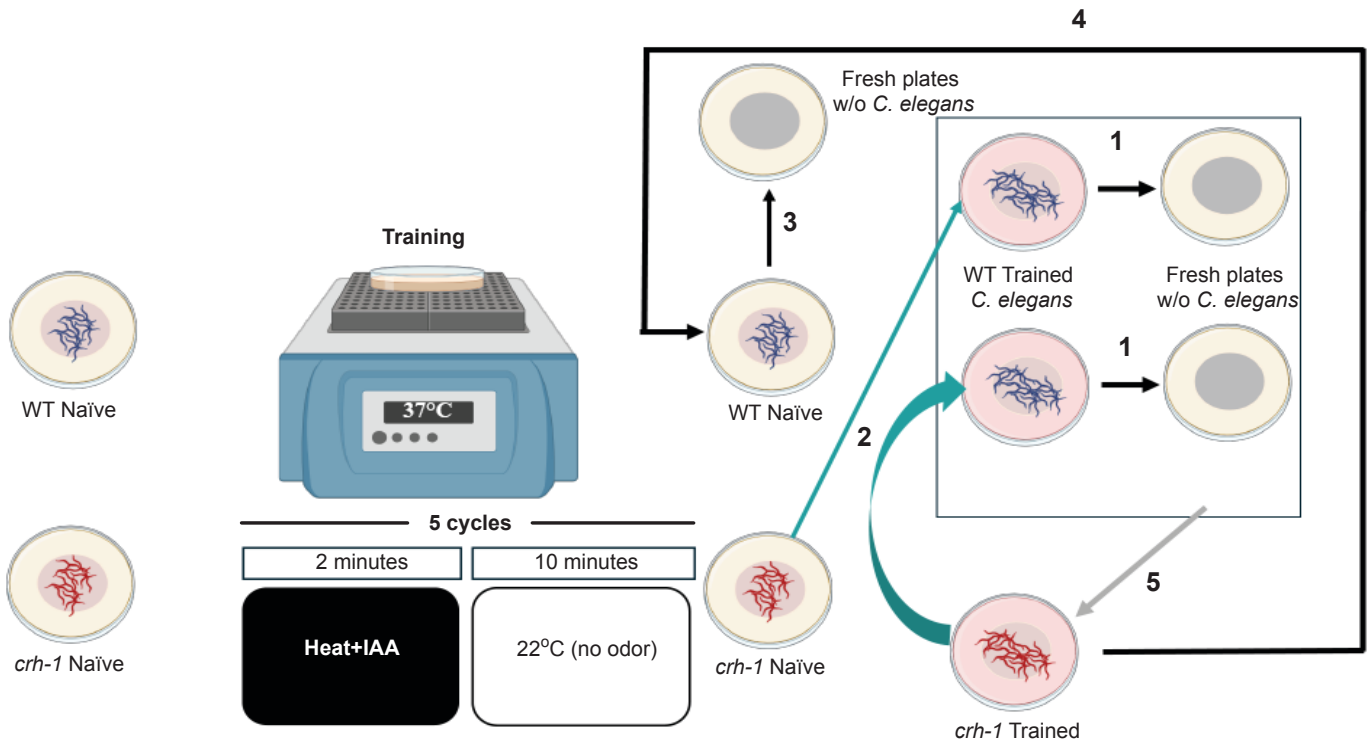

A
IAA trained, chemotaxis wrt heptanone
Chemotaxis index
1
0
-1
ns
ns
ns
Naïve
Trained
Naïve to trained
Trained to naïve
B
IAA trained, chemotaxis wrt diacetyl
Chemotaxis index
1
0
-1
ns
ns
ns
Naïve
Trained
Naïve to trained
Trained to naïve
C
Diacetyl trained, chemotaxis wrt diacetyl
Chemotaxis index
1
0
-1
****
****
ns
Naïve
Trained
Naïve to trained
Trained to naïve
D
Diacetyl trained, chemotaxis wrt IAA
Chemotaxis index
1
0
-1
ns
ns
ns
Naïve
Trained
Naïve to trained
Trained to naïve

Figure 4

A


4
Fresh plates
w/o C. elegans
1
3
Training
WT Trained
C. elegans
Fresh plates
w/o C. elegans
WT Naïve
37°C
WT Naïve
1
2
5 cycles
2 minutes
10 minutes
5
WT Naïve
Heat+IAA
22°C (no odor)
crh-1 Naïve
crh-1 Naïve
crh-1 Trained

B

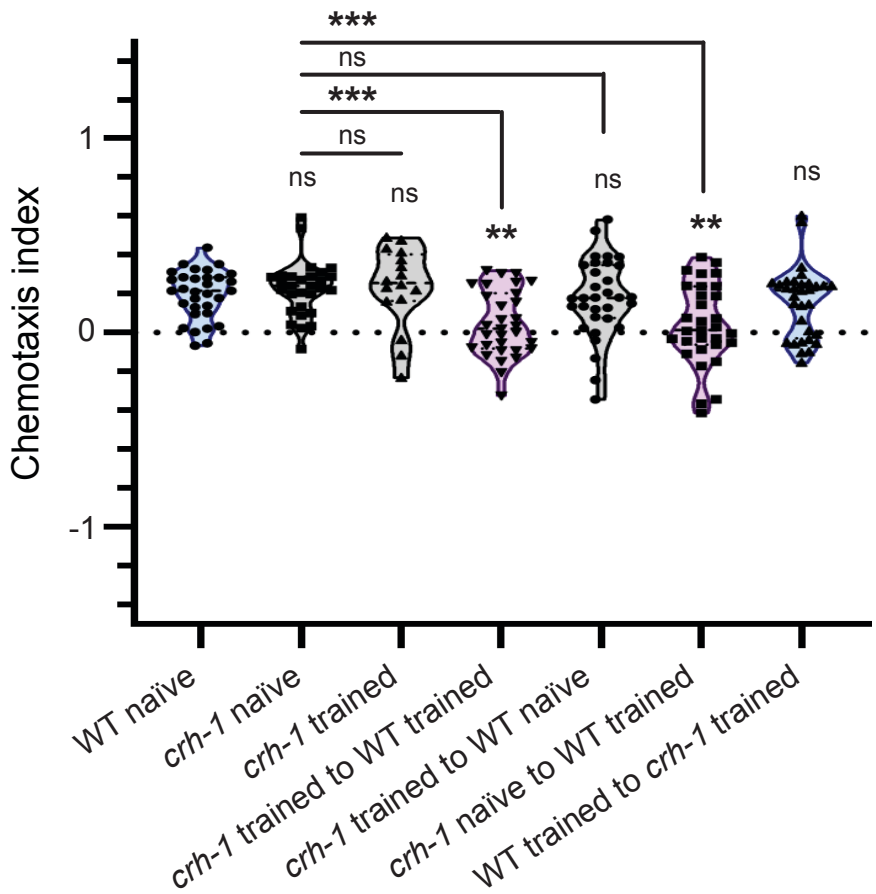

***
ns
***
ns
ns
ns
ns
ns
ns
**
**
1
0
-1
Chemotaxis index
WT naïve
crh-1 naïve
crh-1 trained
crh-1 trained to WT trained
crh-1 trained to WT naïve
crh-1 naïve to WT trained
WT trained to crh-1 trained

Figure 5

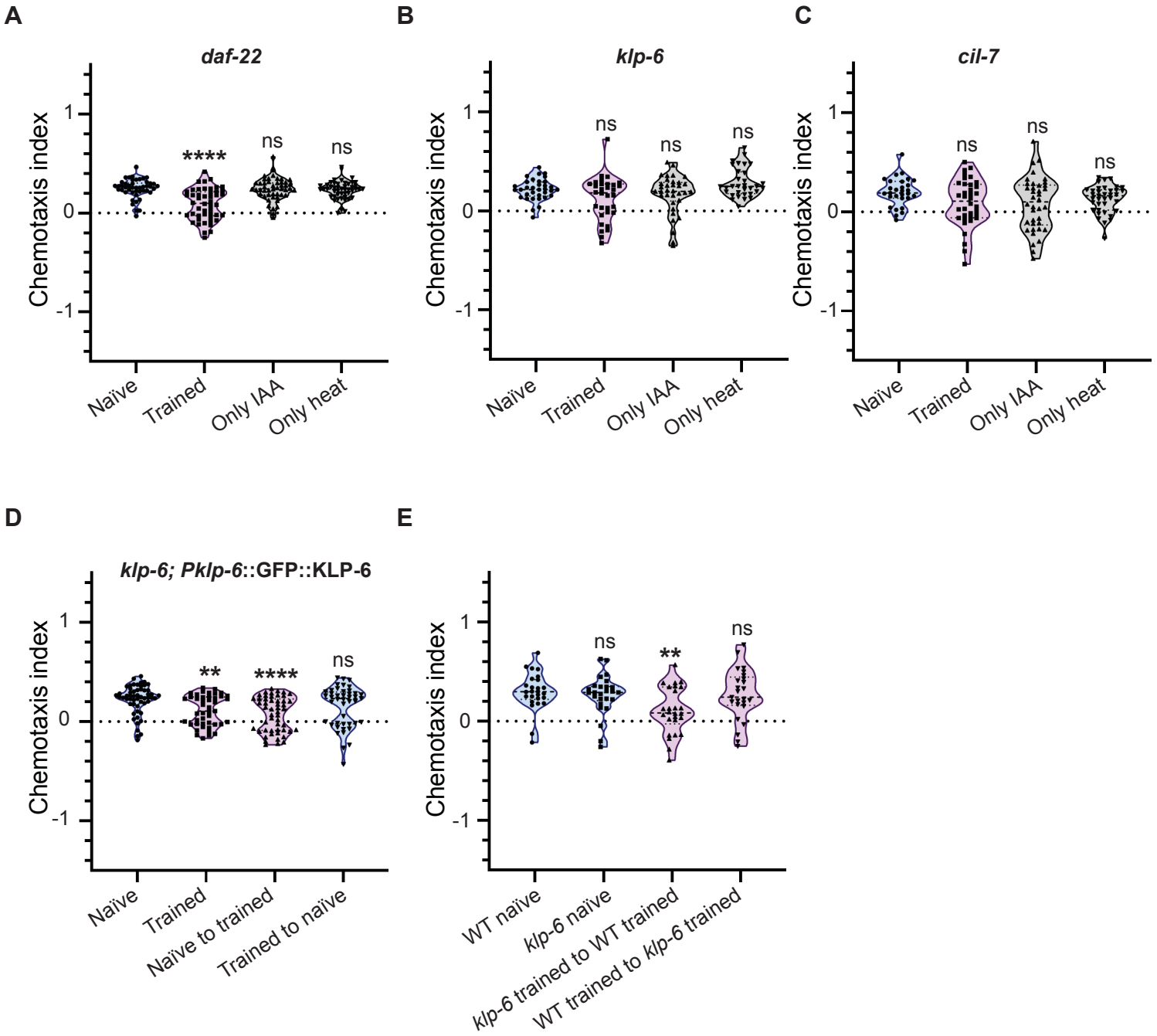
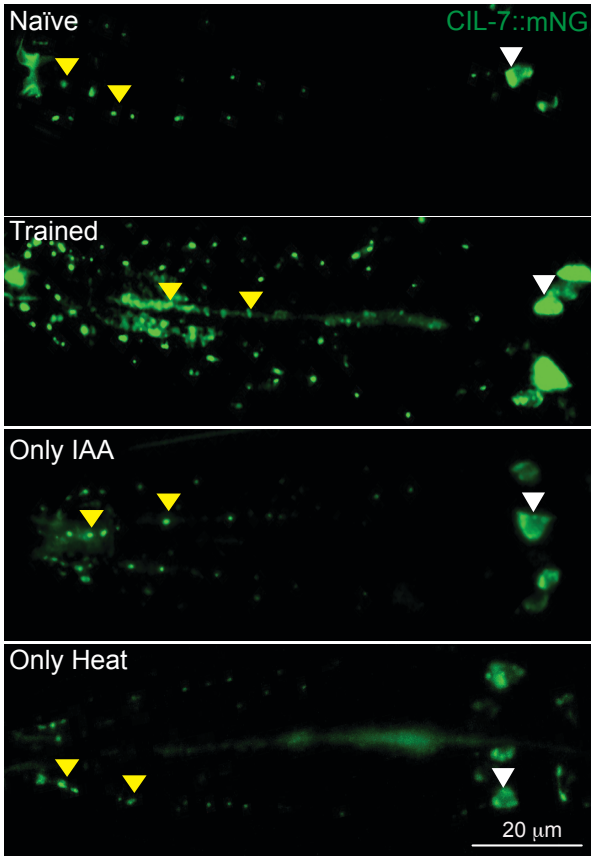

A
daf-22
Chemotaxis index
1
0
-1
****
ns
ns
Naïve
Trained
Only IAA
Only heat
B
klp-6
Chemotaxis index
1
0
-1
ns
ns
ns
Naïve
Trained
Only IAA
Only heat
C
cil-7
Chemotaxis index
1
0
-1
ns
ns
ns
Naïve
Trained
Only IAA
Only heat
D
klp-6; Pklp-6::GFP::KLP-6
Chemotaxis index
1
0
-1
**
****
ns
Naïve
Trained
Naïve to trained
Trained to naïve
E
Chemotaxis index
1
0
-1
ns
**
ns
WT naïve
klp-6 naïve
klp-6 trained to WT trained
WT trained to klp-6 trained

Figure 6

A


Naïve
CIL-7::mNG
Trained
Only IAA
Only Heat
20 µm

B

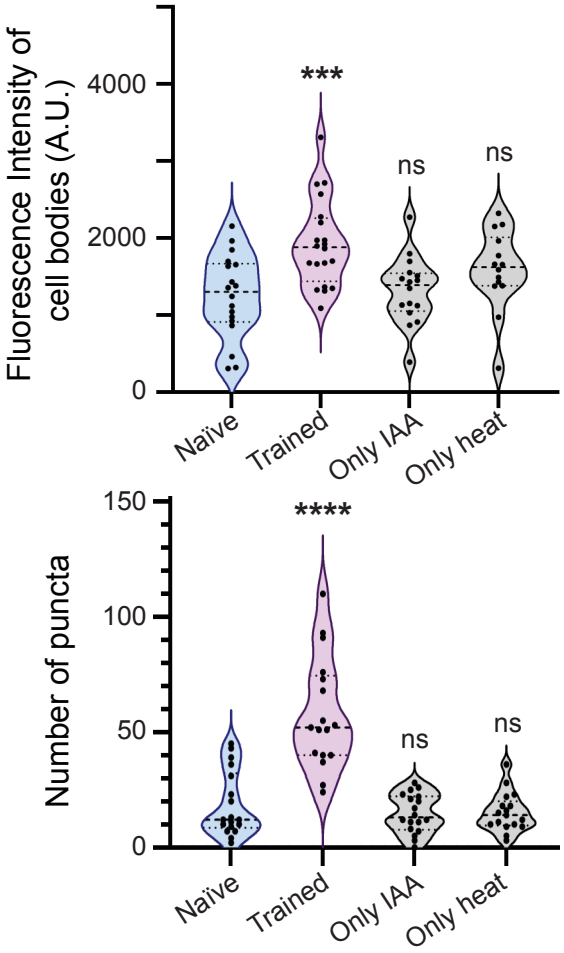
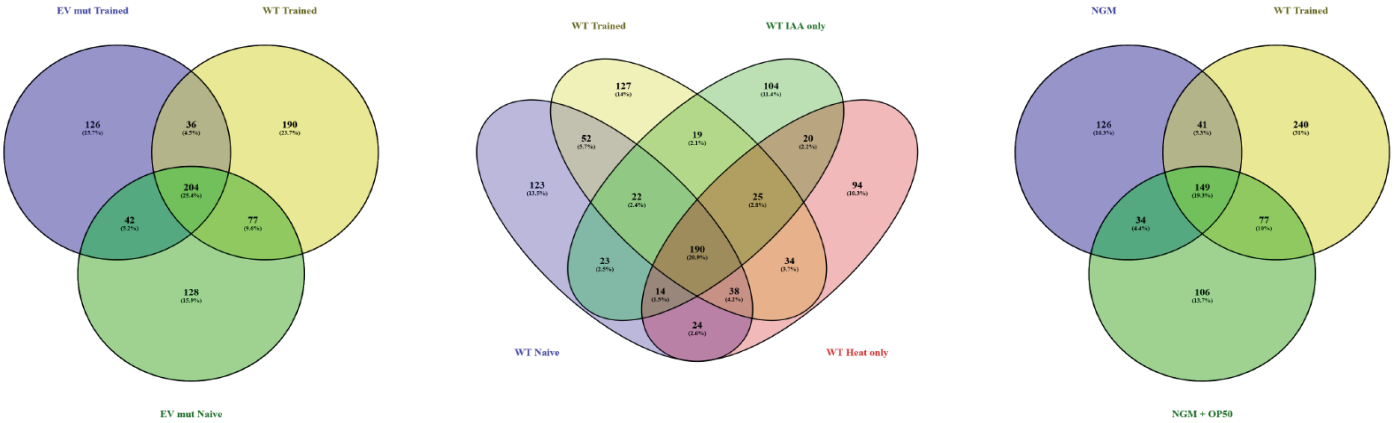

Fluorescence Intensity of cell bodies (A.U.)
4000
2000
0
***
ns
ns
Naïve
Trained
Only IAA
Only heat
Number of puncta
150
100
50
0
****
ns
ns
Naïve
Trained
Only IAA
Only heat

Figure 7

A



Comparison between wild-type trained and *klp-6* groups

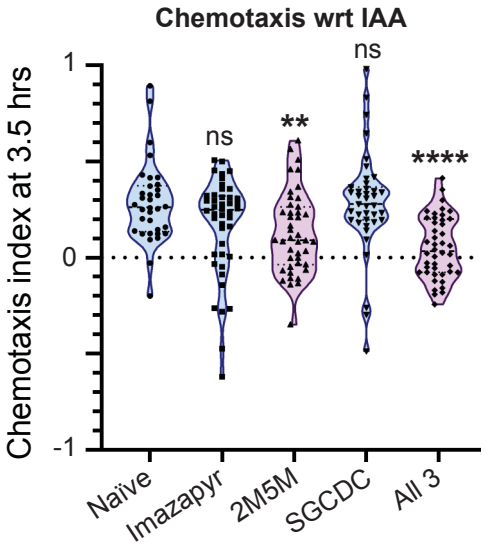
Comparison between wild-type trained and controls

Comparison between wild-type trained and NGM groups

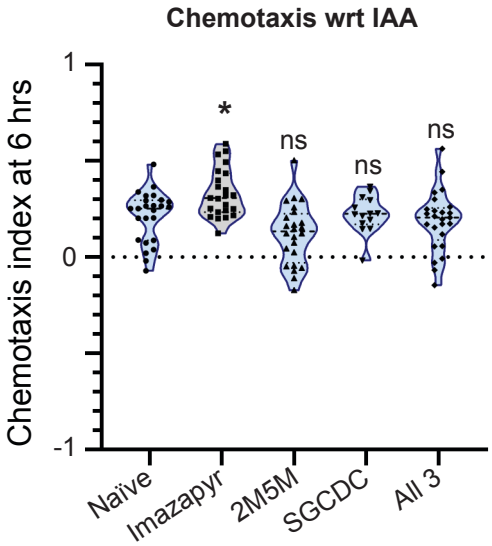
Wild-type Trained-specific candidates filtered for abundance in all three replicates

1. 2,4-Xylidine
2. N~6~-[(2R)-3,4-Dihydro-2H-pyrrol-2-ylcarbonyl]-L-lysine
3. 2-Methoxy-5-methylaniline
4. 4-Methylaminobutanal
5. 6-Amino-2-methyl-2-heptanyl (1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)acetate
6. Imazapyr
7. Glycochenodeoxycholic Acid (sodium salt)
8. 3,6-Bis-O-(3-methylbutanoyl)-β-D-fructofuranosyl 3-O-(2-methylbutanoyl)-4-O-(3-methylbutanoyl)-α-D-glucopyranoside
9. 2-Aminooctanedioic acid

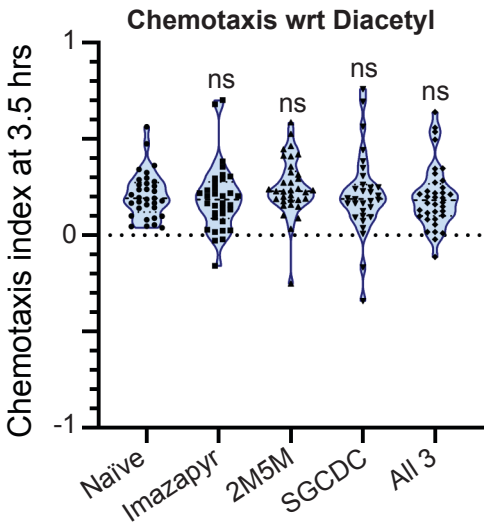
B



C



D



E

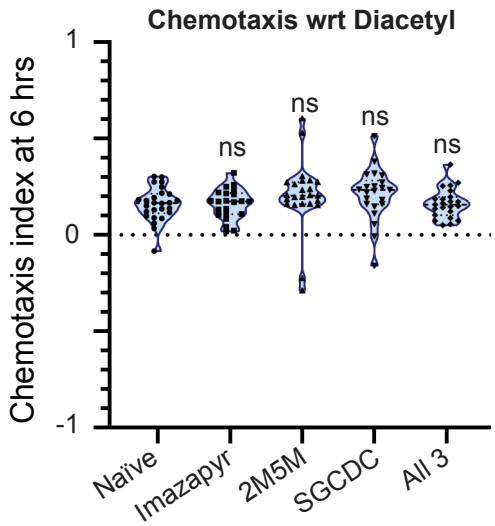


Figure 8

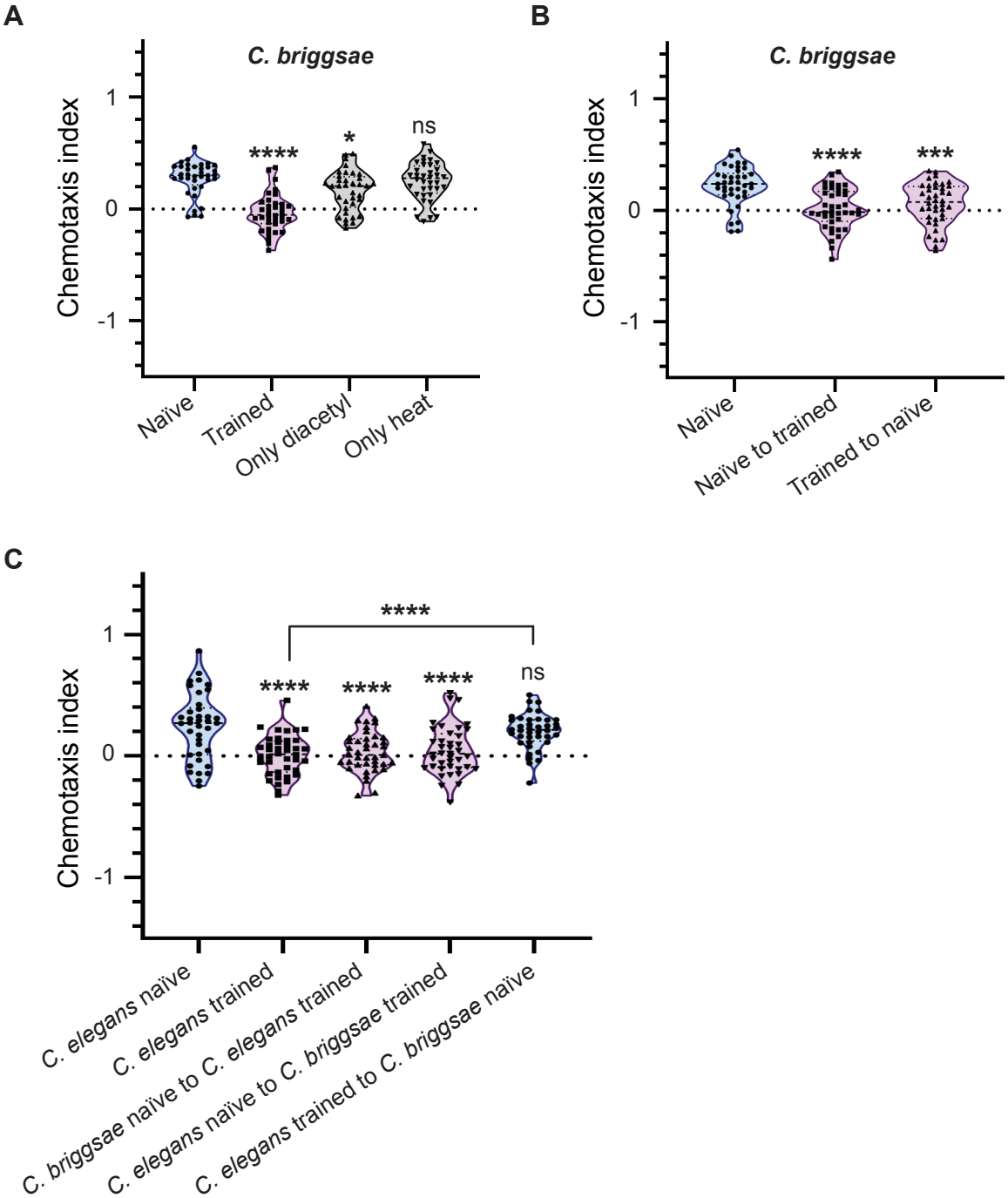
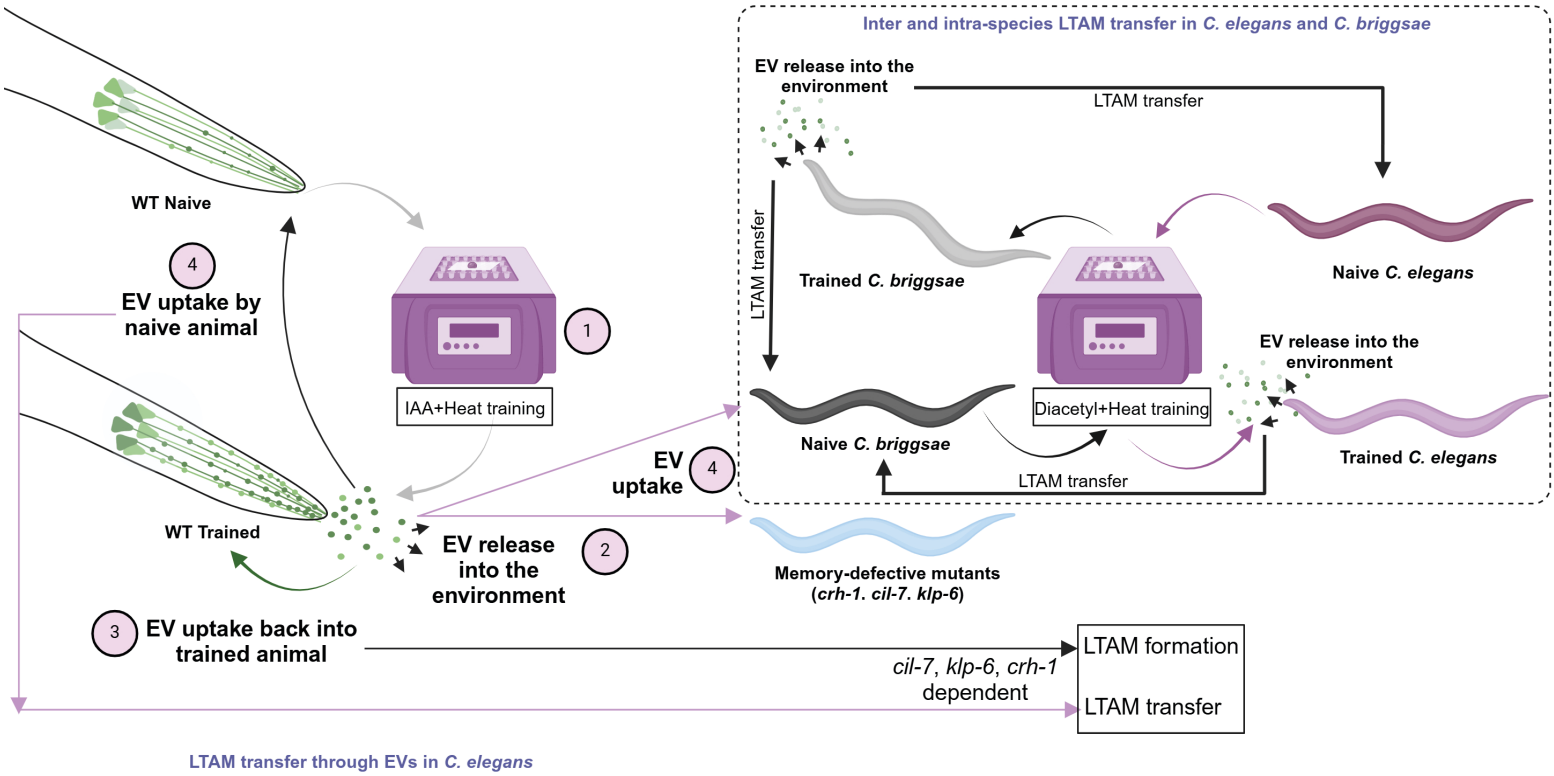

A
C. briggsae
Chemotaxis index
1
0
-1
****
*
ns
Naïve
Trained
Only diacetyl
Only heat
B
C. briggsae
Chemotaxis index
1
0
-1
****
***
Naïve
Naïve to trained
Trained to naïve
C
Chemotaxis index
1
0
-1
****
****
****
****
ns
C. elegans naïve
C. elegans trained
C. briggsae naïve to C. elegans trained
C. elegans naïve to C. briggsae trained
C. elegans trained to C. briggsae naïve

Figure 9


Inter and intra-species LTAM transfer in C. elegans and C. briggsae
EV release into the environment
LTAM transfer
LTAM transfer
Trained C. briggsae
Naive C. elegans
EV release into the environment
Naive C. briggsae
Diacetyl+Heat training
Trained C. elegans
LTAM transfer
WT Naive
4
EV uptake by naive animal
1
IAA+Heat training
EV uptake
4
WT Trained
EV release into the environment
2
Memory-defective mutants (crh-1. cil-7. klp-6)
3
EV uptake back into trained animal
cil-7, klp-6, crh-1 dependent
LTAM formation
LTAM transfer
LTAM transfer through EVs in C. elegans