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

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

89 *Caenorhabditis elegans* can release EVs environmentally through sensory ciliary
90 neurons, which can act as a mode of communication. Previous studies have shown the
91 involvement of EVs in inter-animal communication in the context of mating ((Wang *et al*,
92 2014) and reviewed in (Wang *et al*, 2024a)). There are 27 ciliated EV releasing neurons
93 (EVNs), 21 of these are male-specific neurons in the head and tail, and 6 are inner labial
94 type 2 (IL2) neurons which are present in both males and hermaphrodites (O'Hagan *et*
95 *al*, 2017; Wang *et al*, 2015). It has been previously reported that CIL-7, a myristoylated
96 protein regulates EV biogenesis and is known to be an EV cargo. The kinesin-3 protein,
97 KLP-6, is required for the release of EVs into the environment. Both these genes, *cil-7*
98 and *klp-6*, are expressed in the IL2 neurons of hermaphrodite *C. elegans* (Kahn-Kirby &
99 Bargmann, 2006; Maguire *et al*, 2015; Morsci & Barr, 2011; Peden & Barr, 2005; Wang
100 *et al*, 2021). Here we report that EVs facilitate the transfer of LTAM from trained animals
101 to both naïve and memory-defective *C. elegans*. We also show that *creb1/crh1* mutant
102 animals that show no LTAM formation in this IAA and heat-based paradigm (Dahiya *et*
103 *al*., 2019), show LTAM from exposure to the extrinsic factors (likely EVs) released by
104 trained wild-type (WT) animals. This work adds to the growing body of literature
105 describing the role of EVs in multiple processes including cell death, neurodegenerative
106 diseases and therapeutics (Mohamed *et al*, 2025; Wang *et al*, 2025; Wiersema *et al*,
107 2024; Wright *et al*, 2025).

108 Lastly, we report that LTAM can be transferred across species of *Caenorhabditis*, where
109 *C. elegans* and *C. briggsae* can transfer LTAM from trained animals of one species to
110 naïve animals of the other species. Like most previous studies involving LTAM formation
111 in *C. elegans* and across phyla, we had speculated that the mechanism of LTAM
112 formation as a result of IAA and heat training, was intrinsic to the organism (Dahiya *et*

113 *al.*, 2019). Contrary to this hypothesis, our findings in this study indicate that LTAM can
114 be transferred from one *C. elegans* to another through environmentally released
115 extracellular vesicles (EVs), thus challenging the conventional notion of LTAM being
116 intrinsic to an organism.

117

118

119 **Materials and Methods**

120 **Strains maintenance and transgene generation**

121 All strains were cultured on nematode growth medium (NGM) plates, inoculated with
122 OP50 *E. coli* bacteria, and maintained under standard conditions at 22°C, following
123 established protocols outlined in previous literature (Brenner, 1974). The N2 Bristol strain
124 served as the standard wild-type (WT) control for comparative analyses across all
125 experimental procedures. A synchronous population of young adult *C. elegans* was
126 obtained using previously established protocols (Porta-de-la-Riva *et al*, 2012). The list
127 of strains used in this study is tabulated in Supplementary Table 1 (S1) and primers used
128 for genotyping the strains are tabulated in Supplementary Table 2 (S2). The *klp-6* rescue
129 construct P*klp-6*::GFP::KLP-6 (gift from Maureen Barr, details in (Wang *et al.*, 2014))
130 was microinjected in the *klp-6* mutant *C. elegans* to prepare transgenic lines as
131 previously described (C. Mello and Fire 1995; C. C. Mello et al. 1991).

132

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

134 All assays were performed as previously described in (Dahiya *et al.*, 2019) with minor
135 modifications. Briefly, the training paradigm involves simultaneous exposure of *C.*
136 *elegans* or *C. briggsae* young adults to two cues; heat and a chemoattractant (isoamyl
137 alcohol (IAA)/ Diacetyl (depending on the experiment)), for two minutes followed by ten
138 minutes of rest period at 22°C. This cycle is repeated five times, and the animals are
139 then kept at 22°C for 20 hours (illustrated in Figure 1A). This leads to the formation of
140 long-term associative memory (LTAM), seen in the form of reduced attraction towards

the chemoattractant, which can last for as long as 24 hours. LTAM is quantified at 20-24 hours from the time of training, using a readout called the chemotaxis index (CI), where CI= Displacement along IAA, heptanone or diacetyl (depending on the odorant used) gradient/Total distance traversed. The concentrations of the odorants used are as previously published (Dahiya *et al.*, 2019; Zhang *et al*, 2016). CI is calculated by analysing the videos of the *C. elegans* undergoing chemotaxis with respect to the chemoattractant. The videos were analysed using FIJI, and an associated plugin, Trackmate (Ershov *et al*, 2022; Schindelin *et al*, 2012). Each plot indicates a set of 3-6 replicates with all conditions/genotypes in the graph performed at the same time over multiple days. Each replicate was performed using 8-10 *C. elegans*. A detailed experimental protocol can be found in the Supplementary section.

Exchange assays

Naïve animals were transferred to an empty plate, followed by trained animals being transferred to the plate that previously had naïve *C. elegans.* The final step involved transferring the naïve animals to the plate that had had trained *C. elegans* (illustrated in Figure 2A). This exchange protocol was completed within 30 minutes from the end of training. After exchange, *C. elegans* were kept in a 22°C incubator and LTAM was tested using the chemotaxis assay 20-24 hours after training. The exchange assays were performed between *C. elegans* and *C. briggsae* in a similar manner with naïve *C. elegans* transferred to plates that had contained trained *C. briggsae* and naïve *C. briggsae* transferred to plates that had had trained *C. elegans*. All plates used for training and transfer were 60 mm in diameter.

Imaging experiments

Freshly trained young adult hermaphrodite *C. elegans* were used for imaging as previously described with minor modifications (Tikiyani *et al*, 2018). Briefly, *C. elegans* were mounted on 1% agarose pads with 34 mg/ml 2,3-Butanedione Monoxime (BDM) solution in M9 medium. Images were acquired as Z-stacks on a Zeiss Apotome microscope using the 63X oil objective within four hours of the training paradigm. The

order of imaging of trained and control animals was changed for each of the three replicates. For processing and analysis of images, FIJI (Schindelin *et al.*, 2012) was used to quantify fluorescence intensity of cell body and number of puncta in each condition by selecting specific regions of interest. A threshold was set for analyzing particles of each *C. elegans* to separately quantify cell body intensity and punctal number across the neuronal processes. The summation of mean grey values using "Analyze Particles" from each cell body per worm and the punctal number along each neuronal process was counted using "Analyze Particles". Both cell body intensity and punctal numbers were plotted as independent graphs. Each replicate had 4-7 *C. elegans* that were imaged per condition over three replicates and each dot on the plots indicates a single animal.

Liquid Chromatography-Mass Spectrometry (LC-MS)

The protocol used was modified from a previous study (Nikonorova *et al*, 2022). Sample collection involved four 60 mm plates with approximately 70 *C. elegans*/plate for each sample. Freshly trained WT animals were washed with M9 buffer and collected as one sample. This was repeated for every group of *C. elegans*; naïve, trained, only IAA and only heat. Next differential centrifugation was performed. Firstly, the *C. elegans* 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.

Statistical analysis

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

201 The statistical analyses used for each plot is indicated in the figure legends.

202

203

204 **Results**

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

206 **by the animals**

207 We have previously published a training paradigm to study long-term associative memory
208 (LTAM) in wild-type (WT) *C. elegans* using isoamyl alcohol (IAA) and heat ((Dahiya *et al.*,
209 2019) and illustrated in Figure 1A). Using this paradigm, we replicated the data showing
210 that unlike naïve animals, their trained counterparts showed a significantly lower
211 chemotaxis index (CI) to IAA (Figure 1B, tracks indicated in Figure S1A). To investigate
212 the underlying molecular mechanism of LTAM formation we planned to perform RNA
213 sequencing experiments using naïve and trained WT animals.

214 In order to perform this experiment optimally, adult *C. elegans* had to be synchronized
215 (without eggs and L1 animals) at 20 hours post training for RNA extraction ((Brenner,
216 1974) and illustrated in Figure S1B)). To remove eggs and L1s from the population, the *C.*
217 *elegans* were separated from eggs twice (at 12 and 8 hours) during the twenty-hour
218 resting stage after training and a similar protocol was followed for naïve animals
219 (illustrated in Figure S1B). We initially gently washed the *C. elegans* and eggs with M9 to
220 remove the *C. elegans* from the eggs on the plate at both time points. However, upon
221 testing the animals for LTAM after the washes, we found the animals no longer showed
222 LTAM (Figure S1C). This prompted us to pick animals at the standardized 12-hour time
223 point during the 20-hour resting period to circumvent the effects of washing. Again, we
224 found that the animals removed from the plates at the 12-hour time-point no longer
225 showed LTAM (Figure 1F). This prompted us to hypothesize that trained *C. elegans* may
226 be releasing factor(s) onto the plate that could allow for LTAM formation. In order to test
227 the time-point at which the animals may be releasing unknown factor(s) we tested the
228 animals by picking them from the plate within a few minutes of training (t_0), 6 hours after
229 training (t_6), 12 hours after training (t_{12}), and keeping some animals in the original plate

230 (illustrated in Figure 1C). We found that only the animals on the original plate showed
231 LTAM formation (Figure 1B), while all other animals lost LTAM associated with heat and
232 IAA (Figures 1D-F).

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

237

238 **Naïve *C. elegans* on trained plates gain LTAM**

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

251 Next, to make sure that the factor(s) released on the plate were not produced by bacteria
252 on the plate as the animals are well fed with OP50 *E. coli* throughout their training and
253 resting phases, we performed the same experiments with heat-killed *E. coli.* The *E. coli*
254 were heat-killed as previously described (Qi *et al*, 2017). We observed the same results
255 as previously seen with live *E. coli* (Figure 2C). To confirm the associative nature of the
256 transferred LTAM, we tested WT *C. elegans* with heat and IAA in an unpaired assay. In
257 order to perform this experiment, we exposed naïve *C. elegans* to heat and IAA one after
258 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).

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

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

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

We next decided to test LTAM formation with respect to another chemoattractive odour sensed by a different neuron. Diacetyl is an odour that is sensed by the AWA neuron ((Sengupta *et al*, 1996; Sengupta *et al*, 1994) and illustrated in Figure S3). We trained animals with heat and IAA and found that trained animals and naïve animals transferred to plates that had contained trained animals did not show any defects in chemotaxis with respect to diacetyl (Figure 3B). Our previous work has shown that the heat and chemoattractant based training paradigm may be used with diacetyl and heat in a process similar to that performed with IAA and heat (Dahiya *et al.*, 2019). We next trained the animals with heat and diacetyl and found that they showed LTAM towards diacetyl (Figure 3C). Further, naïve animals placed on a plate that had had trained *C. elegans* showed

288 LTAM towards diacetyl (Figure 3C). This experiment indicates that a release of factor(s)
289 occurs independent of whether the odour is IAA or diacetyl; the nature of these factor(s),
290 however, appears to be extremely specific to the odorant used during training. Finally, we
291 trained animals with diacetyl and tested them with IAA and found no LTAM formation
292 towards IAA (Figures 3D). These data together indicate a high degree of specificity of the
293 factor(s) released for allowing LTAM formation only with respect to the cue present in the
294 training paradigm.

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

297

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

300 A plethora of literature has implicated CREB1 (cAMP Response Element Binding protein
301 1) in long-term memory formation across phyla ((Bartsch *et al*, 1998; Timbers & Rankin,
302 2011; Yin *et al*, 1995; Yin *et al*, 1994) and reviewed in (Flavell & Greenberg, 2008;
303 Johannessen *et al*, 2004; Silva *et al*, 1998; Yin & Tully, 1996)). We have shown that the
304 mutants of the ortholog of mammalian *creb1*, *crh-1* in *C. elegans,* show LTAM defects in
305 the heat and IAA paired assay (Dahiya *et al.*, 2019). We decided to test if *crh-1* mutants
306 allowed for LTAM formation when transferred to a plate that had contained trained WT
307 animals (illustrated in Figure 4A). Our data indicates that *crh-1* mutants that basally show
308 no LTAM, show LTAM when transferred to a plate which had had WT trained animals. We
309 also found that WT trained animals transferred to a plate that had contained trained *crh-1*
310 mutant *C. elegans* do not show LTAM (Figure 4B and tracks indicated in Figure S4). These
311 data allowed us to conclude that *crh-1* mutants may have defects in the synthesis or
312 release of the factor(s) that allow for LTAM formation but appear to be able to uptake these
313 factor(s) to show LTAM.

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

316

317 **Extracellular vesicle are required for LTAM formation**

318 A well-studied mechanism of social behaviour in *C. elegans* is through secreted small
319 molecule signalling by ascarosides (reviewed in (Yang *et al*, 2023)). Pre-exposure to
320 different ascarosides can change the chemotaxis of *C. elegans* to different odours (Wu *et*
321 *al*, 2023). We decided to test for LTAM formation in the *daf-22* mutant *C. elegans* that are
322 defective in ascaroside biosynthesis (Golden & Riddle, 1985). We found that *daf-22*
323 mutants showed no defects in LTAM formation (Figure 5A). These data indicate that the
324 secreted factor(s) may not be *daf-22* dependent. Apart from ascarosides, sensory
325 signalling allowing for behaviours like mate sensing through chemoattraction requires
326 extracellular vesicle (EV) release from *C. elegans* (reviewed in (Wang *et al.*, 2024a)). We
327 next tested mutants in EV biogenesis (*cil-7*) and release (*klp-6*) for LTAM formation (Wang
328 *et al.*, 2021). We found that both mutants showed no LTAM formation (Figure 5B and C).
329 In order to further confirm the role of EVs in the process of LTAM, we attempted to rescue
330 the loss of LTAM phenotype in *klp-6* mutants by expressing KLP-6 specifically in the IL2
331 neurons where KLP-6 has been shown to be expressed in hermaphrodites (Peden & Barr,
332 2005; Wang *et al*, 2024b). We observed that expressing KLP-6 in the mutant *klp-6* animals
333 rescued the loss of LTAM phenotype where both trained *C. elegans* as well as naïve
334 animals transferred to plates that had contained trained animals now showed LTAM
335 formation (Figure 5D). We next decided to test if *klp-6* mutants allowed for LTAM formation
336 when transferred to a plate that had trained WT animals. We found that trained *klp-6*
337 mutants transferred to a plates which had had trained WT animals showed LTAM, while
338 trained WT animals transferred to plates that had contained trained *klp-6* mutant *C.*
339 *elegans* did not show LTAM (Figure 5E and tracks indicated in Figure S5). These data
340 allow us to conclude that EV release may be the basis for LTAM formation in a
341 chemoattractant and heat-based associative training paradigm.

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

344

345 **Changes in EV biogenesis marker expression upon heat and IAA training**

346 Previous work has elegantly shown that an EV cargo, CIL-7, is released from the IL2
347 neurons into the external region around *C. elegans* (Wang *et al.*, 2024b). We tested the
348 same line for changes in fluorescence expression in the IL2 neurons and found that the
349 *cil-7 (my61[cil-7::mNG])I; him-5(e1490)* line showed increased fluorescence in the IL2 cell
350 bodies and an increase in fluorecent puncta along the IL2 neuronal processes in trained
351 WT animals when compared to the control *C. elegans* that were either naïve or trained
352 with only IAA or only heat (Figure 6A). With these data indicating the involvement of EVs
353 in LTAM transfer, we decided to perform a Liquid Chromatography-Mass Spectrometry
354 (LC-MS) experiment to study the differences in the released products upon heat and IAA
355 training when compared to control animals. The experiment allowed us to find 9 consistent
356 hits that were only present in the heat and IAA trained *C. elegans* plates (Figure 6B and
357 S6). These data suggest that multiple ions are differentially released onto the plates upon
358 heat and IAA training. These ions may allow for LTAM formation in *C. elegans*. Hence,
359 studies on these ions may allow us to get better insight into LTAM formation using heat
360 and IAA.
361 Finally, we wanted to test if LTAM formation is conserved across *Caenorhabditis* species.
362 In order to perform this experiment, we investigated LTAM formation in *Caenorhabditis*
363 *briggsae.*
364

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

366 We first tested the *C. briggsae* strain for LTAM formation to heat and IAA and found that
367 *C. briggsae* were surprisingly not attracted to IAA even in naïve conditions (Figure S7A
368 and B). This precluded us from studying LTAM formation in *C. briggsae* using the IAA and
369 heat based associative paradigm. However, since *C. briggsae* naïve animals behaved like
370 *C. elegans* trained animals (Figure S7B), we went on to transfer *C. briggsae* naïve animals
371 to a plate that had contained *C. elegans* trained animals and found that there was no
372 change in *C. briggsae* chemotaxis index. We also transferred trained *C. elegans* to a plate
373 that had contained naïve *C. briggsae* (exchange assay) and found that these animals
374 behaved like naïve *C. elegans* (Figure S7B). These data could not allow us to make many
375 conclusions other than the fact that *C. briggsae* was not attracted to IAA in the naïve
376 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 7A). 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 7B). 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 7C). We also transferred trained *C. elegans* to a plate that had contained naïve *C. briggsae* and unsurprisingly found no LTAM formation (Figure 7C). Together, our data indicates the role of extracellular vesicles in LTAM formation and transfer in *C. elegans* and 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 8. Below, we discuss the significance of these findings.

Novel mechanism for transfer of LTAM from one *Caenorhabditis elegans* to another

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 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.

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

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

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

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

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

455 Studies have shown that CIL-7, a myristoylated protein regulates EV biogenesis along
456 with being an EV cargo and the kinesin-3 protein KLP-6 is required for the release of EVs.
457 Both these genes, *cil-7* and *klp-6,* are expressed in the IL2 neurons in *C. elegans*
458 hermaphrodites (Kahn-Kirby & Bargmann, 2006; Maguire *et al.*, 2015; Morsci & Barr,
459 2011; Peden & Barr, 2005; Wang *et al.*, 2021). Here we report that *cil-7* and *klp-6* mutant
460 animals are LTAM defective, uncovering a previously unreported role of the IL2 neurons
461 in LTAM formation.

462 This raises important questions in understanding the neuronal circuitry involved. Different
463 neurons are responsible for sensing different odorants in *C. elegans*. Our experiments
464 indicate that animals trained with IAA (sensed by the AWC neurons) and heat versus
465 diacetyl (sensed by the AWA neurons) and heat release EVs with different cargo causing

466 cue-specific LTAM formation. Understanding the neuronal circuitry between
467 chemosensory neurons and IL2 neurons may allow for a better understanding of the
468 specificity of LTAM formation.

469 **Dynamic EV cargo for cue-specific LTAM**

470 *C. elegans* show LTAM formation and transfer, specific to the odorant cue used during
471 training. For examining the extrinsic factor(s) released by trained animals, we performed
472 LC-MS to identify candidate molecules present exclusively on the plates of trained animals
473 (Figure 6B). One of these molecules 4-methylaminobutanal, might be of potential interest.
474 IAA is chemically 3-methylbutanol, and a biological pathway for the conversion of 3-
475 methylbutanol to 4-methylaminobutanal may exist. The steps would include oxidation of
476 3-methylbutanol to 3-methylbutanal through an alcohol dehydrogenase (Illikoud *et al*,
477 2018), followed by a transamination step producing 3-methylbutylamine (Encyclopedia of
478 Sensors and Biosensors, 2023). This intermediate could potentially result in 4-
479 methylaminobutanal through additional reactions. This and the other molecules that are
480 released during training (Figures S6), may allow for a better understanding of LTAM
481 formation in *C. elegans*. Since we see specificity of LTAM formation to the odorant used
482 in the training paradigm, it is plausible that different odorants may allow for release of cue-
483 specific cargo. This in turn may give rise to the specificity of the observed LTAM
484 phenomenon.

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

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

494 RNA has been implicated in memory in multiple model organisms. The role of different
495 types of non-coding RNAs like micro-RNAs (miRNAs) and Piwi-interacting RNAs
496 (piRNAs) have been explored and studied in the context of long-term non-associative
497 memory in *Aplysia* (Fiumara *et al*, 2015; Rajasethupathy *et al*, 2012; Rajasethupathy *et*
498 *al*, 2009). Further, it has been shown in *Aplysia* that memory formed from long-term
499 sensitization (non-associative) can be transferred from trained to naïve animals
500 (Bedecarrats *et al.*, 2018). More recently, multiple studies in have defined the role of non-
501 coding RNAs in transgenerational memory as well (reviewed in (Miska & Rechavi, 2021)).
502 Studies have also shown that small non-coding RNAs expressed in *Pseudomonas* strains
503 allow for the transgenerational inheritance of avoidance memory in *C. elegans* (Kaletsky
504 *et al*, 2020; Legue *et al*, 2022; Sengupta *et al*, 2024; Seto *et al*, 2024). Additionally, Moore
505 et al, 2021 has shown that this avoidance memory can also be transferred from one animal
506 to another (trained to naïve *C. elegans*) through virus-like particles encoded by the *Cer1*
507 retrotransposon (Moore *et al.*, 2021). Multiple studies have shown that extracellular
508 vesicles from *C. elegans* carry proteins, non-coding RNAs, miRNAs, lipid nanodomains,
509 enzymes and other chemicals (Nikonorova *et al.*, 2022; Russell *et al*, 2020). Collectively,
510 these findings suggest the involvement of RNA in learning and memory and may allow for
511 the hypothesis that non-coding RNAs or miRNAs could be involved in LTAM uptake from
512 trained to naïve animals through EVs.

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

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Conflict of Interest:

Authors declare no conflict of interests.

554 **Figure legends:**

555

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

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

567

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

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

576

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

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

586

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

589 (A) Schematic representation of exchange assay between wild-type (WT) and *crh-1*
590 mutant *C. elegans.* The numbers along the arrows indicate the sequence of moving *C.*
591 *elegans* across multiple plates. (B) Violin plots indicating CI in *crh-1* mutant animals
592 (naïve and trained) when exposed to factor(s) released by trained WT animals. Multiple
593 comparisons were performed using one-way ANOVA and p-values were adjusted using
594 Dunnett's correction and Sidak's correction (for within group comparisons), "**" indicates
595 $p < 0.01$, "***" indicates $p < 0.001$ and "ns" indicates not significant. Note that in the *crh-1*
596 trained violin plot, only 16 animals are plotted across 3 replicates, although 8 *C. elegans*
597 were taken per replicate. This is because of multiple tracks that overlapped during
598 analyses and could not be separated out.

599

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

602 (A) Violin plots indicating CI in *daf-22* mutant animals defective in ascaroside
603 biosynthesis. (B) Violin plots indicating CI in *klp-6* mutant animals defective in
604 extracellular vesicle release. These animals show no LTAM formation. (C) Violin plots
605 indicating CI in *cil-7* mutant animals defective in extracellular vesicle biosynthesis. These
606 animals also show no LTAM formation. (D) Violin plots indicating CI in the *klp-6* rescuing
607 strain. (E) Violin plots indicating CI in *klp-6* mutant animals when exposed to factor(s)
608 released by WT animals, no LTAM was observed in WT animals exposed to plates that
609 had had trained *klp-6* mutants. Multiple comparisons for all plots were performed using
610 one-way ANOVA and p-values were adjusted using Dunnett's correction, "**" indicates
611 $p < 0.01$, "****" indicates $p < 0.0001$ and "ns" indicates not significant.

612

613 **Figure 6: EV imaging and LC-MS data**

614 (A) The images on the left represent fluorescence imaging of the IL2 neurons using the
615 *cil-7*::mNG marker line in naïve, trained, IAA only and heat only conditions. The panels
616 on the right indicate violin plots showing total fluorescence intensity of cell bodies of IL2
617 neurons (above) and the number of puncta across the IL2 processes (below). The
618 trained animals show increased cell body fluorescence and increased punctal number
619 along the IL2 processes when compared to the control *C. elegans*. White arrowheads
620 indicate cell bodies and yellow arrowheads indicate puncta in all images. Multiple
621 comparisons were performed using one-way ANOVA and p-values were adjusted using
622 Dunnett's correction, "***" indicates $p < 0.001$, "****" indicates $p < 0.0001$ and "ns"
623 indicates not significant. (B) LC-MS data analysed from eight animal groups processed
624 as three replicates per group; WT trained and controls (4 groups), *klp-6* naïve and
625 trained *C. elegans* (2 groups) and empty plates containing NGM alone or NGM+OP50 *E.*
626 *coli* (2 groups). The venn diagrams were created using the Venny 2.1 software

(https://bioinfogp.cnb.csic.es/tools/venny/). The list of molecules present exclusively on WT trained plates in every replicate is indicated at in the bottom panel of 6B.

Figure 7: LTAM formation and transfer is conserved across *Caenorhabditis* species

(A) Violin plots indicating CI in *C. briggsae* trained with the Diacetyl and heat associative learning paradigm. (B) Violin plots indicating CI in *C. briggsae* that underwent exchange from trained plates. This plot indicates conservation of the LTAM transfer phenomenon from naïve to trained animals in *C. briggsae*. Further, no LTAM loss is seen in trained *C. briggsae* when removed from trained plates, unlike in *C. elegans*. (C) Violin plots indicating CI after transferring *C. briggsae* to plates that had contained trained *C. elegans* and transferring *C. elegans* to plates that had contained trained *C. briggsae*. This plot indicates interspecies transfer of LTAM from *C. elegans* to *C. briggsae* and vice versa. 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 $p < 0.05$, "***" indicates $p < 0.001$, "****" indicates $p < 0.0001$ and "ns" indicates not significant.

Figure 8: 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.

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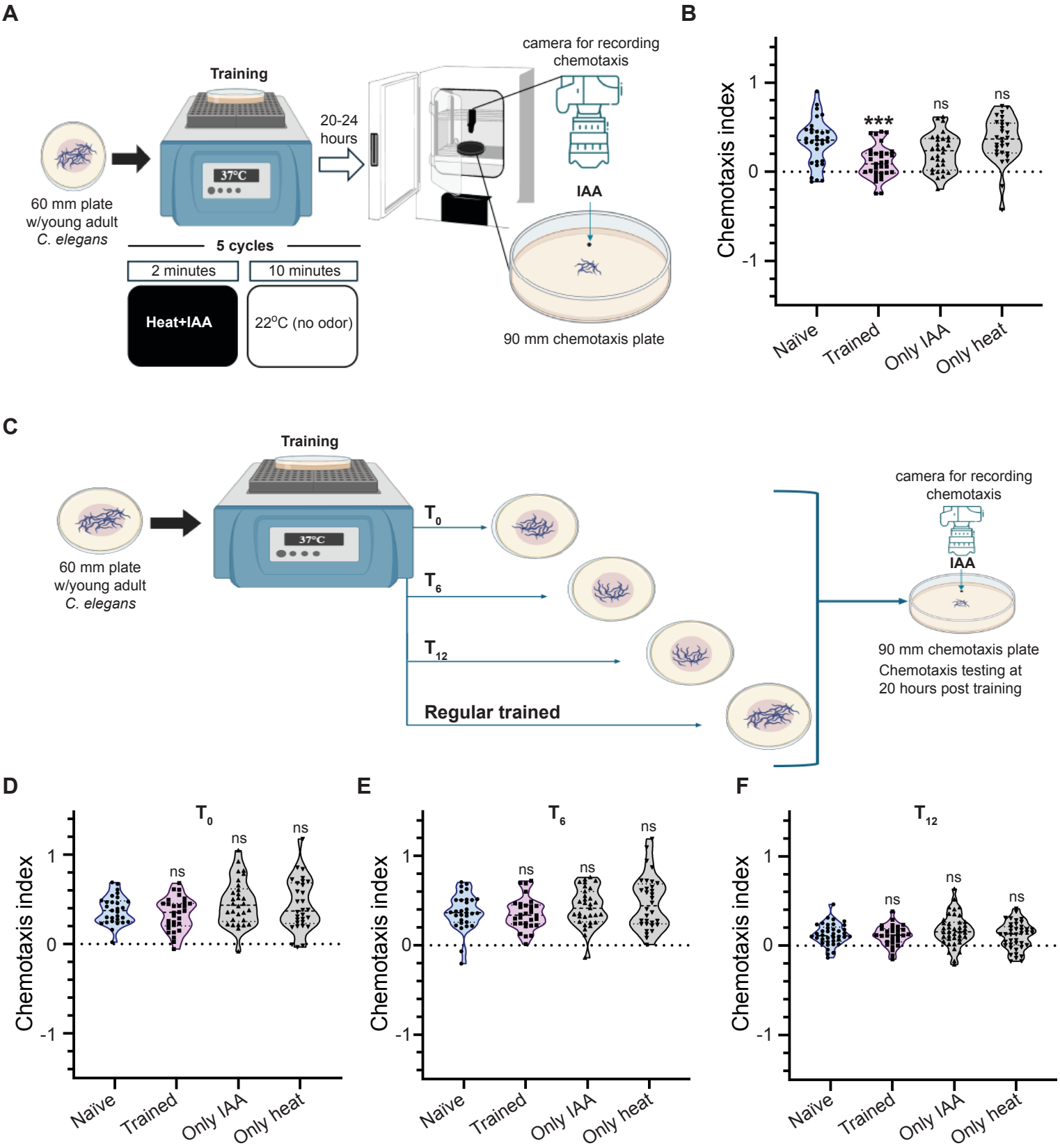
Figure 1


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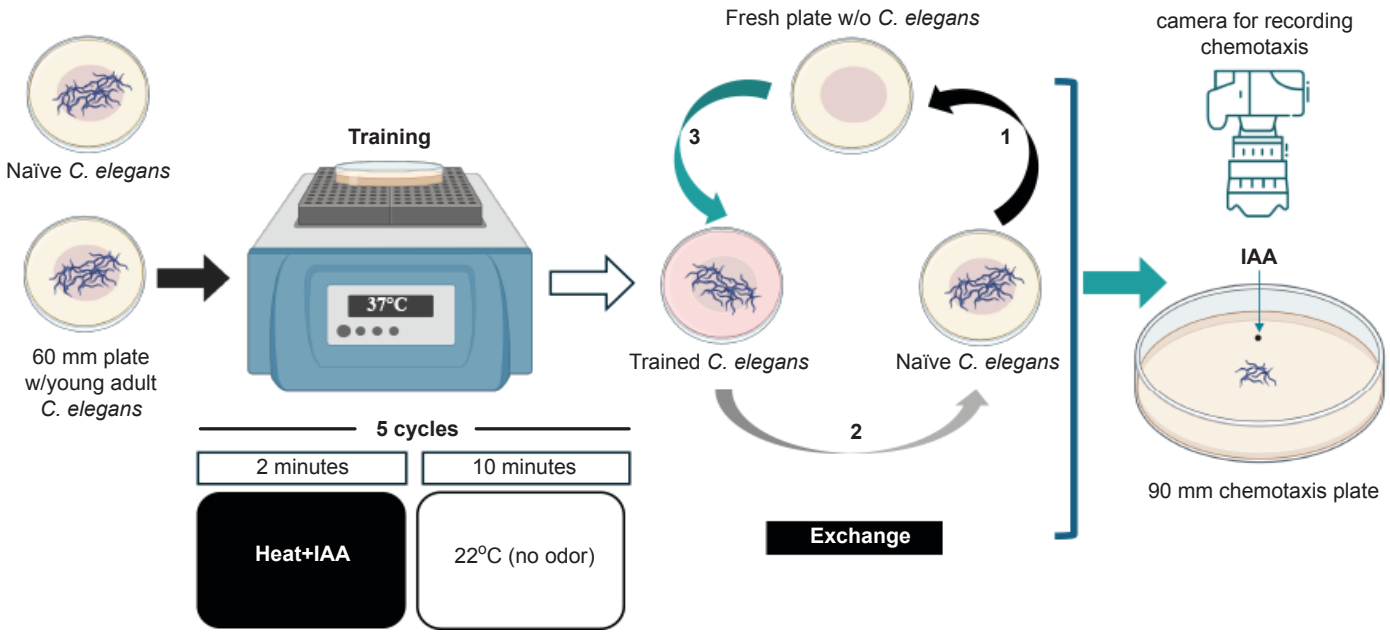
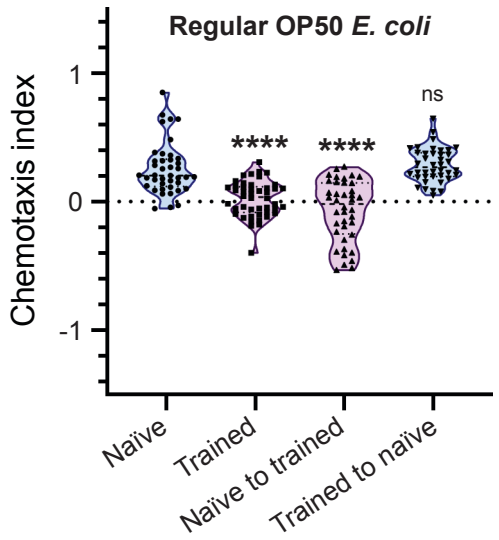
2 minutes
10 minutes
Heat+IAA
22°C (no odor)
B
Chemotaxis index
1
0
-1
***
ns
ns
Naïve
Trained
Only IAA
Only heat
C
Training
37°C
60 mm plate
w/young adult
C. elegans
T0
T6
T12
Regular trained
camera for recording
chemotaxis
IAA
90 mm chemotaxis plate
Chemotaxis testing at
20 hours post training
D
T0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

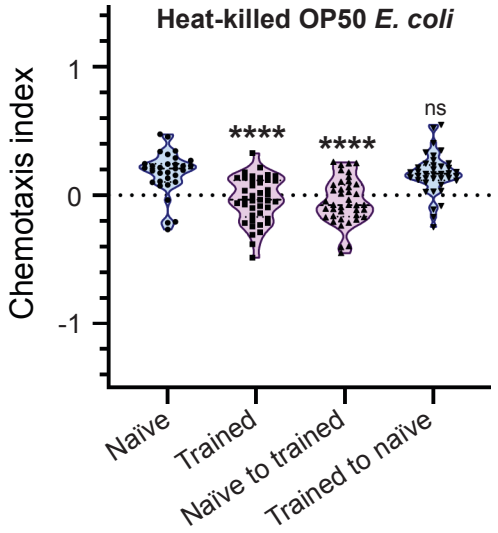

Heat-killed OP50 E. coli
Chemotaxis index
1
0
-1
****
****
ns
Naïve
Trained
Naïve to trained
Trained to naïve

Figure 3

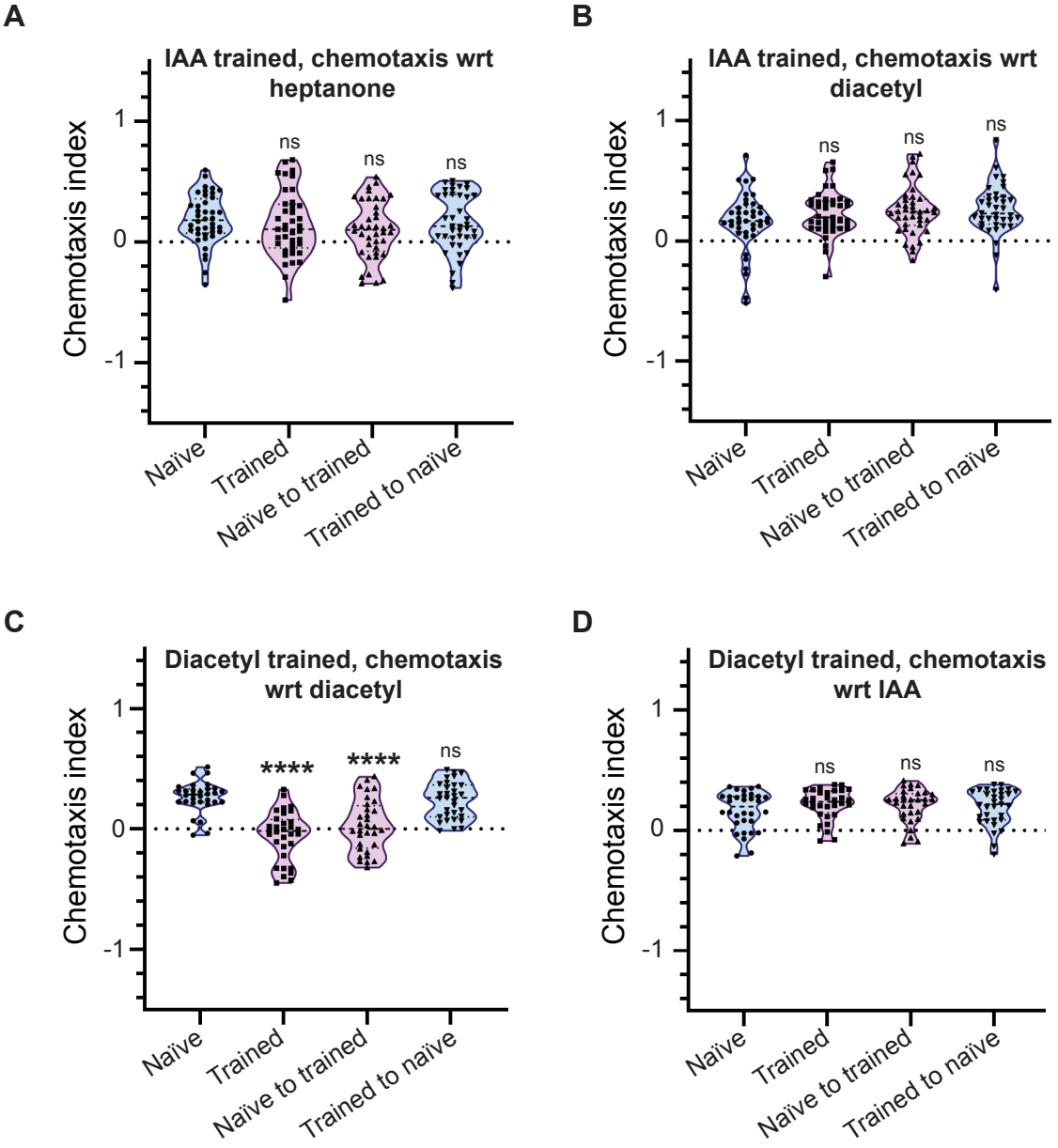
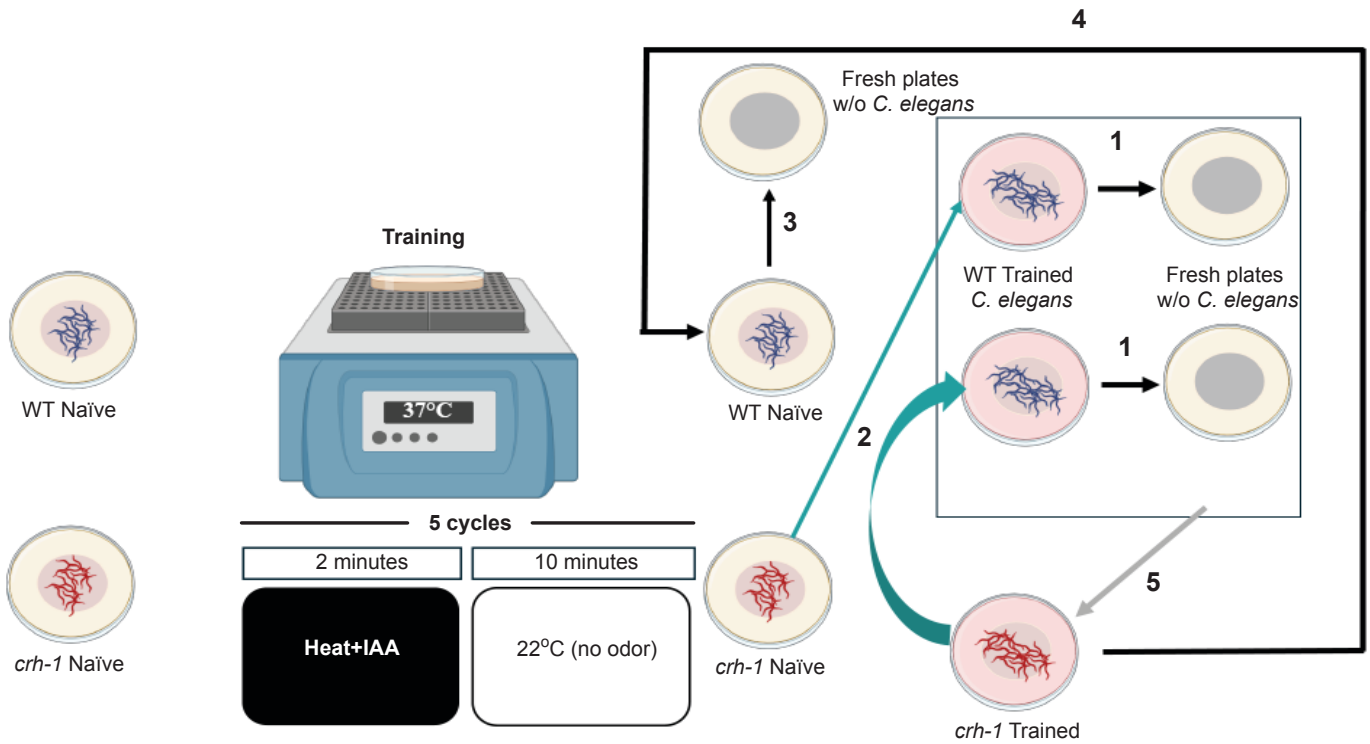

A
IAA trained, chemotaxis wrt heptanone
Chemotaxis index
1
0
-1
ns
ns
ns
Naïve
Trained
Naïve to trained
Trained to naïve
B
IAA trained, chemotaxis wrt diacetyl
Chemotaxis index
1
0
-1
ns
ns
ns
Naïve
Trained
Naïve to trained
Trained to naïve
C
Diacetyl trained, chemotaxis wrt diacetyl
Chemotaxis index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

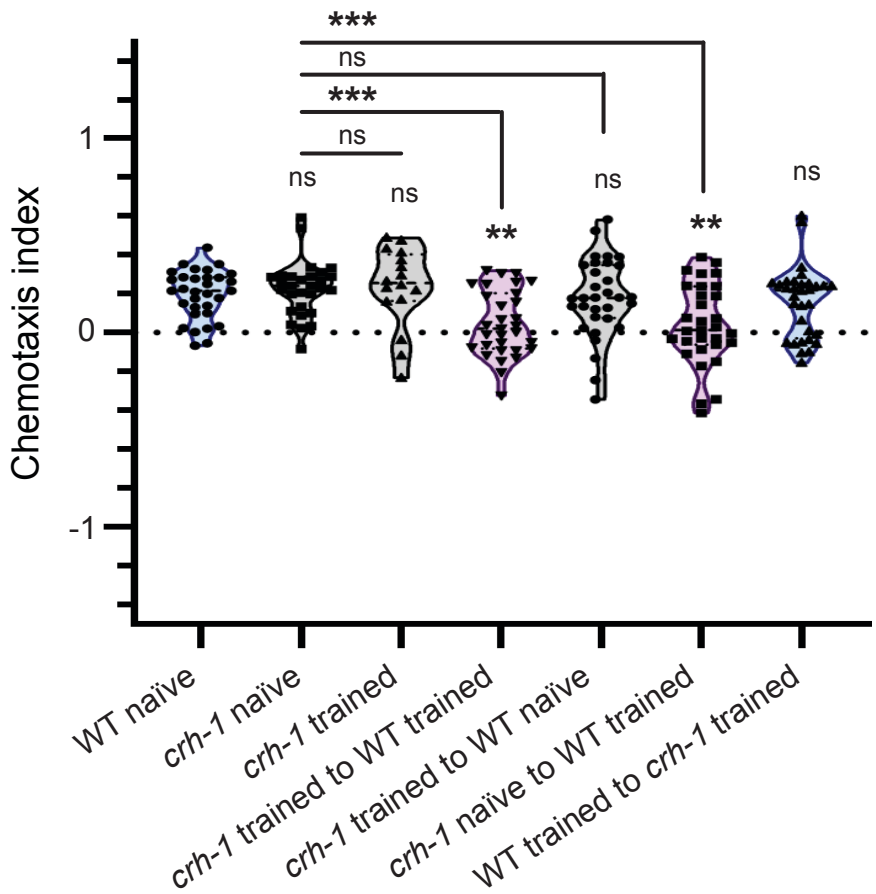

***
ns
***
ns
ns
ns
ns
ns
ns
**
**
1
0
-1
Chemotaxis index
WT naïve
crh-1 naïve
crh-1 trained
crh-1 trained to WT trained
crh-1 trained to WT naïve
crh-1 naïve to WT trained
WT trained to crh-1 trained

Figure 5

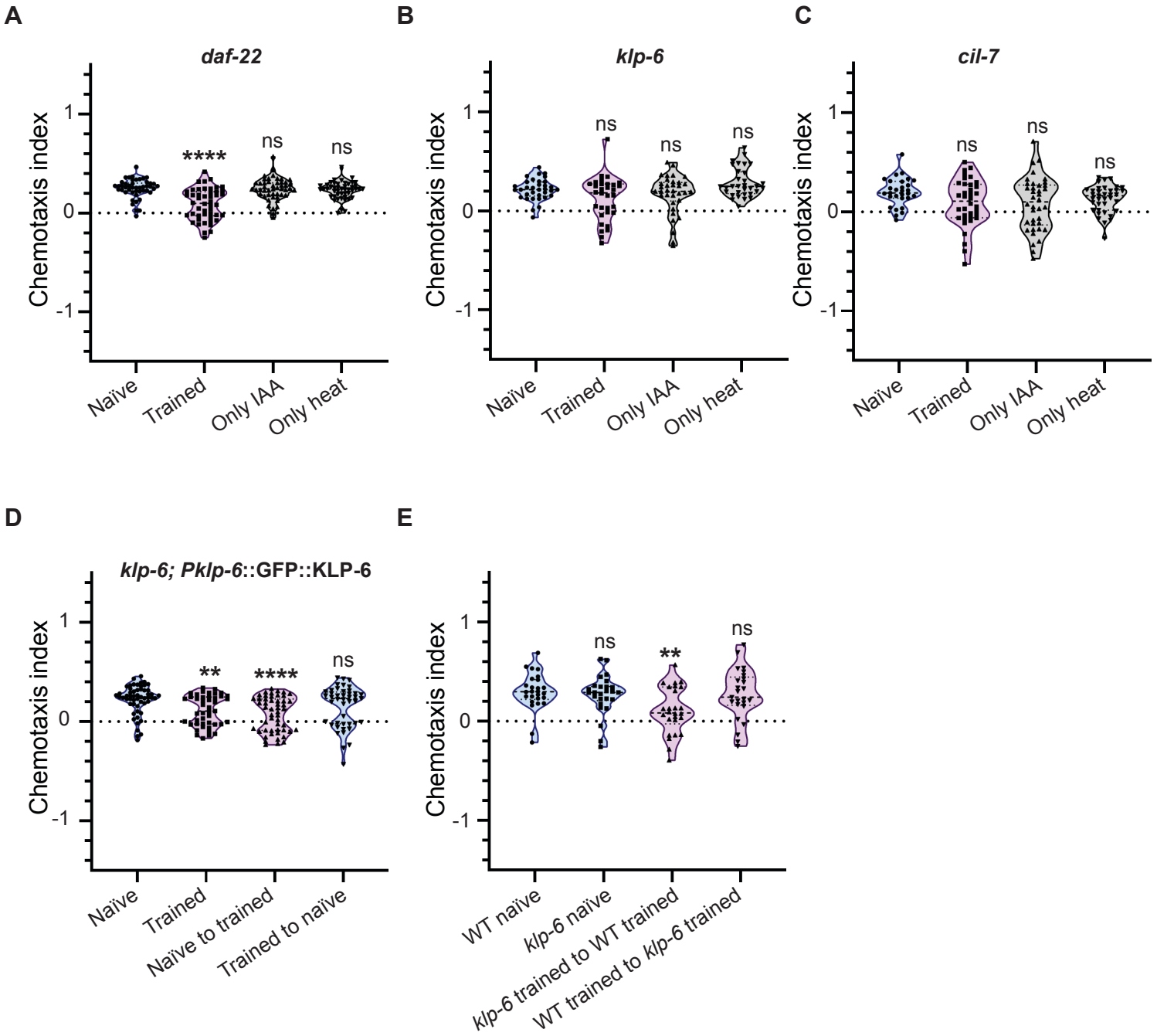
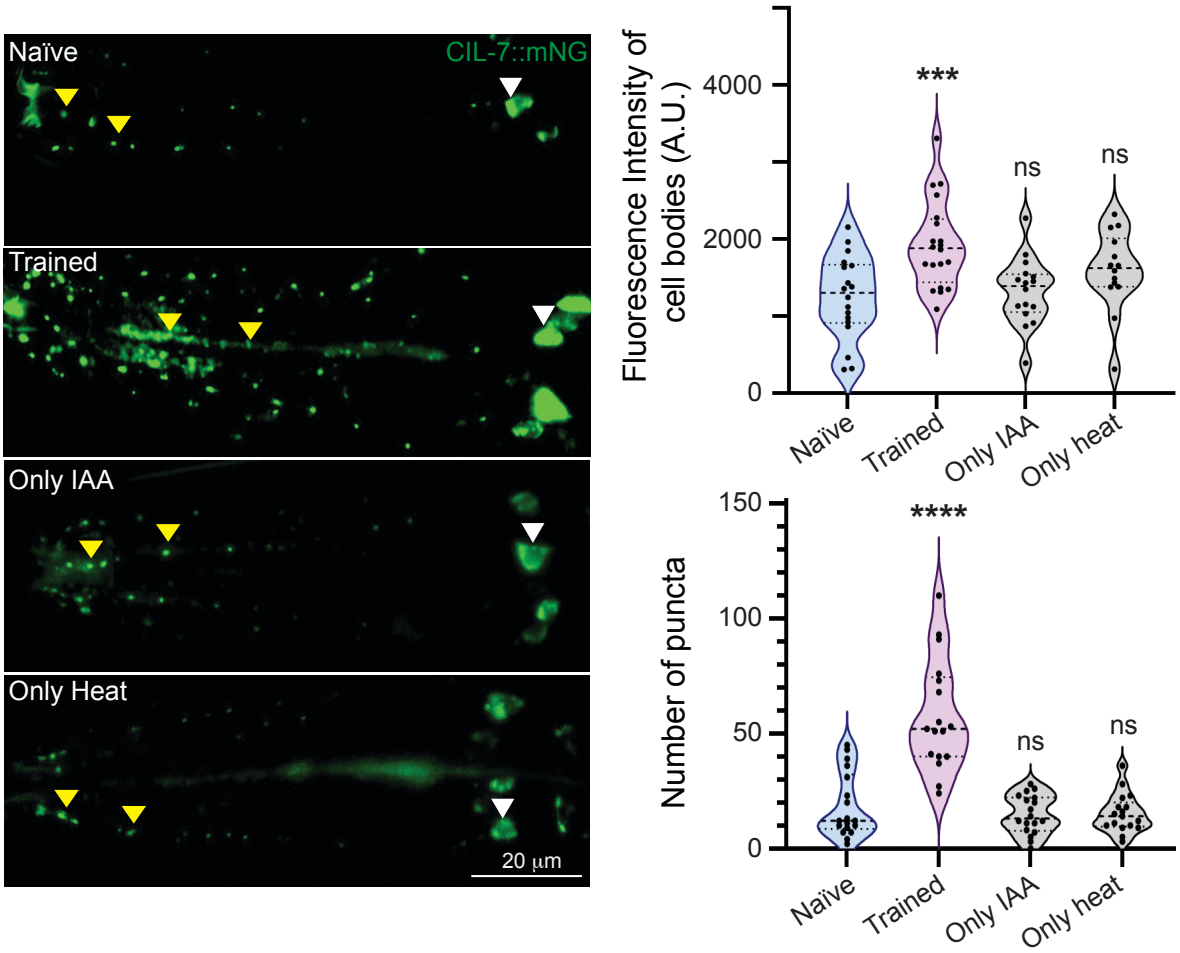
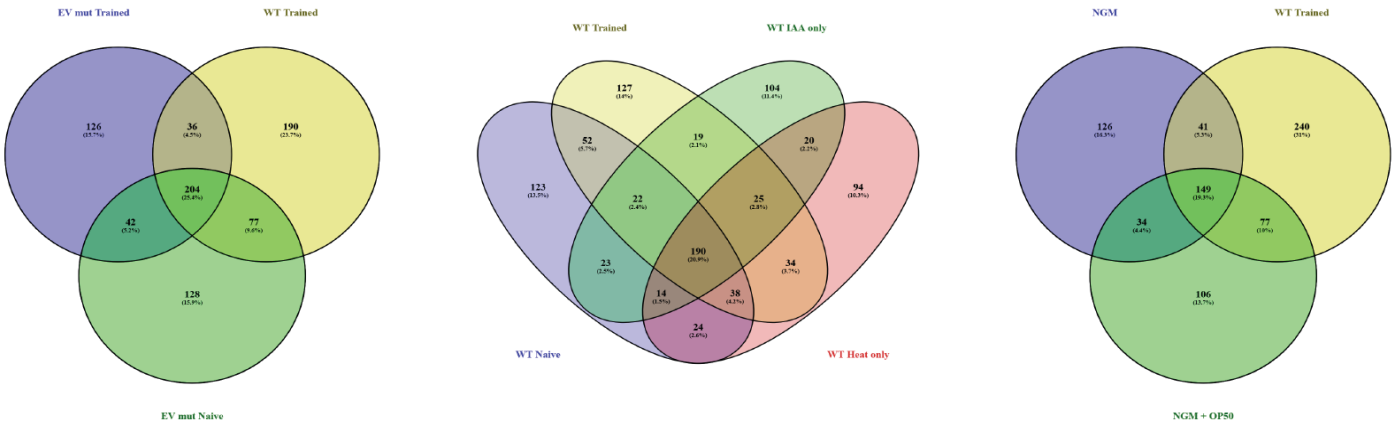

A
daf-22
Chemotaxis index
1
0
-1
****
ns
ns
Naïve
Trained
Only IAA
Only heat
B
klp-6
Chemotaxis index
1
0
-1
ns
ns
ns
Naïve
Trained
Only IAA
Only heat
C
cil-7
Chemotaxis index
1
0
-1
ns
ns
ns
Naïve
Trained
Only IAA
Only heat
D
klp-6; Pklp-6::GFP::KLP-6
Chemotaxis index
1
0
-1
**
****
ns
Naïve
Trained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



B



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

Figure 7

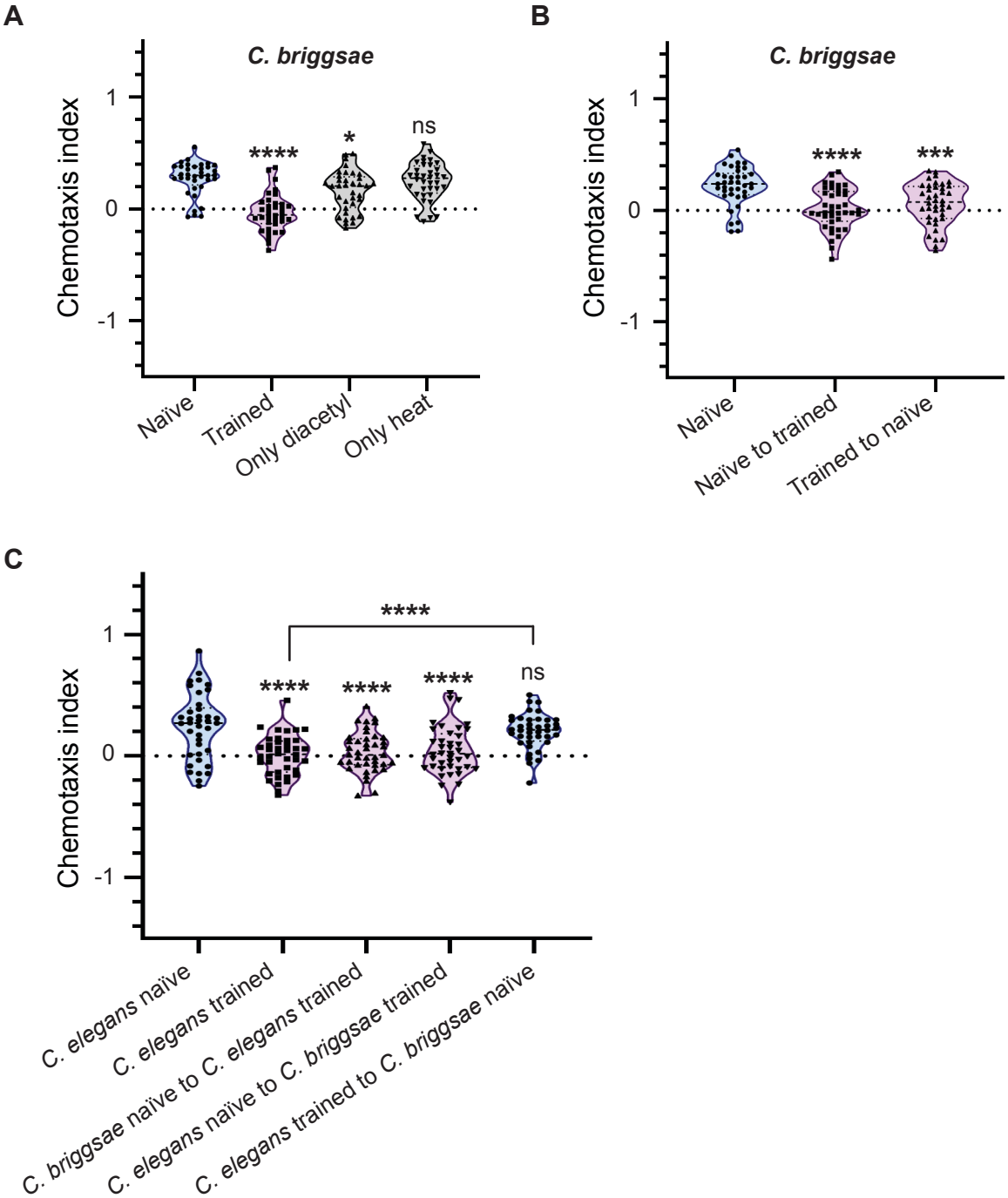
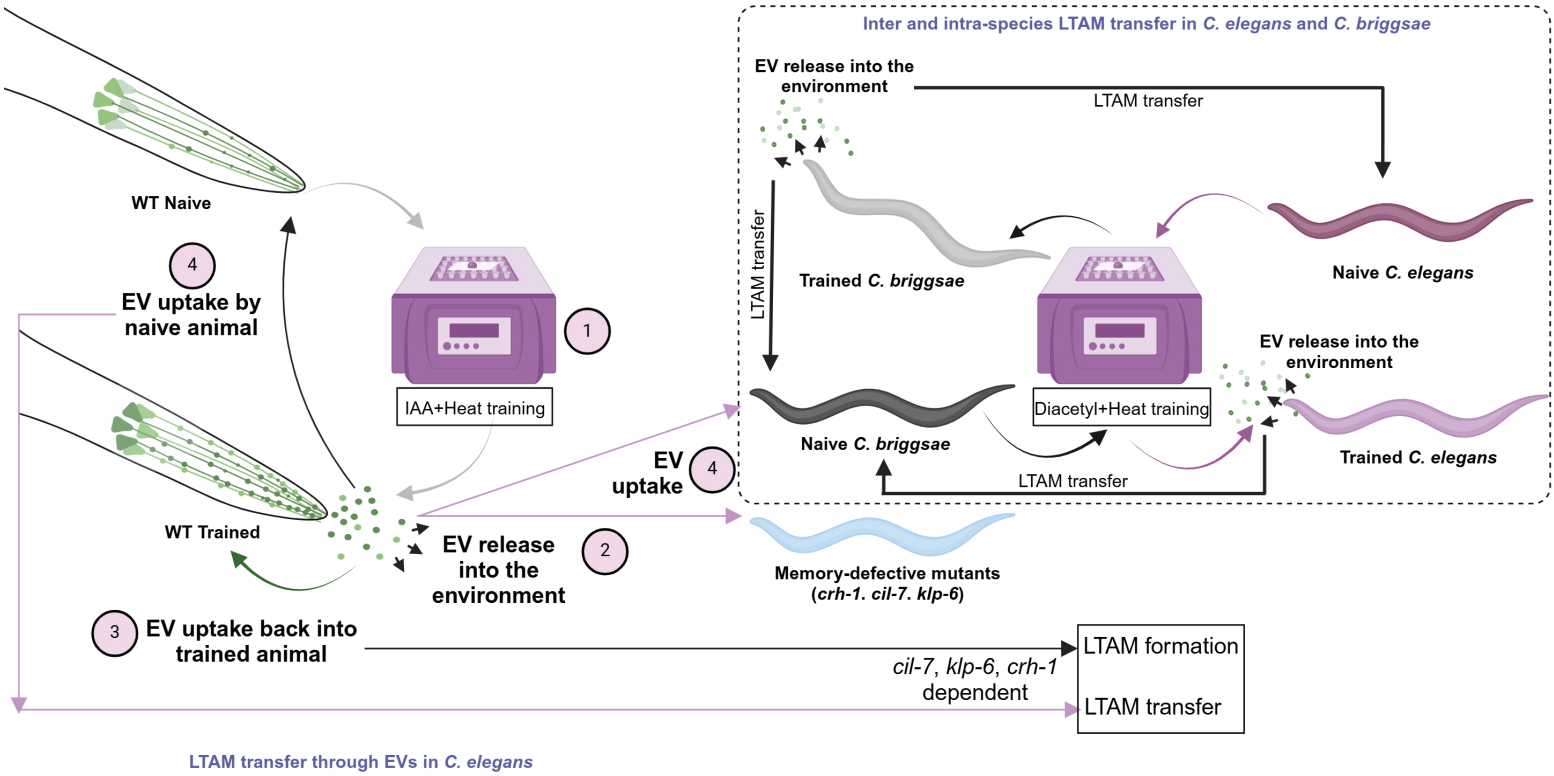

A
C. briggsae
Chemotaxis index
1
0
-1
****
*
ns
Naïve
Trained
Only diacetyl
Only heat
B
C. briggsae
Chemotaxis index
1
0
-1
****
***
Naïve
Naïve to trained
Trained to naïve
C
Chemotaxis index
1
0
-1
****
****
****
****
ns
C. elegans naïve
C. elegans trained
C. briggsae naïve to C. elegans trained
C.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

Figure 8


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