Dopamine signaling regulates predator-driven behavioral changes in *Caenorhabditis elegans*

Amy Pribadi[1,2], Kaila Rosales[2], Kirthi C. Reddy[2] and Sreekanth H. Chalasani[1,2]

[1]Biological Sciences Graduate Program, University of California, San Diego, La Jolla, CA, USA.
[2]Molecular Neurobiology Laboratory, The Salk Institute for Biological Studies, La Jolla, CA, USA.

Address for correspondence: schalasani@salk.edu (S.H.C.).

Abstract

Prey respond to predators by altering their behavior to optimize their own fitness and survival. We probe the interactions between *Caenorhabditis elegans* and its naturally cohabiting predator *Pristionchus uniformis* to reveal the pathways driving changes in prey behavior. *C. elegans*, which normally lays its eggs on a bacteria lawn, alters the location of the eggs away from a lawn occupied by the predator. Also, these eggs are laid close to thin streaks of bacteria, which are likely the result of the prey moving some bacteria away from the dense lawn as they exit the lawn. We confirm that this change in egg location requires a predator capable of biting, but not predator secretions. Next, we find that mutants in dopamine and serotonin synthesis do not display this predator-evoked change in egg location. We also show that dopamine is primarily released from CEP dopaminergic neurons, and requires multiple dopamine receptors (DOP-1, DOP-2, and DOP-3) to alter prey behavior. Predator-exposed prey continue to lay their eggs near the thin bacterial streaks and away from the dense lawn even after the predator is removed, indicating a form of learning. Finally, exogenous dopamine can rescue the behavioral deficits in a dopamine-deficient mutant, suggesting that predator-exposure might cause an increase in prey dopamine. Together, we show that dopamine alters prey foraging strategy upon predator exposure, implying a role for this pathway in defensive behaviors.

Keywords

Predator-prey, *C. elegans*, *P. uniformis*, egg-laying, dopamine.

Introduction

Predator-prey systems offer a rich variety of prey behaviors to explore, from innate to learned responses. Prey responses to predators also vary depending on the predation strategy [1,2], as well as the prey's abilities and the environmental context of both species [3]. Additionally, prey can evaluate the cost/benefit of engaging in these antipredator behaviors, since they might impose additional costs by reducing access to food or mates [4]. While predators kill and consume prey [5,6], prey behavior can also change without receiving direct harm from the predator [7]. Specifically, multiple studies have shown that prey alter their foraging behavior in response to predator presence [8]. For example, reintroducing wolves into Yellowstone National Park affected the foraging behavior of their prey, the elk. In particular, female elk with calves in wolf territory spent less time grazing and more time performing vigilance behaviors [9,10]. This wolf-induced behavioral change in the prey elk affected the growth of the plants upon which the prey grazed, ultimately re-shaping the landscape of the park [11,12]. Similarly, in a laboratory setting, rats confronted with cat odor spend more time in shelter and less time exploring, feeding, or mating [13,14,15]. Prey can also identify features of putative predators and react accordingly; rats in the presence of a moving predator-like robot take more time to acquire the same amount of food because their hesitancy in exploring the cage [16,17]. While large scale ecological studies lack mechanistic insights into the pathways driving prey behaviors, experiments with model organisms often miss the natural ecological context. We wondered whether we could use nematodes to bridge the gap between these two scales linking neural pathways with an ecologically relevant predator-prey interaction.

C. elegans is a nematode that lives in rotting vegetation and eats bacteria found there [18]. With 302 neurons and a mapped connectome [19], it is an excellent model to study behavior through manipulation of genes and circuits potentially to the single-cell level. Considering that much research in prey-predator relationships involve organisms that have vision, the behaviors in olfactory/mechanosensory-dependent organisms like *C. elegans* should be viewed separately than the behaviors exhibited by other organisms. With this in mind, we considered that *C. elegans*-specific behaviors that may not necessarily mimic behaviors which are traditionally associated with predator response in many prey with vision like freezing [20,21]. Much is known already about *C. elegans* foraging behavior including decisions about how to find food [22,23],

how to stay near food [23], and evaluating food quality [24]. We hypothesized that foraging behavior in *C. elegans* might change following predator exposure, and existing knowledge in the field about *C. elegans* foraging could help contextualize any change in foraging.

Previous studies have shown that nematodes of the *Pristionchus* genus can predate on other nematodes like *C. elegans* [25] and are found in necromenic association with beetles [26,27] as well as in rotting vegetation along with *Caenorhabditis* [28,29]. Members of the *Pristionchus* genus exhibit mouth polyphenism, which affects its physical ability to bite [30]. *Pristionchus pacificus* exhibit either a two-toothed Eurystomatous (Eu) mouthform or a single-toothed Stenostomatous (St) mouthform [31]. The Eu mouthform enables biting, and potential killing of nematode prey like *C. elegans* [32,33]. Moreover, environmental conditions such as food availability and media type can alter the Eu/St ratio [34]. Under standard laboratory culture conditions on solid NGM with *E. coli* OP50 as food, the majority of *P. pacificus* exhibit the Eu mouthform [34]. While *P. pacificus* is a relatively well-studied species within *Pristionchus,* it is uncertain whether *C. elegans* actually interacts with *P. pacificus* in nature. In contrast, the gonochoristic species *Pristionchus uniformis* has been found in the same sample with wild *C. elegans* isolates [28] so it is likely that the two species may encounter each other in nature. *P. uniformis* was first characterized as a St-only species [35], but was recently recharacterized as possessing either a bacterivorous St or predatory Eu mouthform [36]. We found that in standard growth conditions most *P. uniformis* strain JU1051 are Eu (**Fig. 1- S1a**).

Given the intertwined role of spatial food location on prey behavior (as well as the influence of prey on food location), we wondered whether we could observe the effect of predator presence on foraging behavior in *C. elegans*, and then use the molecular tools developed in *C. elegans* to discover what mechanisms might underlie these changed behaviors. In this study, we show that *C. elegans* avoids a bacteria lawn that is occupied by its naturally cohabiting predator *P. uniformis* [28] and lays its eggs away from that lawn. We show that this behavior requires dopamine and serotonin and multiple dopamine receptors. Furthermore, we find that *C. elegans* avoids a dense bacteria lawn for many hours even after the predator is removed, and this avoidance is exaggerated when there is a less-dense alternate source of food available. Finally, we use exogenous dopamine to reveal insights into the underlying mechanism affecting this long-lasting behavioral change.

Results

C. elegans avoids bacteria lawns habited by *Pristionchus* predators

We recently showed that *P. pacificus* bites *C. elegans* adults even though it is difficult to consume them. This biting of adult *C. elegans* prey forces these animals to leave the bacteria lawn, resulting in greater access to the lawn by the predator [37]. Using a modified version of the protocol in this previous study [37], we placed three predators and three *C. elegans* on an assay plate containing a small, dense bacterial lawn. There was a small area of bare agar between the bacterial lawn and the corral. A ¼" filter paper corral was placed around the lawn to constrain the animals' movements. Control plates had six *C. elegans* to maintain a consistent number of animals between plates with and without predators. The area inside the corral was imaged at the relevant time points to observe the position of eggs relative to the bacterial lawn. Changes in egg location were analyzed in relation to the location of the lawn. Since *Pristionchus* also lay eggs, we used a *C. elegans* strain with an integrated GFP marker that expresses in eggs (*Pelt-2*::GFP) (**Fig. 1a**).

To observe whether predator type affects *C. elegans* prey behavior, we chose several different types of predators: *P. pacificus* strains PS312 and RS5194, a stenostomatous-only *P. pacificus* mutant TU445 *eud-1(tu445)* [33], and an isolate of *P. uniformis*, JU1051. *P. pacificus* strain RS5194 is more aggressive than PS312 as characterized by an increased probability of bite per encounter [37] so both strains were included in this analysis. The stenostomatous-only mutant was included to demonstrate whether biting was required for predators to alter *C. elegans* behavior. Finally, we wondered whether *P. uniformis*, which may interact with C. *elegans* in nature, could induce different behavioral changes than *P. pacificus*. *P. uniformis* males and females were considered separately, while only hermaphrodite *P. pacificus* were used. We first tested if short-term exposure could increase off-lawn egg-laying. After six hours of exposure, no obvious changes in egg location could be seen (**Fig. 1- S1b**). To prevent eggs hatching into L1s, which secrete pheromones that promote lawn-leaving [38] this assay only ran for six hours.

We hypothesized that increasing predator exposure time would increase the difference between egg location in predator-exposed animals. To maximally increase exposure time, we conducted a long-term assay with L4 *C. elegans* and J4 *Pristionchus* instead of adults and stopped the assay after 20 hours of exposure. The juveniles developed into adulthood over the course of the assay, and *C. elegans* start laying eggs approximately 8-10 hours after the L4 stage

[39] so eggs were laid in the latter part of the assay and most did not hatch during the 20-hour assay duration. We found that all *P. pacificus* except for the stenostomatous *eud-1* mutant were able to increase the median distance of eggs laid from the lawn edge (**Fig. 1b**). *P. uniformis* males also induced a change in egg location. These experiments showed that *C. elegans* change their egg location to be farther outside the lawn edge when paired with a predator for 20 hours, and a predator that cannot bite does not induce egg location change.

Next, we tested whether *Pristionchus* biting-induced injury was required for the change in *C. elegans* egg location. We used a *C. elegans* reporter strain expressing GFP (green fluorescent protein) under the control of a *nlp-29* promoter. This strain (*Pnlp-29*::GFP) has been shown to increase GFP expression upon wounding the cuticle with a microinjection needle, a laser beam, or fungal infection [40,41]. We paired each of the predators we tested in our egg location assay with this reporter strain and monitored GFP fluorescence relative to the co-injection marker (*Pcol-12*::*dsRED*) (**Fig. 1c-d**). We found that both isolates of *P. pacificus* (PS312 and RS5194) were able to increase GFP fluorescence in this reporter strain within four hours (**Fig. 1- S1c**). In the 20-hour assay, exposure to *P. pacificus* PS312 adults increased GFP fluorescence in *C. elegans* prey. *Pnlp-29*::GFP reporter animals exposed to *P. pacificus* RS5194 for 20 hours were killed and could not be measured. (**Fig. 1c-d**). In contrast, the stenostomatus *eud-1* mutant was unable to increase GFP fluorescence even after 20 hours. Neither *P. uniformis* males and females were able to increase GFP fluorescence in this *Pnlp-29*::GFP reporter strain (**Fig. 1c-d**). However, *C. elegans* do recoil and reverse upon receiving bites from *P. uniformis* (**Supplementary Video 1**), confirming *P. uniformis* biting can be sensed by the prey. It is possible that these bites are causing some low level of harm without damaging the cuticle sufficiently to increase expression from the *Pnlp-29*::GFP reporter. Regardless, these data suggest extensive injury is not required to induce *C. elegans* to lay its eggs away from the bacteria lawn. We planned to use the *C. elegans* egg location assay for the remainder of our study and so chose a predator that does not lay eggs (*P. uniformis* males) in our assays (**Fig. 1e**). Moreover, we also suggest that change in egg location is not a result of extensive injury to the prey.

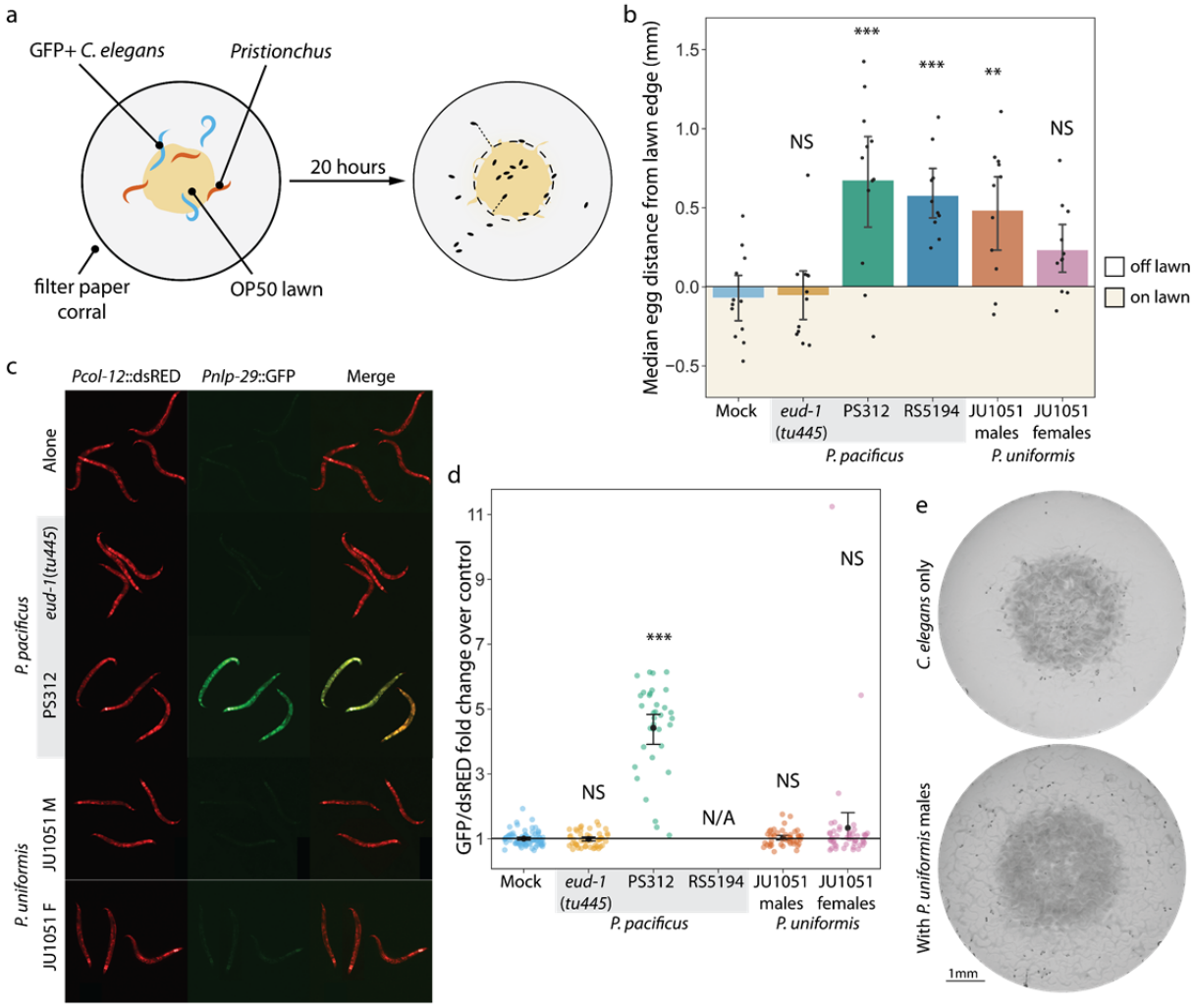

Figure 1 || Predator biting influences prey egg location. a. Egg position assay setup. Three *C. elegans* expressing *Pelt-2*::GFP are paired with three *Pristionchus* in a ¼" arena with a small OP50 lawn in the center. The animals are allowed to interact and *C. elegans* eggs are identified through GFP expression. Egg locations are marked and distances to the lawn edge are calculated. **b.** Overnight (20-hour) egg location assay results with various predators. Each data point represents the median distance of the eggs to the lawn edge for one assay plate. n≥10. **C.** *C. elegans* expressing *Pnlp-29*::GFP paired with various predators after 20 hours.. Reporter strain also includes *Pcol-12*::dsRED co-injection marker. **d.** Fluorescence of *Pnlp-29*::GFP C. elegans after 20-hour exposure to various predators. RS5194-exposed animals all died following predator exposure. GFP measurements are normalized to *Pcol-12*::dsRED co-injection marker. Fold-change of fluorescence is shown relative to the mean of the *C. elegans*-only control. n≥34. **e.** Representative images of assay plates after the 20-hour assay, with *C. elegans*-only control on top and *C. elegans* paired with *P. uniformis males* on the bottom. Error bars represent 95% CI. Pairwise comparisons were performed with a Dunnett's test between each predator type compared to mock no-predator condition. * p<0.05, ** p<0.01, ***p<0.001, NS=p>0.05.

To test whether the location of the *C. elegans* matched the location of their eggs, we used an imaging setup (WormWatcher) and monitored whole-body fluorescent *C. elegans* positions over the entire 20-hour assay period (**Fig. 2a**). This assay also gave an insight into the location of the *C. elegans* during the beginning of the assay before egg-laying starts. We found that *C. elegans* leave the bacteria lawn occupied by *P. uniformis* males by around 6 hours and by 10 hours spend most of the remaining assay duration off of this lawn (**Fig. 2b**). Together, these data show that *C. elegans* avoids the bacteria lawn occupied by *P. uniformis* males after at least six hours of interaction, and that the egg locations change matches the body locations in the latter part of the 20-hour assay.

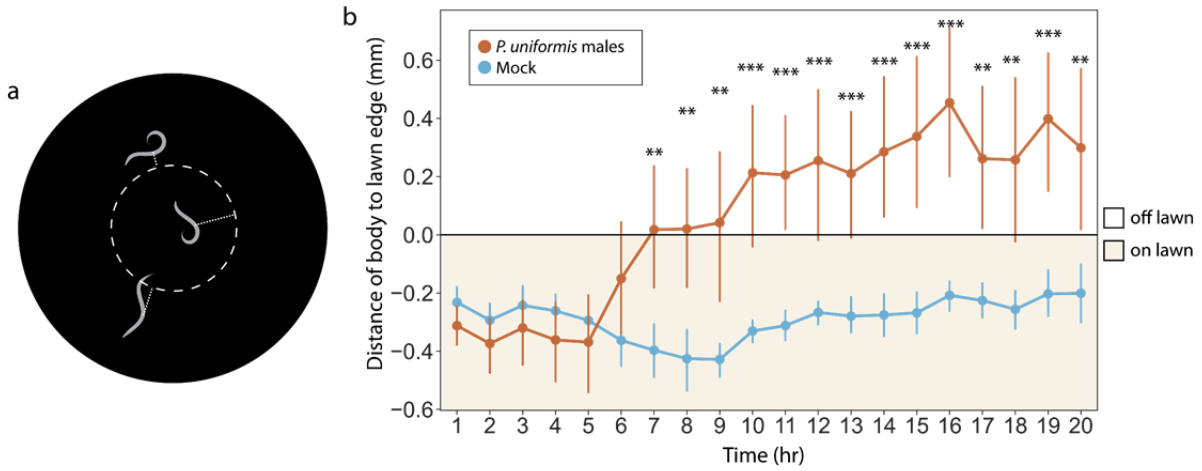

Figure 2 || Prey location matches their change in egg location. **a.** Schematic for WormWatcher experiments for location tracking. Midpoint of the fluorescent *C. elegans* body is identified and distance from midpoint to lawn edge is calculated. **b.** Location traces of fluorescent *C. elegans* with or without a predator present over the course of the 20-hour assay. Images are acquired every four minutes. Data was then binned into hourly averages (per arena). A two-way mixed effects ANOVA showed a significant interaction between predator exposure and time (p=5.80E-29). Pairwise comparisons between predator exposure conditions at each time point are indicated on graph (*p<0.05, **p<0.01, ***p<0.001; Bonferroni adjustment). n=12 arenas per condition.

We next tested what ratio of predators (*P. uniformis* males) to prey (*C. elegans*) was able to alter the location of prey eggs. While maintaining the same arena size and total number of animals (six), we altered the ratio of predators and prey. We found that the presence of a single predator was able to shift egg location and adding additional predators resulted in a stronger shift (**Fig. 1-S2a**). We also found that *P. uniformis* males did not change the total number of *C. elegans* eggs, but only altered their location away from the bacteria lawn (**Fig. 1-S2b**). These data are consistent with results in our previous study using *P. pacificus* [37]. We have

previously shown that *P. pacificus* secretions are aversive to *C. elegans* [42]. Given this, we tested whether *P. uniformis* was secreting an aversive chemical that drives *C. elegans* away from the bacteria lawn. We conditioned bacteria lawns with *P. uniformis* males and sterile *C. elegans* (to simulate changes in lawn caused by animal movement) and tested whether naïve *C. elegans* would alter their egg location on these lawns. We observed no difference in *C. elegans* egg location between *P. uniformis*- or sterile *C. elegans*-conditioned bacteria lawns (**Fig.1-S3**), suggesting that *P. uniformis* males likely do not secrete a *C. elegans* aversive signal that can account for a change in their egg location.

Change in bacterial topography alone contributes to, but does not account for extent of egg location change

We observed that arenas containing *C. elegans* and *P. uniformis* males, but not controls had streaks of bacteria outside the main lawn (**Fig. 3a**). Given the duration of our assay, these streaks might represent bacteria that sticks on the *C. elegans* body and gets deposited onto the agar as it exits the lawn. Over the duration of the assay, these streaks grow and are visible to the naked eye by the end of the 20-hour period. We first tested whether the presence of streaks outside the main lawn alone could account for the change in egg location. We artificially streaked bacteria lawns at the beginning of the assay and monitored the location of the eggs in these predator-free arenas (**Fig. 3a**). While artificial streaking did increase the average distance from the lawn edge where the eggs are laid, the distance of eggs from animals exposed to *P. uniformis* males was significantly different from the distance of eggs on the artificially streaked lawn (**Fig. 3b**). These data showed that bacteria streaks outside the main lawn are not the only driver of egg location change during predator exposure, but do contribute to the phenotype.

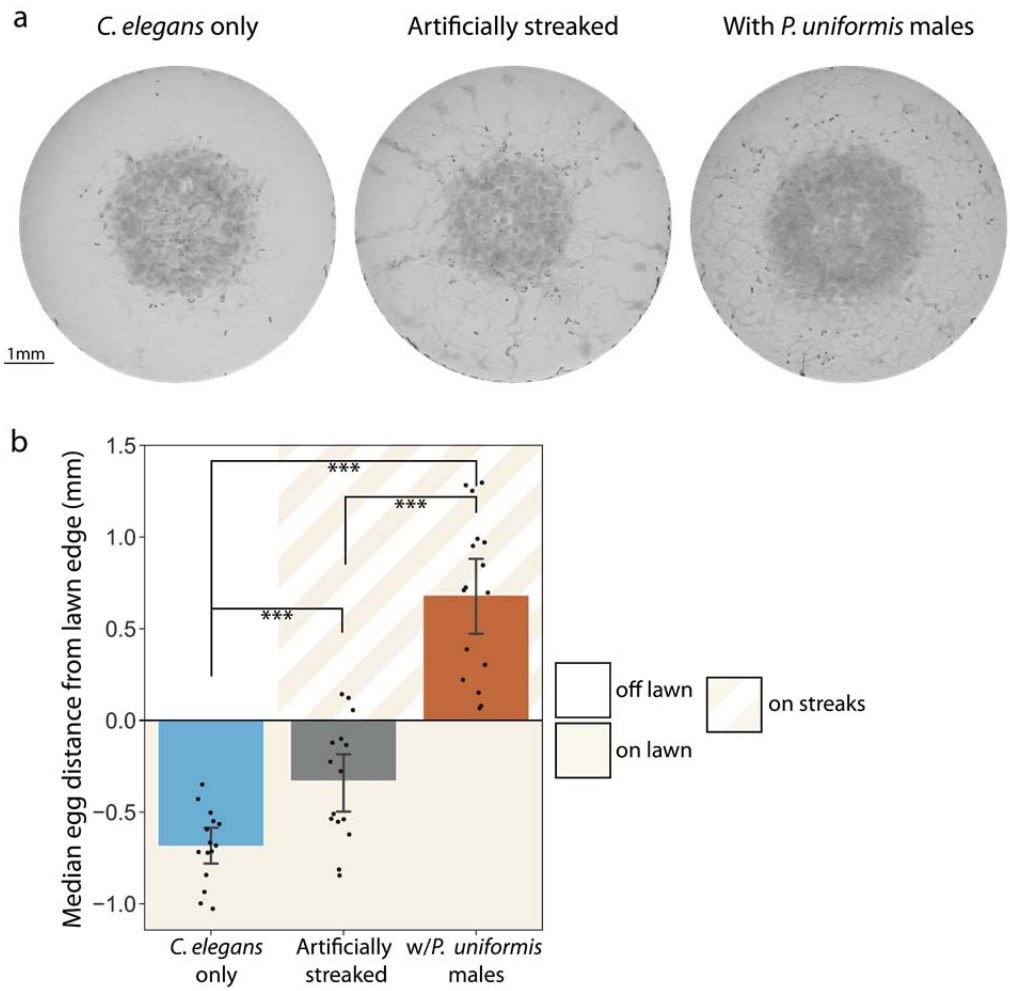

Figure 3 || Bacterial topography changes alone do not account for egg location changes. a. Representative images of assay plates after the 20-hour assay, with *C. elegans*-only control on the left, an artificially streaked lawn with only *C. elegans* (see Methods) and *C. elegans* paired with *P. uniformis* males on the right is). **b.** Egg location on the artificially streaked lawn, compared to a *C. elegans*-alone control and a lawn with *C.* elegans and *P. uniformis.* Significance determined with a one-way ANOVA followed by Tukey's HSD for all pairwise comparisons. * $p<0.05$, ** $p<0.01$, ***$p<0.001$, NS=$p>0.05$. $n\geq12$

Change in *C. elegans* egg location requires dopamine and serotonin signaling

Biogenic amines are known to modulate behaviors over long time scales, and the change in egg location behavior takes over six hours to take effect and appears to last for many hours. We hypothesized that biogenic amines may play a role in the egg-laying location change. We tested mutants in various biogenic amines synthesis and release machinery. Consistent with a role for biogenic amines, we found that mutants in the *C. elegans* homolog of the mammalian vesicular monoamine transporter (VMAT), *cat-1* [43] had did not shift egg location in response to *P.*

uniformis males (**Fig. 4a**). We also found that mutants in *cat-2* (which encodes tyrosine hydroxylase for dopamine synthesis [44,45]) and *tph-1* (tryptophan hydroxylase for serotonin synthesis [46]) had reduced changes in egg location upon exposure to *P. uniformis* males, but *tdc-1* (tyrosine decarboxylase required for tyramine synthesis [47]) and *tbh-1* (tyramine beta hydroxylase for octopamine synthesis [47]) displayed wildtype egg location behavior (**Fig. 4a**). These data show that mutants in both serotonin and dopamine synthesis contribute to the egg location change in response to *P. uniformi*s males. As serotonin has been previously shown to play a major role in modifying egg-laying behavior [48,49], we focused our analysis on the role of dopamine in this behavior. We tested an additional allele of *cat-2 (n4547)* and found that these mutants also did not respond to predator, confirming a role for *cat-2* and dopamine signaling in this behavior (**Fig. 4b**).

In *C. elegans*, CAT-2 is expressed by eight neurons (four CEPs, two ADEs and PDEs), and dopamine signaling has been previously shown to affect modulation of locomotion as well as learning [50]. Additionally, analysis of the dopamine transporter promoter has identified specific elements that drive expression of transgenes in subsets of these dopaminergic neurons [51]. Using these cell-selective promoter elements, we expressed full-length coding sequence of the *cat-2* cDNA under either CEP- or ADE/PDE- specific promoters. We found that both transgenic animals restored responses to *P. uniformis* males, but CEP-specific expression was able to increase off-lawn egg laying to near wildtype levels (**Fig. 4c**). These results indicate dopamine synthesis from CEPs likely play a major role in altering egg location upon exposure to *P. uniformis* males.

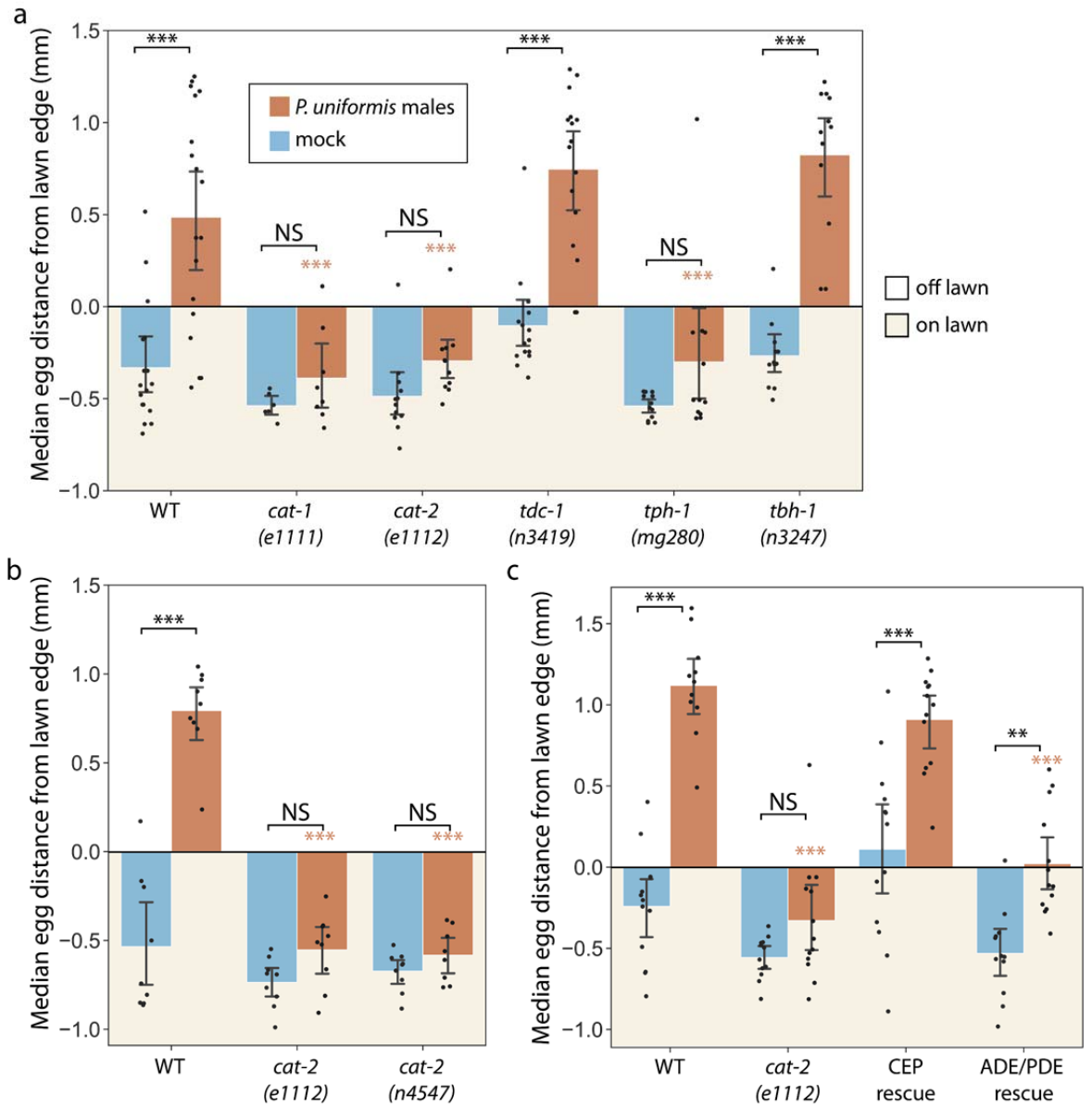

Figure 4 || Dopamine synthesis is required for predator-induced egg location change. a. Egg locations of mutants in biogenic amine-relate genes. **b.** Egg location of a second *e1112* and a second *cat-2* allele *n4547*. **c.** Transgenic rescue of *cat-2* using a CEP-expressing promoter p27 or ADE/PDE-expressing promoter dat-1 p19 in a *cat-2(e1112)* background. Error bars represent 95% confidence interval. Pairwise comparisons are shown between predator/mock conditions within the same strain (black asterisks), as well as between WT and mutants of the same predator/mock condition (blue asterisk = comparison between WT/mock and mutant/mock; orange asterisk = comparison between WT/predator and mutant/predator, non-significant differences are not marked). A two-way ANOVA was conducted followed by multiple pairwise comparisons for all relevant pairs (See Materials and Methods). * $p<0.05$, ** $p<0.01$, ***$p<0.001$, NS=$p>0.05$. $n\geq9$

To test whether *cat-2* mutants' body location throughout the assay reflected their egg location changes, we monitored the location of these animals over the course of the entire 20-hr

assay period using the WormWatcher imaging setup (**Fig 5a**). To observe effects between all three variables (genotype, predator exposure, and time), a three-way mixed ANOVA was performed and found a significant three-way interaction (p=5.88E-5). To specifically investigate the effect of the *cat-2* mutation on egg distance in the predator-exposed or mock conditions, two-way mixed ANOVAs were conducted on either predator-exposed or mock data. In the mock data, no significant two-way interaction was found between genotype and time (p=0.377), but a significant two-way interaction was found in the predator-exposed data (p=2.82E-5). These results imply that the *cat-2* mutation affects egg location over time only in the predator-exposed condition. While the *cat*-2 mutants trended toward smaller distances in the latter half of the assay, significant differences between *cat-2* and wild-type predator-exposed distances were only found at a few late time points. This could be due to incomplete knockdown of dopamine synthesis in *cat-2(e1112)* [52], or due to an additional role for dopamine in altering egg laying dynamics [53] during predator exposure.

As reducing the amount of available dopamine in the *cat-2* mutant depressed locomotor response to predator, we hypothesized that increasing dopamine would increase the response to predator. Excess dopamine at synapses is removed by the dopamine transporter DAT-1 and mutants in this gene likely have more dopamine at their synapses [54,55]. We found that *dat-1(ok157)* mutants, with and without predator exposure, had increased distances from the lawn edge at most time points starting from 6 hours compared to wild-type (**Fig 5b**). A significant interaction between genotype and time was found only in the no-predator condition (p=0.0140) but not in the predator-exposed condition (p=0.940). A three-way mixed ANOVA was performed and failed to find a significant three-way interaction. Taken together, these results imply that *dat-1* increases distances from the lawn overall, and due to the effect on the baseline (no-predator) condition, the predator-evoked behavioral change in the *dat-1* mutants is similar to wild-type. These results suggest that dopamine likely acts to alter locomotor behavior, consistent with its known role linking environmental changes to locomotor changes [50]. Combining the location tracking results from *cat-2(e1112)* and *dat-1(ok157)*, dopamine may be involved in this assay by encouraging movement away from the lawn, and potentially increasing egg-laying off-lawn (dopamine can increase egg-laying, even in off-food conditions [56]), which would highlight the effect of predator exposure on egg location.

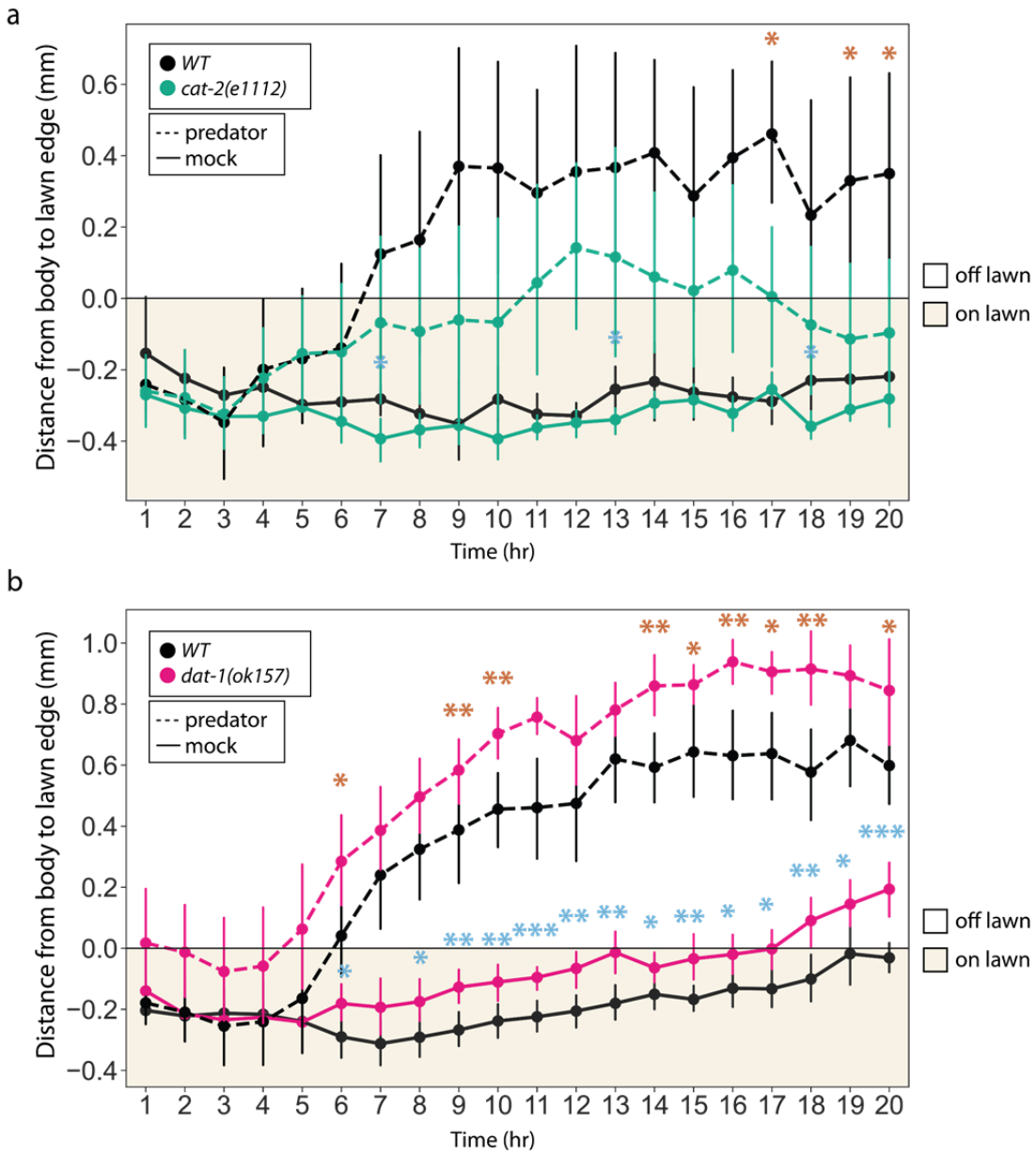

Figure 5 || Deficient and excessive dopamine affect location relative to the lawn. Pairwise tests for predator-exposed between genotype are indicated with orange asterisks (*p<0.05, **p<0.01, ***p<0.001; Bonferroni adjustment). Dashed lines represent the predator-exposed condition, unbroken lines represent no-predator controls. Images are acquired every four minutes and distances from each worm's midpoint to the lawn edge is calculated. Data was then binned hourly. Line represents mean, error bars are 95% CI. **a.** Location traces of fluorescent *C. elegans* carrying the *cat-2(e1112)* allele compared to wild-type. A three-way mixed ANOVA found a significant three-way interaction between genotype, predator exposure, and Time (p=5.88E-5) but there was significant two-way interaction between genotype and Condition. n=8 **b.** Location traces of fluorescent *C. elegans* carrying the *dat-1(ok157)* allele compared to wild-type. A two-way mixed ANOVA was conducted for the predator-exposed conditions. A significant interaction was not found for both genotype and Time (p=0.940). In Control data, a significant effect was found for genotype and Time (p=0.0140). n=12 (See Materials and Methods; ANOVA results provided in **Appendix**)

The *C. elegans* genome encodes at least four dopamine receptors (*dop-1*, *-2*, *-3*, and *-4*) with viable mutants in each. We tested single mutants in each of these four receptors and found that they all displayed wild-type egg location changes in response to predator (**Fig. 6-S4**). Next, we tested mutant combinations and found that each double mutant was able to alter its egg location in response to predator, similar to wild-type animals. However, we did observe a slight decrease in the *dop-1; dop-3* double mutant response to predator (**Fig. 6a**). A triple mutant in *dop-1, dop-2*, and *dop-3* as well as a quadruple mutant in all four dopamine receptors both displayed strongly reduced off-lawn egg-laying behavior, although they still retained some sensitivity to predator presence (**Fig. 6b**) Therefore, *dop-1, dop-2*, and *dop-3* appear to combine to drive the egg location behavior change in response to predator, while *dop-4* appears dispensable to this phenotype.

While the triple and quadruple mutants showed reduced off-lawn egg laying, they were not able to abolish the behavior as seen in *cat-2* dopamine-deficient mutants. This suggests that there may be additional pathways through which *cat-2* modulates this behavior. These pathways could include additional dopamine receptors or developmental abnormalities present in CAT-2-deficient animals but not present in the receptor-deficient animals. Together, these results show that dopamine released from CEP neurons is likely sensed by DOP-1, -2, and -3 receptors altering egg location upon exposure to *P. uniformis* males.

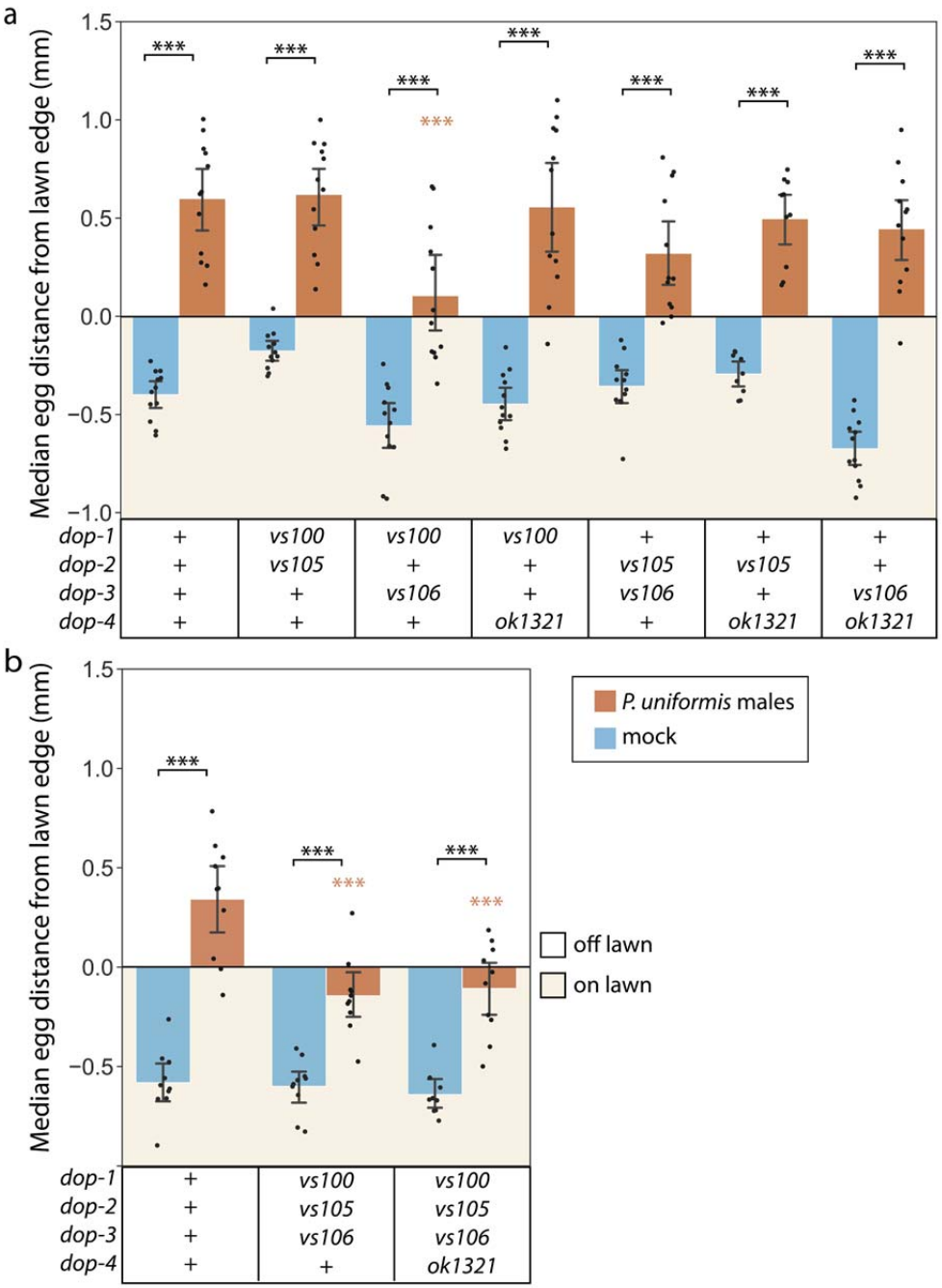

Figure 6 || DOP-1, DOP-2, and DOP-3 contribute to egg location change. a. Egg locations of double mutants in every combination between *dop-1*, *dop-2*, *dop-3*, and *dop-4*. **b.** Egg locations of a triple mutant in *dop-1*, *dop-2*, and *dop-3* and a quadruple mutant in all four dopamine receptors. Error bars represent 95% confidence interval. Pairwise comparisons are shown between predator/mock conditions within the same strain (black asterisks), as well as between WT and mutants of the same predator/mock condition (blue asterisk = comparison between WT/mock and mutant/mock; orange asterisk = comparison between WT/predator and mutant/predator, non-significant differences are not marked). A two-way ANOVA was conducted followed by pairwise multiple comparisons for all relevant pairs (See Materials and Methods). * $p<0.01$, ** $p<0.01$, ***$p<0.001$, NS=$p>0.05$. $n\geq9$

Egg location change lasts many hours even without the predator

Next, we tested whether egg location changes persist even in the absence of predators. We "trained" *C. elegans* prey in our egg location assay setup with *P. uniformis* males for 20 hours and transferred only the prey to a test arena. As shown above, the presence of bacterial streaks during the assay affects prey behavior. While the streaked area alone did not account for the egg location change, we wondered whether trained *C. elegans* prey might respond to the change in bacterial topography differently.

To determine what aspects of the bacterial environment the trained *C. elegans* might use to navigate, the test arenas were either completely covered with bacteria (filled), or a small lawn (small) with bare agar around it, or an artificially streaked lawn (streaked) (**Fig. 7a**). We transferred "trained" *C. elegans* prey to test arenas, then monitored the location of prey eggs over the next 6 hours. We found that there was no difference in egg location on filled lawn plates, indicating that predator-exposed and control animals disperse similarly on bacteria lawns (**Fig. 7b,c**). In contrast, on small lawn plates, predator-exposed animals laid most of their eggs on the lawn but laid more eggs off-lawn as well as close to the lawn edge compared to controls (**Fig. 7b, c, Fig. 7-S5**). However, on the streaked lawn plates, predator-exposed, but not controls laid their eggs throughout the arena both on the lawn and outside (**Fig. 7b, c**). Additionally, this altered egg-laying behavior lasts at least 6 hours even without the predator.

To determine whether the change in behavior persists even if transferred to a different environment, we transferred 20-hour "trained" *C. elegans* to a rest plate completely filled with food before testing them on an assay plate that had a lawn and artificial smears (**Fig. 7d**). We found that *C. elegans* exposed to predators continued to lay their eggs off lawn after a 1-hour rest, but the difference in behavior was reduced when the duration of rest was increased. However, differences in egg location behavior could still be detected even after a 24-hour rest period (**Fig. 7e**). Collectively, these data indicate that *C. elegans* alters its egg location upon predator exposure for at least six hours even without predators, a behavior that is also retained even after exploring a different context during a rest period.

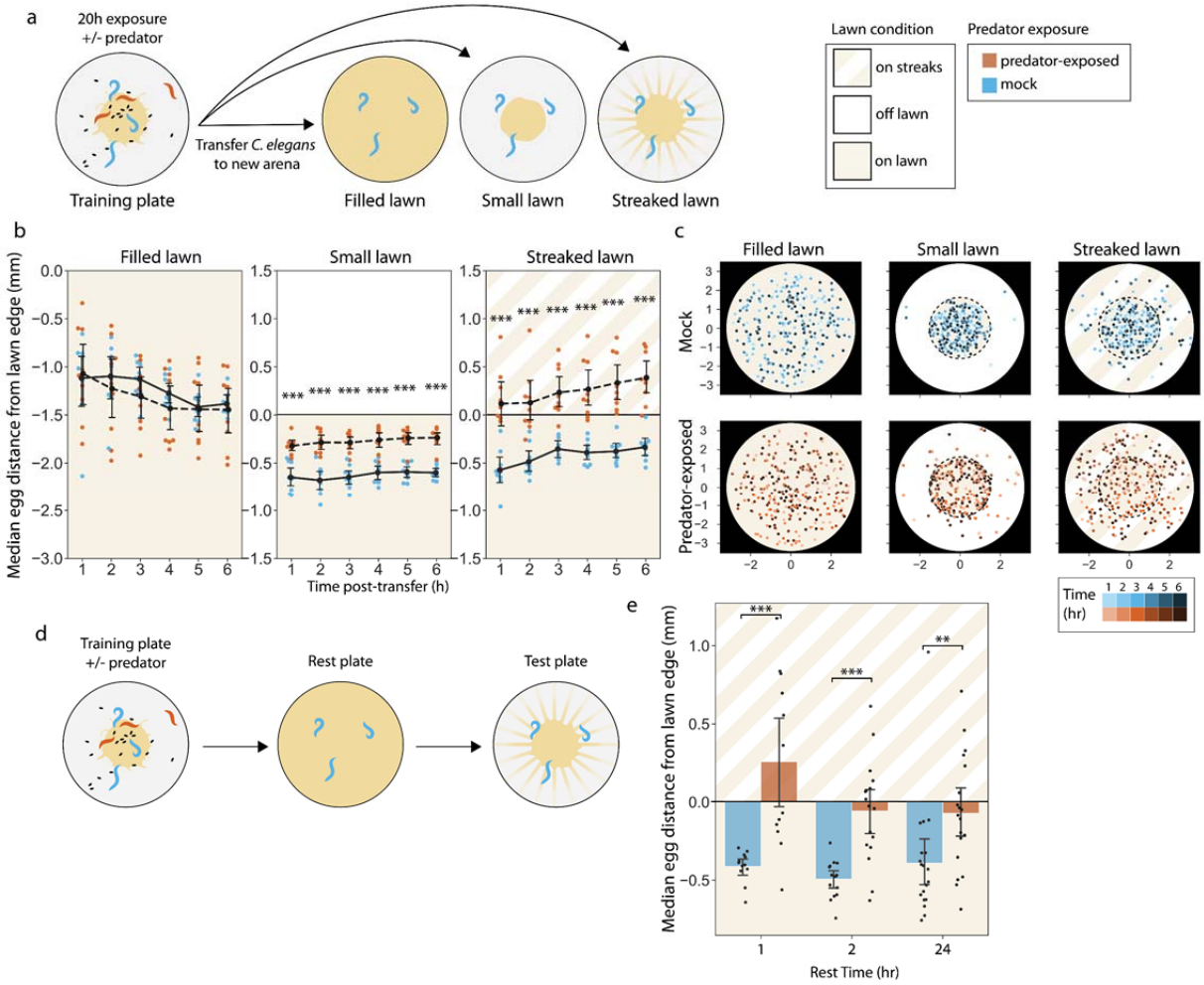

Figure 7 || Predator-exposed *C. elegans* have persistently altered egg locations on streaked lawns.2 a. After a 20-hour exposure to predator as in the egg location assay, *C. elegans* are transferred to one of three types of test plates: a "filled" lawn, a "small" lawn, or a "streaked" lawn (see Materials and Methods). n≥8 **b.** Egg locations of *C. elegans* after predator exposure for six hours after transfer to test plate. Error bars represent 95% CI. Two-way mixed ANOVAs were performed for each lawn type and significant differences between pairwise comparisons between predator-exposed/mock at each time point are shown (See Materials and Methods; ANOVA results provided in **Appendix**) **c.** Sample egg locations relative to the food (dashed line circle). 50 eggs were randomly sampled and plotted from each time point to create representative plots. **d.** Alternate experimental setup including an additional rest step with variable times. **e.** Egg locations of trained C. elegans transferred to a rest plate for 1, 2, or 24 hours and then transferred to the "streaked" lawn test plate. n≥12. Error bars represent 95% CI. A two-way ANOVA was conducted followed by multiple comparisons for pairs between predator exposure/mock at each rest time (See Materials and Methods). * $p<0.05$, ** $p<0.01$, ***$p<0.001$, NS=$p>0.05$.

Exogenous dopamine rescues the behavioral deficits of dopamine-deficient mutants

Based on the location data from the *cat-2* and *dat-1* mutants (**Fig. 5**), increasing dopamine levels chronically can increase distance off-lawn but does not seem to alter the timing of behavioral change. These results suggest that dopamine is responsible for the locomotion-related effects of

predator exposure. We hypothesized that dopamine release in the latter portion of the assay was required for execution of the changed egg location behavior. If this were the case, then exogenous dopamine should be able to rescue the behavior of *cat-2* mutants after predator exposure. To test this hypothesis, we exposed wildtype and *cat-2* mutant *C. elegans* to three *P. uniformis* males for 20 hours (training) and then transferred them to a plate with a lawn and artificial smears along with different amounts of exogenous dopamine (**Fig. 8a**). In previous studies, 2mM exogenous dopamine could rescue basal slowing upon encountering food [57] and density pattern discrimination of PDMS pillars [58] in *cat-2* mutants. We found that adding 2mM exogenous dopamine restored behavioral response to predator in *cat-2* mutants (**Fig. 8b**).

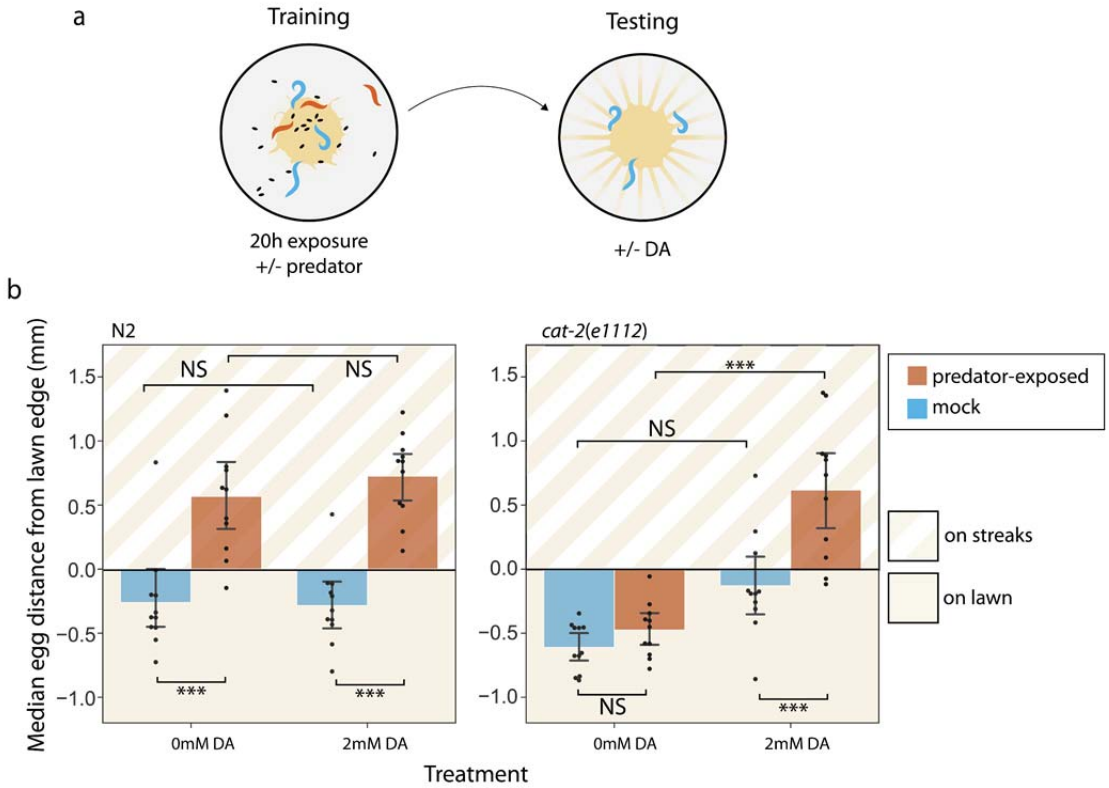

Figure 8 || Exogenous dopamine rescues egg location change in predator-exposed *cat-2* animals. **a.** Assay setup for exogenous dopamine rescue of predator-induced trained behavior. Training occurs with or without predator for 20 hours, then the trained *C. elegans* are transferred to a streaked lawn test plate with or without 2mM dopamine. **b.** Effect of 2mM exogenous dopamine on egg location of WT and *cat-2(e1112)* animals with or without predator exposure. Significant differences between pairwise comparisons were tested using a Tukey's HSD following three-way ANOVA (see Appendix). * $p<0.05$, ** $p<0.01$, ***$p<0.001$, NS=$p>0.05$. $n\geq10$

Discussion

In this study, we show that *C. elegans* responds to its predator *P. uniformis* by leaving a bacteria lawn that they both co-habit and laying its eggs outside of that lawn. Moreover, we find that this change in egg location involves dopamine signaling from CEP neurons and to a lesser extent, ADE/PDE neurons, and multiple dopamine receptors. Finally, we show that *C. elegans* continues to avoid the bacteria lawn even after the predator threat is removed. When given the option to inhabit less-dense bacterial streaks, predator-exposed *C. elegans* will lay eggs on these streaks while mock-treated *C. elegans* prefer to lay eggs in the dense bacterial lawn.

We find that biogenic amine signaling plays an important role in mediating a change in prey egg location in response to predators. Biogenic amines like dopamine and serotonin have been previously shown to play a role in driving responses to predator threat in honey bees [59], ants [60], and fruit flies [61]. Consistently, we find that mutants in dopamine and serotonin synthesis enzymes do not shift their egg location upon predator threat. Since the role of serotonin in defensive responses has been characterized in both vertebrate and invertebrate systems [62,63,64,65], we focused our study on dopamine signaling in this phenomenon. We show that prey responses to predator threat require dopamine synthesis, which is likely released from CEP neurons and to a lesser extent, ADE/PDE neurons. CEP neurons have been previously implicated in learning the size of bacteria lawns. We previously showed that *C. elegans* learns the size the of the lawn by using high threshold sensory neurons that detect lawn edges, which in turn signal to CEP neurons to release dopamine. In this paradigm, we speculated that information about lawn size was stored in amount of dopamine released from CEP neurons [66]. In our current 20-hour assay, we also observe that the bacteria lawn is streaked outside the main lawn, likely caused by *C. elegans* dragging bacteria on its body while it exits the lawn after encountering the predator. The streaking of the lawn itself is not enough to drive the level of shift in egg location during predator exposure. Taken together, we suggest that predator encounters likely alters dopamine signaling from CEP neurons driving the shift in egg location away from the dense bacteria lawn and into the streaks. These results are consistent with studies in the fruit fly where dopamine acts as a reinforcement signal for fear learning in the mushroom body [67]. PDE neurons are involved increasing egg-laying during roaming, and dopamine release can increase the probability of egg-laying in the absence of food [53], so dopamine from PDE in this predator-prey assay could also encourage egg-laying off-lawn.

We also observe a role for multiple dopamine receptors in this prey response to predator threat. The *C. elegans* genome encodes at least four dopamine receptors [68]. While DOP-1 and DOP-4 is the *C. elegans* homolog of the mammalian D1-like receptors, DOP-2, and DOP-3 are D2-like receptor homologs [69,70]. We find that both *dop-1*; *dop-2*; *dop-3* triple mutant animals and quadruple (*dop-1;dop-2;dop-3;dop-4*), implying that *dop-4* has minimal contribution to this phenotype. Studies in mammals where pharmacology and receptor knockouts have shown that knockouts in D1- and D2-like receptors can have opposing effects on behavior [69,71,72,73]. However, we show a *dop-1* (D1-like), *dop-2* (D2-like) and *dop-3* (D2-like) triple mutant can reduce response to predator. This suggests that in some cases *dop-1* (D1-like) and *dop-3* (D2-like) can act additively to affect prey response to predators. While we did not identify the site of action of these receptors, we suggest that DOP-1, -2, and -3 receptors act downstream of dopamine release to alter prey egg location in predator exposed animals.

Finally, we show that exogenous dopamine after predator exposure is able to rescue the predator-evoked behavioral deficit in the dopamine-deficient *cat-2* animals. These results imply that dopamine acts downstream of predator exposure. However, this exogenous dopamine does not affect animals that have not experienced predators. We speculate that sensing predator bites likely alters dopamine signaling, similar to mammals where aversive stimuli has been shown to stimulate dopamine [74]. This increase in dopamine signaling, in turn, is likely to alter prey egg location away from the lawn.

Ideas and speculation

A strategy utilized by prey that reduces predation risk is known as a prey refuge. The use of prey refuges can stabilize predator-prey interactions but often come at a cost of decreased feeding and reproduction for the prey [75]. In this case, the predator-exposed *C. elegans*'s strategy of shifting egg location to streaks away from a central lawn could reduce risk of predation by reducing encounter probability with the *Pristionchus* predator, but this question was not directly addressed in this study. Additionally, whether restricting consumption primarily to the streaked bacteria instead of the dense lawn is detrimental to the health or fitness of the *C. elegans* prey has yet to be tested. Regardless, predator exposure does appear to alter *C. elegans* foraging decisions to no longer favor the largest amount of food, but instead choose a variable and minimal amount. This strategy could fit into prey refuge theory [75].

Dopamine synthesis is required for egg location change in response to a predator in this study. Dopamine has been shown to modulate learned responses to aversive stimuli in *C. elegans*. For example, dopamine release sensitizes aversive responses to multiple soluble repellent stimuli including copper and glycerol [76]. Dopamine also delays habituation to repeated taps [77,78] and state-dependent olfactory adaptation during ethanol exposure [79]. Dopamine is generally thought of as the food sensor, so in this way it which provides context for appropriate behavior – it may be more beneficial to alter learning rate when on food versus when off food. Consistently, we observe a large change in off-food egg location, with minimal effect on food exploration.

Finally, while dopamine has been generally thought to represent reward [80], recent studies have demonstrated a more complex role for this biogenic amine. Dopamine release can be both phasic or tonic and can signal via different dopamine receptors to alter behaviors [81]. For example, phasic dopamine is associated with reward prediction, but can also affect responses to painful stimuli [74]. To reconcile these roles of dopamine, one model proposed that dopamine codes for salience regardless of valence – a dopamine spike alerting the animal to something important and unexpected so that the animal may adjust its expectations [82]. Dopamine release does not appear to be required for the acquisition of memory in the current study, as exogenous dopamine added after the acquisition period rescues *cat-2(e1112)* behavior. However, this addition of exogenous dopamine does little to *C. elegans* that have not experienced predators. We propose a model where bites from predators alter the dopaminergic circuit to respond to dopamine differently. One possibility is that tonic dopamine levels increase in predator-exposed animals due to prolonged biting. If basal dopamine is already high, food-associated dopamine release may need to be higher to stand out from background. The high contrast of the areas with bacterial variability can result in more dopamine release [66] so they may become preferred over the constant, though denser, environment. This increase in dopamine either from predator bites or exploring variable bacterial topography might reinforce each other leading to change in prey behavior. Also, other changes like dopamine receptor expression may be altered during the training period to affect response to dopamine.

In summary, after a period of predator exposure, *C. elegans* switches foraging strategy to move away from dense food areas to high variability areas that still have some food. Exogenous dopamine after predator training enhances off-lawn movement and off-lawn egg-laying in a *cat-2* mutant background. This study lays the foundation for studying prey behavior in *C. elegans*, and

future studies can use this system to study how dopamine affects foraging decisions based on experience.

Materials and Methods

Egg location assay

Assay plates are created by spotting 0.5ul of OP50 liquid culture (OD600=0.5) on 35mm standard NGM plates [39]. The bacterial lawns are allowed to grow at 20°C for 30 hours, then stored for up to one month at 4°C. Whatman filter paper with ¼" punch forms the "corral" and encircles the lawn, allowing approximately 1.5mm of clean agar in between the lawn edge and the corral edge. All animals are allowed to crawl on a clean section of agar to clean them of bacteria and picked to the assay plate using a sanitized eyelash, placed next to the lawn on a clean area of agar. Three predators are picked first, staged by overall size and pigment development as J4s. Then three *C. elegans* L4s are picked to the assay plate. The animals are allowed to interact for a determined amount of time, 20 hours for an overnight assay, at 20°C. For short-term exposure (6 hours and under), gravid *C. elegans* adults and adult predators are used by picking L4s or J4s the day before to plates with plenty of food. The juveniles are allowed to grow overnight into adulthood and then used in the same assay setup. After their interaction, corrals and all adults are removed from the plate and the area inside the corral is imaged using an AxioZoom V16 (ZEISS).

For the streaked lawn variation, streaks are formed by gently dragging a sanitized eyelash through the center of the lawn in radial streaks ten times, followed by two concentric circular streaks halfway between the lawn and the corral edge. The streaked lawn is then used immediately.

Injury assay

Injury assays are set up in the same way as the egg location assays, using a *C. elegans* strain containing the array *frIs7* [*Pnlp-29*::GFP + *Pcol-12*::*DsRed*]. After the set interaction time, worms are immobilized by placing the plates on ice and imaged on an AxioZoom V16 (ZEISS) within one hour, with exposure times kept constant for fluorescence imaging (25ms).

Learning assay

C. elegans are trained using the 20-hour egg location assay. At the same time as the animals used for training are transferred to their assay plates, test plates are set up. Three types of test plates

are used: a filled lawn (10ul of OP50 (OD600=0.5), a streaked lawn (same as the streaked lawn variant of the egg location assay), and a small lawn (same as the original assay plate). The training plates with animals on them and the test plates are incubated at 20°C for 20 hours, during which the *C. elegans* is exposed to JU1051 males and the smears on the test plates are allowed to grow. (The bacteria on the other test plates is also allowed to grow at this time so that the bacteria is at a similar metabolic state and density across test plates, and streaks are already present). Filter paper corrals like those used in the egg location assay are centered over the test plate lawns.

After the *C. elegans* are incubated in their training conditions for 20 hours, they are carefully removed with an eyelash pick from their training plates to a clean section of an NGM plate. The animal is allowed to crawl for a few seconds to remove bacteria and then picked to a test plate halfway between the central lawn and the corral edge. For the filled test lawns, the animals are placed in an equivalent position relative to the corral edge. The test plates are then imaged every hour on an AxioZoom V16 for 6 hours. For the variant including rest plates, the *C. elegans* are picked from their training plate to rest plates ("filled lawn" plates) for the set rest time. They are then transferred to a streaked lawn test plate and egg locations are observed.

Exogenous dopamine assay

When adding exogenous dopamine to the learning assay, a 200mM stock of dopamine hydrochloride (Code 122000100 Lot: A0427132, CAS: 62-31-7, Acros Organics) in water was prepared. Two hours before the trained worms needed to be transferred to the test plates, 50µl of the dopamine stock or water as a control was gently applied onto the streaked lawn test plate. The plates were allowed to diffuse and dry with the lids off for two hours, at which time the trained worms were transferred to the test plates. The trained worms were allowed to lay eggs for two hours before their plates were imaged.

Egg location image quantification

Egg location images are quantified in FIJI with the experimenter blinded to the condition by randomizing the file order and obscuring the filenames (using the Filename_Randomizer macro found at https://imagej.nih.gov/ij/macros/Filename_Randomizer.txt). Eggs are manually selected with the multipoint tool and lawns are selected as circles. Distances from each egg from to the lawn edge are calculated in Python. All assays are performed with their relevant controls over at least three separate days.

WormWatcher assays

Assays conducted in the WormWatcher (Tau Scientific Instruments, West Berlin, New Jersey, USA) were performed on a single 6cm 2.5% agar NGM plate in a 12-arena setup. The 12-well corral was created by cutting a 3x4 array of ¼" circles into a plastic sheet using a Cricut machine. OP50 was spotted in the 3x4 pattern using the same concentration and allowed to grow for the same amount of time as in the egg location assay. The increased agar percentage on the WormWatcher plates helped prevent worms from escaping under the plastic edges of the corral. The assays were set up like the egg location assays, with three L4 *C. elegans* and three J4 JU1051 males or six L4 *C. elegans* in control arenas. The positions of predator-containing and/or mutant-containing arenas was alternated on different assay days. The WormWatcher was set to acquire fluorescent frames with a green LED excitation light every four minutes. A reference darkfield image was acquired before and after every experiment to reference the positions of the arenas and the size and positions of the lawns. After the experiment was completed, each arena was inspected and imaged to determine whether any worms escaped away or into it. Custom code was written to segment the *C. elegans* and arenas in each position and the median distance from the lawn edge to the mid-point of each worm body per well was recorded. Data from arenas were discarded if two worms had escaped from an arena, or if a *P. uniformis* was seen in a control arena.

***Pristionchus* mouthform analysis**

Pristionchus mouthform analysis was performed as reported in [34]. Briefly, *Pristionchus* were egg-prepped via bleaching and eggs were either cultured on standard solid NGM plates or in liquid culture. After eggs reached adulthood, they were immobilized on agarose slides with sodium azide. The slides of different strains from different culture conditions were mixed and their labels obscured while they were observed. The slides were scored as either Eu (wide mouth, two teeth) or St (narrow mouth, one tooth) while the experimenter was blinded to strain.

Statistical methods

For experiments involving two variables (usually predator exposure and genotype), a two-way ANOVA was conducted using the statsmodels module in Python. The two-way ANOVA was conducted with a linear OLS model to determine interactions. For pairwise comparisons, predator/mock conditions within genotype and wild-type and mutants in the same predator/mock

condition were tested with OLSResults.t_test_pairwise. p-values were adjusted on the subset of the relevant comparisons using Holm-Šídák's method.

For experiments involving three variables where one is time over multiple time points, mixed ANOVAs were performed in R using the **rstatix** package and anova_test(). A three-way mixed ANOVA was performed to determine three-way interactions and subsequent two-way mixed ANOVAs were performed for relevant two-way interactions. Post-hoc tests were conducted in R using pairwise_t_test().

Full statistical results for main figures can be found in the **Appendix**.

Table 1: List of strains and their genotypes used in this study.

Strain Name	**Genotype**	**Figure**	**Notes**
N2	Wild-type	1e; 3a,b; 4a,b,c; 6a,b; 7b,e; 8b;	
CX7389	*kyIs392 [Pstr-2::GFP::rab-3; Pttx-3::lin-10::dsRed; Pelt-2::GFP]*	1b	Fluorescent eggs
CZ6326	*frIs7 [nlp-29p::GFP + col-12p::DsRed] IV*	1c,d;	Injury reporter
ARM112	*wamSi112 [eft-3p::mScarlet::unc-54 3'UTR + Cbr-unc-119(+)] II; unc-119(ed3) III*	2b	WT whole-body fluorescent strain
IV983	*cat-2(e1112) wamSi112[eftp-3::mScarlet::unc-54 3'UTR + Cbr-unc-119(+)] II*	5a	Created for this study
IV988	*wamSi112[eftp-3::mScarlet::unc-54 3'UTR + Cbr-unc-119(+)] II; dat-1(ok157) III*	5b	Created for this study
CB1111	*cat-1(e1111) X*	4a	
CB1112	*cat-2(e1112) II*	4a,b,c; 8b	
MT13113	*tdc(n3419) II*	4a	
MT15434	*tph-1(mg280) II*	4a	
MT9455	*tbh-1(n3247) X*	4a	
MT15620	*cat-2(n4547) II*	4b	
IV111	*cat-2(e1112) II; ueEx51 [p27::cat-2-sl2-GFP; Pelt-2::GFP]*	4c	
IV552	*cat-2(e1112) II; ueEx355 [Pdat-1p19::cat-2-sl2-GFP; Pelt-2::GFP]*	4c	

LX645	*dop-1(vs100) X*	S4	
LX702	*dop-2(vs105) V*	S4	
LX703	*dop-3(vs106) X*	S4	
RB1254	*C52B11.3(ok1321) X*	S4	
LX705	*dop-1(vs100) dop-3(vs106) X*	6a	
LX706	*dop-2(vs105) V; dop-1(vs100) X*	6a	
IV984	*dop-4(ok1321) dop-1(vs100) X*	6a	Created for this study
IV985	*dop-2(vs105) V; dop-4(ok1321) X*	6a	Created for this study
IV986	*dop-4(ok1321) dop-3(vs106) X*	6a	Created for this study
LX734	*dop-2(vs105) V; dop-1(vs100) dop-3(vs106) X.*	6b	
CF2805	*dop-2(vs105) V; dop-4(ok1321) dop-1(vs100) dop-3(vs106) X*	6b	
CF1903	*glp-1(e2144) III*	S3	
JU1051	*P. uniformis* wild isolate	1b,c,d; 2b; 3b; 4a,b,c; 5a,b; 6a,b; 7b,e; 8b; S1a,b,c; S2a,b; S3a,b; S4	From Marie-Anne Félix
PS312	*P. pacificus* California isolate	1b,c,d; S1a,b,c	From Ralf J. Sommer
RS5194	*P. pacificus* Japanese isolate	1b,c,d; S1b,c	From Ralf J. Sommer
TU445	*P. pacificus eud-1(tu445) X*	1b,c,d; S1b,c	From Ralf J. Sommer

Acknowledgements

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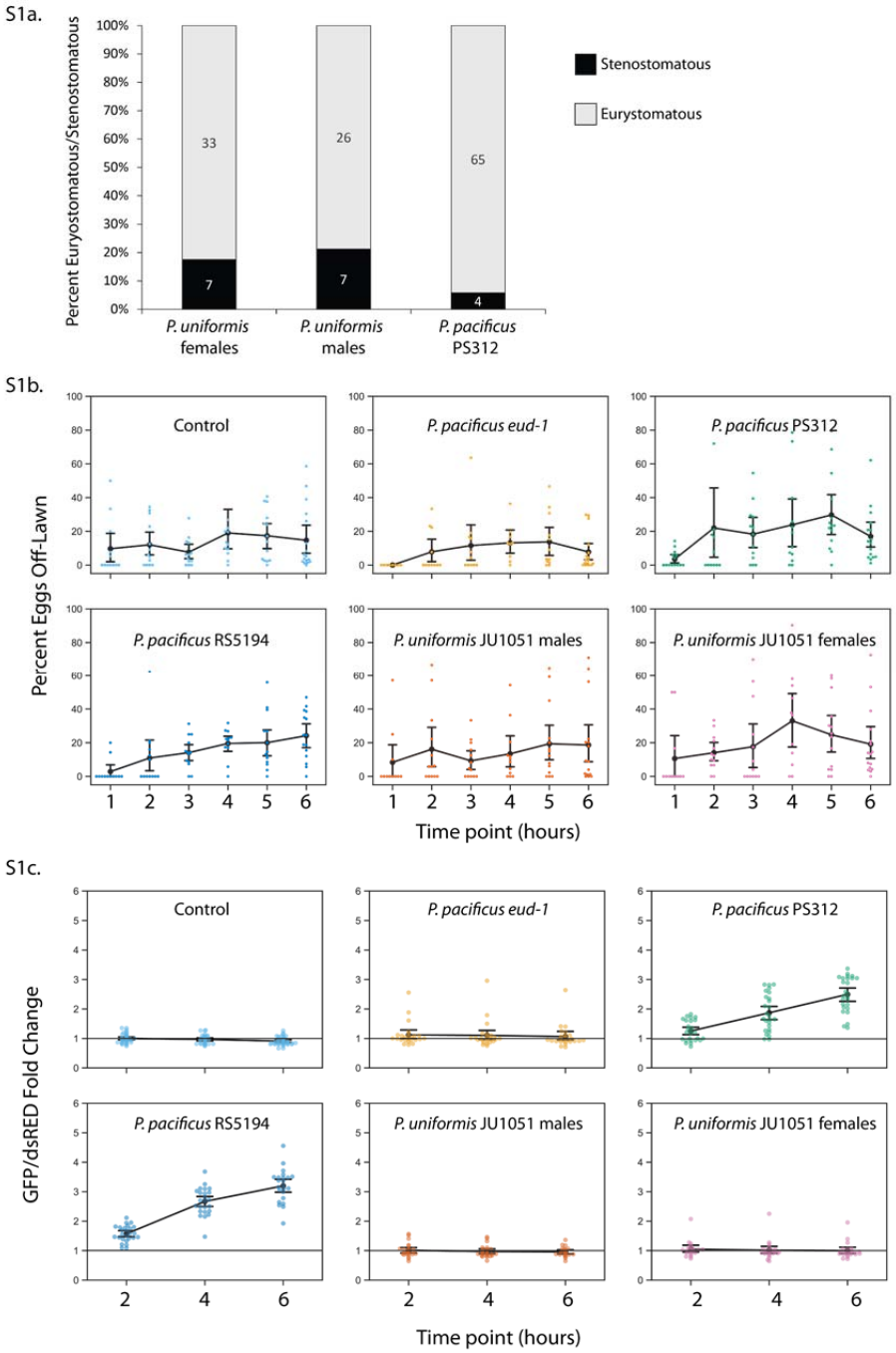
615 **Supplementary Figures**



616
617 **Figure 1-S1 a.** Mouthform ratios for *P. uniformis* (JU1051) females and males, and *P. pacificus*
618 (PS312) grown on standard NGM with OP50. Chi-squared test for proportion of Eu between all
619 possible strain pairings failed to find a significant difference (p>0.05, Holm-Sidak p-value
620 correction). **b.** Expression of *Pnlp-29*::GFP in *C. elegans* paired with various predators.
621 Expression of GFP was compared to dsRED coinjection marker and measured at 2, 4, and 6 hours.
622 Error bars indicate 95% CI. **c.** Percent eggs off-lawn for short-term (6 hours total) exposure to
623 various predators. Error bars indicate 95% CI.

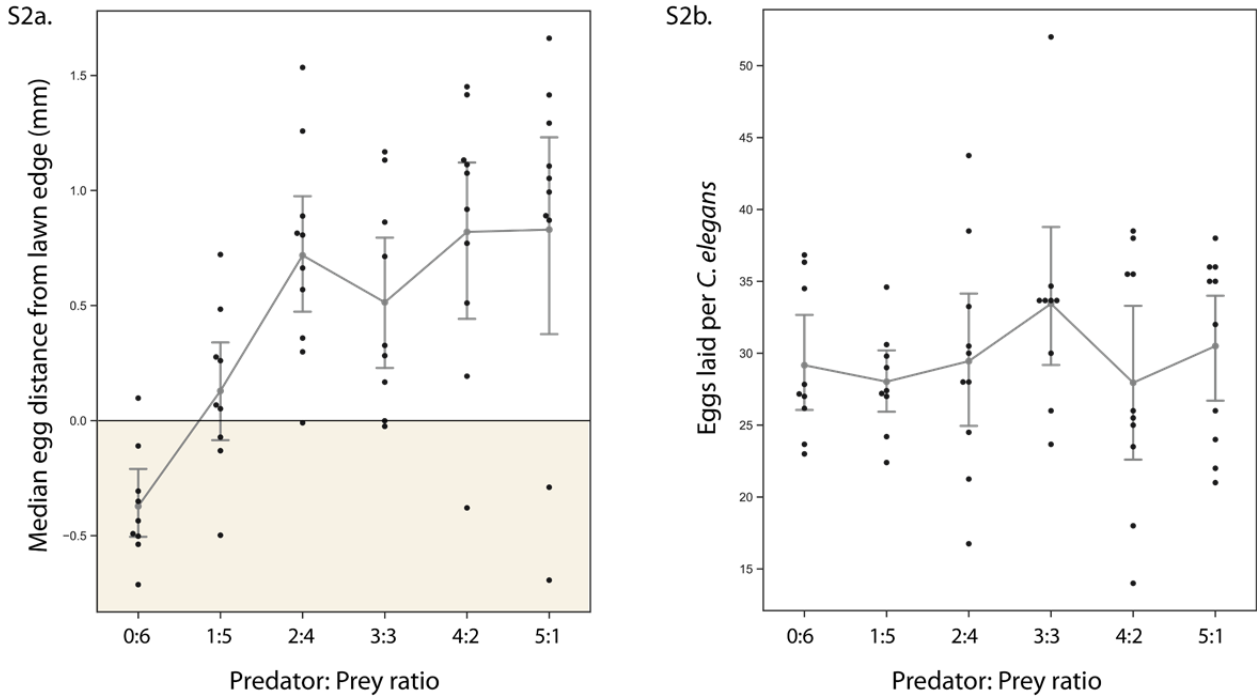


Figure 1-S2 a. Egg locations of arenas containing varying ratios of *P. uniformis* males (predators) and *C. elegans* (prey). **b.** Eggs laid per *C. elegans* in arenas with varying ratios of *P. uniformis.* Error bars represent 95% CI.

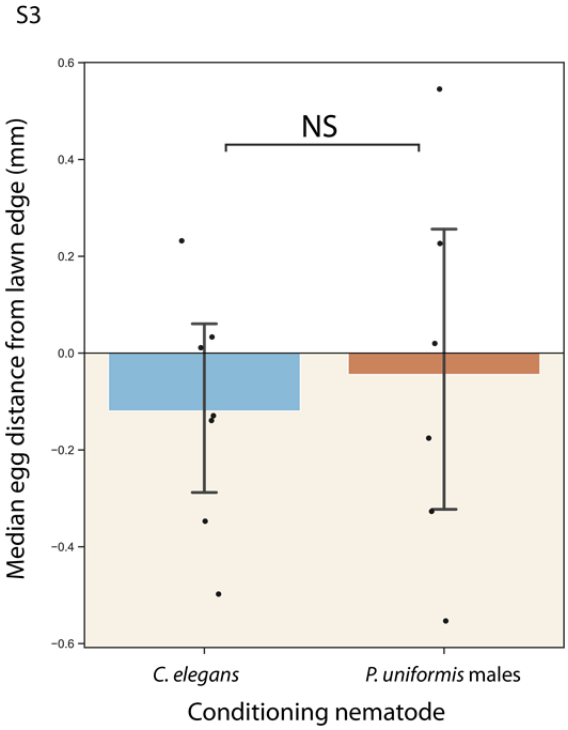


Figure 1-S3 Egg location of *C. elegans* transferred to arenas that were previously conditioned with either sterile *C. elegans* or *P. uniformis* males. Error bars represent 95% CI. T-test showed no significant difference in means (p=0.6812133).

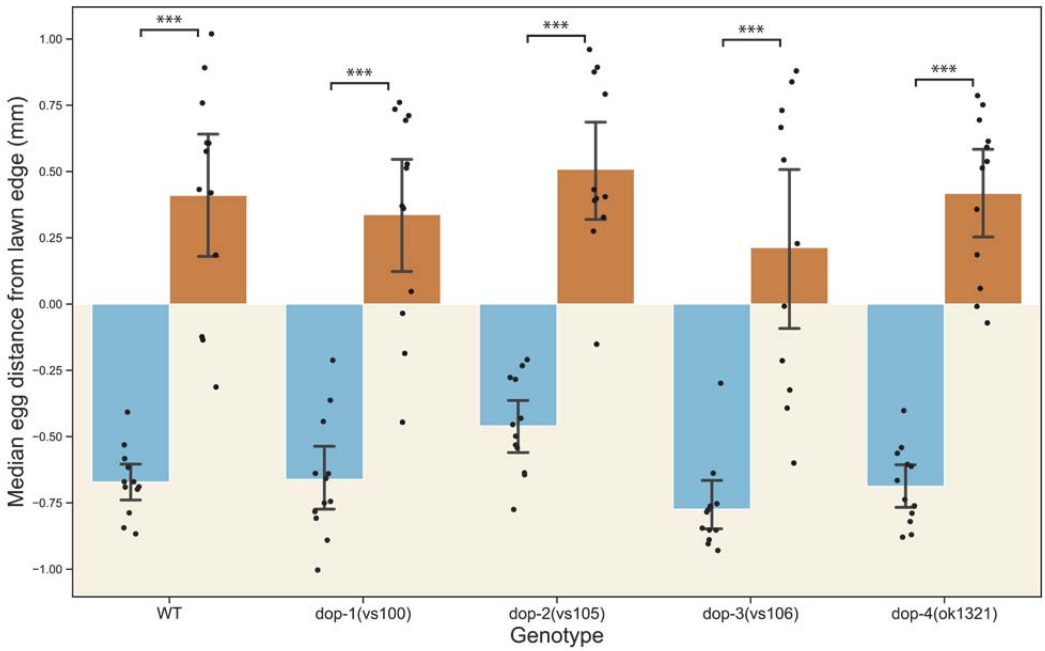


Fig. 4-S4 Egg locations of single dopamine receptors. Error bars represent 95% CI. A two-way ANOVA was conducted followed by pairwise multiple comparisons for all relevant pairs. Pairwise comparisons are shown between predator/mock conditions within the same strain (black asterisks); no significant differences were found between predator or mock conditions of mutants compared to control (See Materials and Methods). * $p<0.05$, ** $p<0.01$, ***$p<0.001$, NS=$p>0.05$.

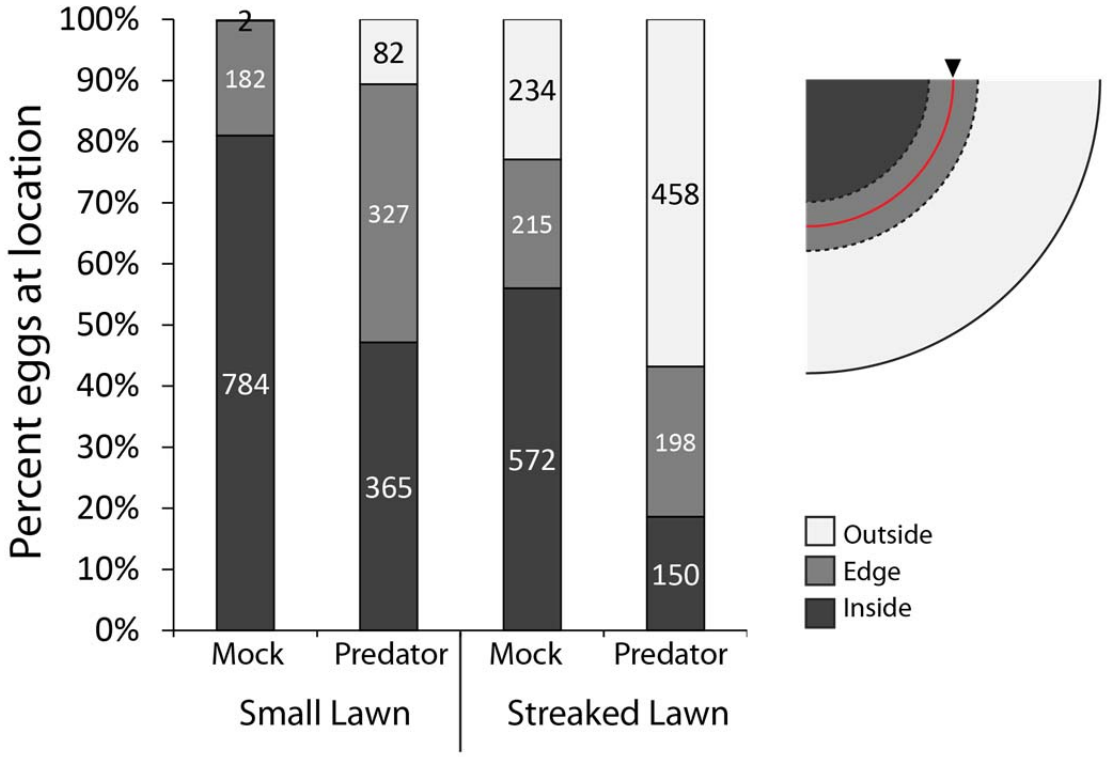


Fig. 7-S5 After transfer to the small lawn (with a region of bare agar), a greater proportion of eggs is found near the edge when *C. elegans* are predator-exposed (p=6.641E-26 after chi-squared test for each "Edge" proportion compared pairwise between each lawn/training type, Holm-Sidak p-value correction for multiple comparisons). "Edge" is considered within 0.5mm of the lawn edge (approximately half a body length of an adult *C. elegans*). Schematic to the right shows how the locations were binned; eggs within 0.5mm of the edge are considered "Edge". Red line (arrowhead) indicates actual lawn edge. "Even" lawns were not included in this analysis because the entire corral is inside the lawn. Data shown is pooled from the 6-hour timepoint; numbers in bars are the number of eggs.

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Appendix

Figure 1

1b Dunnet's Test

Comparison	Diff	Lwr.ci	Upr.ci	pval
Eud-1-mock	0.05994631	-1.2438292	1.363722	0.99998
JU1051F-mock	1.05760999	-0.2754675	2.390688	0.16491
JU1051M-mock	1.90643082	0.5733533	3.239508	0.00217
PS312-mock	2.57742201	1.2736465	3.881198	5.5e-05
RS5194-mock	2.21263783	0.8452265	3.580049	0.00041

1d Dunnet's Test

Comparison	Diff	Lwr.ci	Upr.ci	pval
JU1051F-mock	0.080353759	-0.01628664	0.1769942	0.1376
JU1051M-mock	0.00637192	-0.09359999	0.1063438	0.9997
PS312-mock	0.828386956	0.71938067	0.9373932	<2e-16
Eud-1-mock	-0.002731126	-0.10063235	0.0951701	1.0000

Figure 2

2b Two-way mixed ANOVA

Effect	DFn	DFd	F	p	p<.05	ges
Predator exposure	1	22	14.867	0.000857	*	0.284
Time	19	418	14.756	6.27E-36	*	0.217
Predator exposure:Time	19	418	11.855	5.80E-29	*	0.183

Figure 3

3b one-way ANOVA

	Sum_sq	Df	F	PR(>F)
C(Predator exposure)	15.769007	2.0	69.921033	3.054466e-14
Residual	4.848808	43.0	NaN	NaN

3b Tukey's HSD

Group1	Group2	Meandiff	p-adj	Lower	Upper	reject
Non-streaked	Predator	1.3697	0.0	1.0767	1.6627	True
Non-streaked	streaked	0.3561	0.0157	0.0584	0.6537	True
Predator	streaked	-1.0136	0.0	-1.306	-0.7207	True

Figure 4

4a Two-way ANOVA

	Sum_sq	df	F	PR(F)
C(Genotype)	16.1442	5	25.28599	4.50E-18

C(Predator exposure)	14.45813	1	113.2259	1.04E-19
C(Genotype):C(Predator exposure)	4.762955	5	7.46002	3.15E-06
Residual	17.7493	139	NaN	NaN

4a Subset of pairwise comparisons (Holm-Sidak adjustment)

	coef	**std err**	**t**	**P>|t|**	**Conf. Int. Low**	**Conf. Int. Upp.**	**adj_p**
N2predator-N2alone	0.817519	0.120853	6.764589	3.42E-10	0.578572	1.056467	5.13E-09
CB1111predator-CB1111alone	0.150138	0.192986	0.777975	4.38E-01	-0.23143	0.531707	6.84E-01
CB1112predator-CB1112alone	0.192106	0.149163	1.287894	2.00E-01	-0.102815	0.487027	6.72E-01
MT13113predator-MT13113alone	0.850011	0.128428	6.618598	7.27E-10	0.596087	1.103935	1.02E-08
MT15434predator-MT15434alone	0.239359	0.145884	1.640747	1.03E-01	-0.04908	0.527797	5.33E-01
MT9455predator-MT9455alone	1.09174	0.145884	7.483616	7.49E-12	0.803301	1.380178	1.20E-10
N2predator-CB1111predator	0.873982	0.151841	5.755905	5.28E-08	0.573765	1.174198	6.34E-07
N2predator-CB1112predator	0.78038	0.136757	5.706323	6.69E-08	0.509987	1.050773	7.36E-07
N2predator-MT13113predator	-0.260853	0.12278	-2.124561	3.54E-02	-0.50361	-0.018096	2.77E-01
N2predator-MT15434predator	0.786198	0.133173	5.903573	2.59E-08	0.522891	1.049505	3.37E-07
N2predator-MT9455predator	-0.338627	0.133173	-2.542755	1.21E-02	-0.601934	-0.07532	1.15E-01
N2alone-CB1111alone	0.206601	0.169686	1.217545	2.25E-01	-0.128899	0.542101	6.72E-01
N2alone-CB1112alone	0.154966	0.134731	1.150191	2.52E-01	-0.111421	0.421353	6.72E-01
N2alone-MT13113alone	-0.228361	0.126587	-1.803991	7.34E-02	-0.478646	0.021923	4.57E-01
N2alone-MT15434alone	0.208037	0.134731	1.544096	1.25E-01	-0.05835	0.474424	5.51E-01
N2alone-MT9455alone	-0.064407	0.134731	-0.478038	6.33E-01	-0.330793	0.20198	6.84E-01

4b Two-way ANOVA

	Sum_sq	df	F	PR(F)
C(Genotype)	6.86661	2	67.088291	1.69E-14
C(Predator exposure)	3.896118	1	76.131865	2.15E-11
C(Genotype):C(Predator exposure)	4.227096	2	41.299658	4.45E-11
Residual	2.405268	47.0	NaN	NaN

4b Subset of pairwise comparisons (Holm-Sidak adjustment)

	coef	std err	t	P>|t|	Conf. Int. Low	Conf. Int. Upp.	adj_p
N2predator-N2alone	1.328189	0.106642	12.45471	1.70E-16	1.113654	1.542725	8.51E-16
CB1112predator-CB1112alone	0.182901	0.106642	1.715096	0.092915	-0.03163	0.397436	0.253647

MT15620predator-MT15620alone	0.089973	0.109924	0.818502	0.417201	-0.13117	0.311111	0.417201
N2predator-CB1112predator	1.34684	0.106642	12.62959	1.02E-16	1.132305	1.561375	7.17E-16
N2predator-MT15620predator	1.376716	0.109924	12.5243	1.39E-16	1.155578	1.597854	8.34E-16
N2alone-CB1112alone	0.201551	0.106642	1.889984	0.064936	-0.01298	0.416086	0.235521
N2alone-MT15620alone	0.1385	0.106642	1.298739	0.200369	-0.07604	0.353035	0.360591

4c Two-way ANOVA

	Sum_sq	df	F	PR(F)
C(Genotype)	17.339769	3	47.543454	1.20E-18
C(Predator exposure)	13.248295	1	108.975447	2.74E-17
C(Genotype):C(Predator exposure)	4.174479	3	11.445892	1.91E-06
Residual	11.184566	92	NaN	NaN

4c Subset of pairwise comparisons (Holm-Sidak adjustment)

	coef	std err	t	P>|t|	Conf. Int. Low	Conf. Int. Upp.	adj_p
N2predator-N2alone	1.360919	0.142841	9.527501	2.25E-15	1.077224	1.644614	2.03E-14
CB1112predator-CB1112alone	0.227669	0.13958	1.631101	0.106288	-0.04955	0.504887	0.201279
IV111predator-IV111alone	0.799866	0.13676	5.848682	7.50E-08	0.528248	1.071483	5.25E-07
IV552predator-IV552alone	0.552573	0.13958	3.958823	0.000148	0.275355	0.829791	0.000889
N2predator-CB1112predator	1.44865	0.142841	10.14169	1.15E-16	1.164955	1.732345	1.15E-15
N2predator-IV111predator	0.209813	0.142841	1.468857	0.145283	-0.07388	0.493508	0.201279
N2predator-IV552predator	1.098636	0.142841	7.691316	1.57E-11	0.814941	1.382331	1.26E-10
N2alone-CB1112alone	0.3154	0.13958	2.259636	0.026204	0.038182	0.592618	0.100769
N2alone-IV111alone	-0.35124	0.13676	-2.5683	0.011831	-0.62286	-0.07962	0.05777
N2alone-IV552alone	0.29029	0.13958	2.079739	0.040331	0.013072	0.567508	0.116179

Figure 5

5a Three-way mixed ANOVA

Effect	DFn	DFd	F	p	p<.05	ges
Genotype	1	29	3.136	0.087		0.071
Predator exposure	1	29	17.072	0.000279	*	0.293
Time	19	551	12.069	4.04E-31	*	0.11
Genotype:Predator exposure	1	29	1.097	0.304		0.026
Genotype:Time	19	551	2.451	0.000606	*	0.025
Predator exposure:Time	19	551	14.577	9.71E-38	*	0.13
Genotype:Predator exposure:Time	19	551	2.844	5.88E-05	*	0.028

5a Two-way mixed ANOVA (mock exposure data)

Effect	DFn	DFd	F	p	p<.05	ges

Genotype	1	14	3.304	0.091		0.087
Time	19	266	3.376	5.08E-06	*	0.126
Genotype:Time	19	266	1.074	0.377		0.044

5a Two-way mixed ANOVA (predator exposure data)

Effect	DFn	DFd	F	p	p<.05	ges
Genotype	1	15	2.196	0.159		0.096
Time	19	285	15.745	3.09E-34	*	0.225
Genotype:Time	19	285	3.06	2.82E-05	*	0.053

5b Three-way mixed ANOVA

Effect	DFn	DFd	F	p	p<.05	ges
Genotype	1	29	3.136	0.087		0.071
Predator exposure	1	29	17.072	0.000279	*	0.293
Time	19	551	12.069	4.04E-31	*	0.11
Genotype:Predator exposure	1	29	1.097	0.304		0.026
Genotype:Time	19	551	2.451	0.000606	*	0.025
Predator exposure:Time	19	551	14.577	9.71E-38	*	0.13
Genotype:Predator exposure:Time	19	551	2.844	5.88E-05	*	0.028

5b Two-way mixed ANOVA (mock exposure data)

Effect	DFn	DFd	F	p	p<.05	ges
Genotype	1	21	16.352	5.85e-04	*	0.187
Time	6.92	145.41	22.836	1.53e-20	*	0.434
Genotype:Time	6.92	145.41	2.620	1.40e-02	*	0.081

5b Two-way mixed ANOVA (predator exposure data)

Effect	DFn	DFd	F	p	p<.05	ges
Genotype	1	20	19.383	2.75e-04	*	0.186
Time	6.02	120.38	57.827	3.31e-33	*	0.689
Genotype:Time	6.02	120.38	0.292	9.40e-01		0.011

Figure 6

6a Two-way ANOVA

	Sum_sq	df	F	PR(F)
C(Genotype)	3.199489	6	8.549158	5.48E-08
C(Predator exposure)	30.561714	1	489.972446	5.83E-49
C(Genotype):C(Predator exposure)	1.124231	6	3.003988	8.44E-03
Residual	9.293779	149	NaN	NaN

6a Subset of pairwise comparisons (Holm-Sidak adjustment)

	coef	std err	t	P>|t|	Conf. Int. Low	Conf. Int. Upp.	adj_p
N2predator-N2alone	0.998047	0.101959	9.788667	8.86E-18	0.796574	1.19952	1.51E-16

IV984predator-IV984alone	1.003656	0.101959	9.843677	6.36E-18	0.802183	1.205129	1.15E-16
IV986predator-IV986alone	1.119432	0.101959	10.97919	6.41E-21	0.917958	1.320905	1.22E-19
IV985predator-IV985alone	0.790176	0.114752	6.885968	1.50E-10	0.563425	1.016926	2.25E-09
LX704predator-LX704alone	0.676126	0.101959	6.631328	5.74E-10	0.474653	0.8776	8.04E-09
LX705predator-LX705alone	0.662444	0.101959	6.497133	1.15E-09	0.460971	0.863917	1.50E-08
LX706predator-LX706alone	0.797203	0.101959	7.818828	8.96E-13	0.59573	0.998676	1.43E-11
N2predator-IV984predator	0.042094	0.101959	0.41285	0.68031	-0.15938	0.243567	0.98331
N2predator-IV985predator	0.102095	0.106936	0.954733	0.341259	-0.10921	0.313402	0.916115
N2predator-IV986predator	0.153903	0.101959	1.50945	0.133301	-0.04757	0.355376	0.650076
N2predator-LX704predator	0.278695	0.101959	2.733388	0.007028	0.077221	0.480168	0.074648
N2predator-LX705predator	0.493749	0.101959	4.842605	3.18E-06	0.292276	0.695222	3.81E-05
N2predator-LX706predator	-0.02161	0.101959	-0.21195	0.832433	-0.22308	0.179863	0.98331
N2alone-IV984alone	0.047703	0.101959	0.467861	0.640568	-0.15377	0.249176	0.98331
N2alone-IV985alone	-0.10578	0.110129	-0.96048	0.338372	-0.32339	0.11184	0.916115
N2alone-IV986alone	0.275287	0.101959	2.699969	0.007737	0.073814	0.47676	0.074731
N2alone-LX704alone	-0.04323	0.101959	-0.42395	0.672213	-0.2447	0.158247	0.98331
N2alone-LX705alone	0.158146	0.101959	1.551072	0.123006	-0.04333	0.35962	0.650076
N2alone-LX706alone	-0.22245	0.101959	-2.18179	0.030692	-0.42393	-0.02098	0.244637

6b Two-way ANOVA

	Sum_sq	df	F	PR(F)
C(Genotype)	0.859563	2	10.669651	1.28E-04
C(Predator exposure)	6.01972	1	149.444069	4.75E-17
C(Genotype):C(Predator exposure)	0.625394	2	7.762933	1.10E-03
Residual	2.13488	53	NaN	NaN

6b Subset of pairwise comparisons (Holm-Sidak adjustment)

	coef	std err	t	P>|t|	Conf. Int. Low	Conf. Int. Upp.	adj_p
N2predator-N2alone	0.923142	0.089756	10.28501	3.12E-14	0.743114	1.10317	2.18E-13
LX734predator-LX734alone	0.455138	0.089756	5.07083	5.17E-06	0.27511	0.635166	2.07E-05
CF2805predator-CF2805alone	0.533539	0.092216	5.785774	3.96E-07	0.348578	0.7185	2.38E-06
N2predator-LX734predator	0.486606	0.089756	5.421427	1.48E-06	0.306578	0.666634	7.41E-06
N2predator-CF2805predator	0.449489	0.089756	5.007895	6.46E-06	0.269461	0.629517	2.07E-05
N2alone-LX734alone	0.018602	0.089756	0.207247	0.836611	-0.16143	0.19863	0.836611
N2alone-CF2805alone	0.059885	0.092216	0.649407	0.51888	-0.12508	0.244847	0.768523

Figure 7

7b Three-way mixed ANOVA (filled lawn excluded due to different scale of dependent variable)

Effect	DFn	DFd	F	p	p<.05	ges
Lawn	1	29	39.575	7.19E-07	*	0.522

Condition	1	29	63.438	8.76E-09	*	0.636
Time	5	145	13.674	6.20E-11	*	0.086
Lawn:Condition	1	29	4.609	0.04	*	0.113
Lawn:Time	5	145	3.822	0.003	*	0.026
Condition:Time	5	145	0.321	0.9		0.002
Lawn:Condition:Time	5	145	0.479	0.791		0.003

7b Two-way mixed ANOVA (filled lawn)

Effect	DFn	DFd	F	p	p<.05	ges
Condition	1	15	0.424	0.525		0.017
Time	5	75	5.486	0.000234	*	0.119
Condition:Time	5	75	0.549	0.739		0.013

7b Two-way mixed ANOVA (small lawn)

Effect	DFn	DFd	F	p	p<.05	ges
Condition	1	16	64.057	5.51E-07	*	0.733
Time	5	80	3.771	0.004	*	0.069
Condition:Time	5	80	0.487	0.785		0.009

7b Pairwise comparisons (small lawn)

Time	.y.	group1	group2	n1	n2	p	p.signif	p.adj	p.adj.signif
1	MedianDist	control	predator	9	9	0.000199	***	0.000199	***
2	MedianDist	control	predator	9	9	0.000162	***	0.000162	***
3	MedianDist	control	predator	6	9	0.00022	***	0.00022	***
4	MedianDist	control	predator	9	9	1.95E-05	****	1.95E-05	****
5	MedianDist	control	predator	9	9	5.04E-06	****	5.04E-06	****
6	MedianDist	control	predator	9	9	2.62E-06	****	2.62E-06	****

7b Two-way mixed ANOVA (streaked lawn)

Effect	DFn	DFd	F	p	p<.05	ges
Condition	1	13	24.672	0.000258	*	0.611
Time	5	65	9.019	1.50E-06	*	0.108
Condition:Time	5	65	0.325	0.896		0.004

7b Pairwise comparisons (streaked lawn)

Time	.y.	group1	group2	n1	n2	p	p.signif	p.adj	p.adj.signif
1	MedianDist	control	predator	9	9	0.000199	***	0.000199	***
2	MedianDist	control	predator	9	9	0.000162	***	0.000162	***
3	MedianDist	control	predator	6	9	0.00022	***	0.00022	***
4	MedianDist	control	predator	9	9	1.95E-05	****	1.95E-05	****
5	MedianDist	control	predator	9	9	5.04E-06	****	5.04E-06	****
6	MedianDist	control	predator	9	9	2.62E-06	****	2.62E-06	****

7e Two-way ANOVA

	Sum_sq	df	F	PR(F)
C(Time)	0.585315	2	2.5545	8.34E-02
C(Predator exposure)	4.757932	1	41.530275	5.61E-09
C(Time):C(Exposure)	0.463237	2	2.021716	1.38E-01

Residual	10.310884	90	NaN	NaN

7e Subset of pairwise comparisons (Holm-Sidak adjustment)

	coef	std err	t	P>|t|	Conf. Int. Low	Conf. Int. Upp.	adj_p
1 hourspredator-1 hoursalone	0.670282	0.138182	4.850726	5.13E-06	0.39576	0.944805	1.54E-05
2 hourspredator-2 hoursalone	0.434679	0.119903	3.625242	0.000478	0.19647	0.672888	0.000956
24 hourspredator-24 hoursalone	0.319349	0.107035	2.983586	0.003667	0.106705	0.531993	0.003667

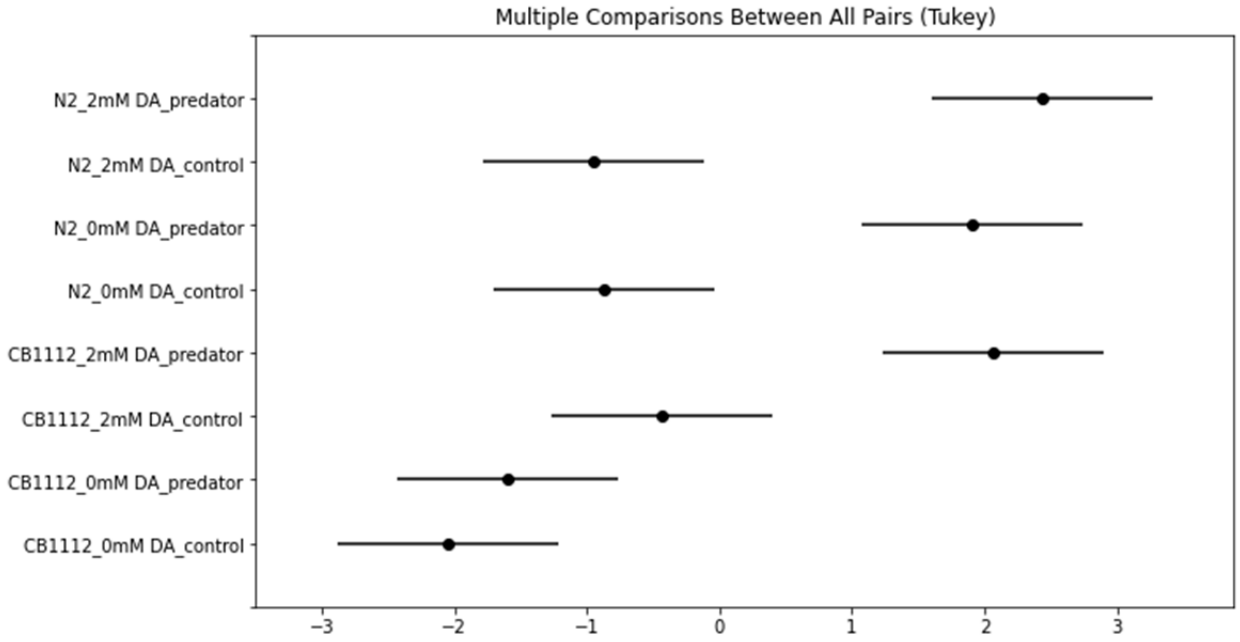
Figure 8

8b Three-way ANOVA

	sum_sq	**df**	**F**	**PR(>F)**
Intercept	0.031666	1	0.226782	0.635218
C(Genotype)	2.502656	1	17.92318	6.09E-05
C(Predator exposure)	10.15736	1	72.74358	7.41E-13
C(Treatment)	4.004103	1	28.67604	8E-07
C(Genotype):C(Predator exposure)	1.256809	1	9.000843	0.003597
C(Genotype):C(Treatment)	2.848986	1	20.40348	2.14E-05
C(Predator exposure):C(Treatment)	0.862029	1	6.173565	0.015054
C(Genotype):C(Predator exposure):C(Treatment)	0.255116	1	1.827055	0.180285
Residual	11.17059	80	NaN	NaN

8b Tukey's HSD

group1	**group2**	**meandiff**	**p-adj**	**lower**	**upper**	**reject**
CB1112_0mM DA_control	CB1112_0mM DA_predator	0.4528	0.9897	-1.2127	2.1184	FALSE
CB1112_0mM DA_control	CB1112_2mM DA_control	1.6148	0.0641	-0.0507	3.2804	FALSE
CB1112_0mM DA_control	CB1112_2mM DA_predator	4.1205	0	2.4549	5.786	TRUE
CB1112_0mM DA_control	N2_0mM DA_control	1.1769	0.3633	-0.4887	2.8424	FALSE
CB1112_0mM DA_control	N2_0mM DA_predator	3.9583	0	2.2928	5.6239	TRUE
CB1112_0mM DA_control	N2_2mM DA_control	1.098	0.4546	-0.5676	2.7635	FALSE
CB1112_0mM DA_control	N2_2mM DA_predator	4.4857	0	2.8201	6.1512	TRUE
CB1112_0mM DA_predator	CB1112_2mM DA_control	1.162	0.3798	-0.5035	2.8275	FALSE
CB1112_0mM DA_predator	CB1112_2mM DA_predator	3.6676	0	2.0021	5.3332	TRUE
CB1112_0mM DA_predator	N2_0mM DA_control	0.7241	0.8752	-0.9415	2.3896	FALSE
CB1112_0mM DA_predator	N2_0mM DA_predator	3.5055	0	1.84	5.1711	TRUE
CB1112_0mM DA_predator	N2_2mM DA_control	0.6451	0.928	-1.0204	2.3107	FALSE
CB1112_0mM	N2_2mM DA_predator	4.0328	0	2.3673	5.6984	TRUE

DA_predator						
CB1112_2mM DA_control	CB1112_2mM DA_predator	2.5056	0.0003	0.8401	4.1712	TRUE
CB1112_2mM DA_control	N2_0mM DA_control	-0.4379	0.9916	-2.1035	1.2276	FALSE
CB1112_2mM DA_control	N2_0mM DA_predator	2.3435	0.0009	0.678	4.009	TRUE
CB1112_2mM DA_control	N2_2mM DA_control	-0.5169	0.9779	-2.1824	1.1487	FALSE
CB1112_2mM DA_control	N2_2mM DA_predator	2.8708	0	1.2053	4.5364	TRUE
CB1112_2mM DA_predator	N2_0mM DA_control	-2.9436	0	-4.6091	-1.278	TRUE
CB1112_2mM DA_predator	N2_0mM DA_predator	-0.1621	1	-1.8277	1.5034	FALSE
CB1112_2mM DA_predator	N2_2mM DA_control	-3.0225	0	-4.688	-1.357	TRUE
CB1112_2mM DA_predator	N2_2mM DA_predator	0.3652	0.9972	-1.3003	2.0307	FALSE
N2_0mM DA_control	N2_0mM DA_predator	2.7815	0	1.1159	4.447	TRUE
N2_0mM DA_control	N2_2mM DA_control	-0.0789	1	-1.7445	1.5866	FALSE
N2_0mM DA_control	N2_2mM DA_predator	3.3088	0	1.6433	4.9743	TRUE
N2_0mM DA_predator	N2_2mM DA_control	-2.8604	0	-4.5259	-1.1949	TRUE
N2_0mM DA_predator	N2_2mM DA_predator	0.5273	0.9753	-1.1382	2.1929	FALSE
N2_2mM DA_control	N2_2mM DA_predator	3.3877	0	1.7222	5.0532	TRUE

8b Visual comparison of universal confidence interval of each group mean all using plot_simultaneous()