

# Molecular Requirements for *C. elegans* Transgenerational Epigenetic Inheritance of Pathogen Avoidance

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## eLife Assessment

This **fundamental** study concerns a model for transgenerational epigenetic inheritance, the learned avoidance by *C. elegans* of the PA14 pathogenic strain of *Pseudomonas aeruginosa*. The authors test the impact of procedural alterations made in another study, by Gainey et al., which claimed that transgenerational inheritance in this paradigm lacks robustness, despite this observation having been reported in multiple papers from the Murphy lab. The authors of the present study show that by following a non-standard avoidance protocol, Gainey et al. likely biased their measurements in a way that made it hard to observe learned avoidance. The authors also highlight the importance of bacterial growth conditions, showing that expression of the trigger molecule, the bacterial P11 RNA, which is necessary and sufficient to drive the transgenerational inheritance of the avoidance phenotype, is influenced by temperature. As expression of P11 was not verified by Gainey et al., this provides another explanation for the inability to observe transgenerational epigenetic inheritance. Together, the authors provide **compelling** and powerful arguments that the original phenomenon is robust and that it can be reproduced in the Murphy lab by following their original protocol precisely, including the use of azide to immobilize the worms at the food source. Overall, this study not only provides guidance for investigators in this experimental paradigm, but it also provides additional understanding of the differences between naïve preference, learned preference, and transgenerational epigenetic inheritance. The present study is therefore of broad interest to anyone studying genetics, epigenetics, or learned behavior.

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## Abstract

*C. elegans*' major food source is bacteria, and worms are naturally attracted to many bacterial species, including pathogenic *Pseudomonas*; in fact, worms prefer PA14 as well as wild bacteria over the lab *E. coli* strain (OP50) standardly used in the laboratory setting. Many labs

have shown that despite this natural attraction to PA14, prior exposure to PA14 causes the worms to instead avoid PA14. This behavioral switch can happen on a relatively fast time scale, even within the duration of the choice assay. Here we show that accurate assessment of the animals' true first choice requires the use of a paralytic (azide) to trap the worms at their initial choice, and to prevent the switch from attraction to avoidance of PA14 within the assay period. We previously discovered that exposure of *C. elegans* to 25°C plate-grown PA14 at 20°C for 24hrs not only leads to these animals switching from attraction to avoidance of PA14, but also to their progeny avoiding PA14 in the naïve state, and this avoidance persists through the F4 generation. Other types of PA14 training can also cause P0 and/or F1 avoidance, but do not induce transgenerational (F2 and beyond) inheritance. We also previously showed that the transgenerational (P0-F4) learned avoidance is mediated by P11, a small RNA produced by PA14. P11 is both necessary and sufficient for transgenerational epigenetic inheritance of avoidance behavior. P11 is highly expressed in our standard growth conditions (25°C on surfaces), but not in other conditions, suggesting that reported failure to observe F2-F4 avoidance is most likely due to the absence of P11 expression in PA14 in the experimenters' growth conditions. Through mutant analyses, we have tested many genes – including germline regulators, small RNA uptake, RNA interference/processing, chromatin modifiers, and neuronal genes - for their involvement in transgenerational inheritance of learned pathogen avoidance, allowing us to better understand the molecular requirements for this process. We previously found that wild *C. elegans* strains also show TEI of learned pathogen avoidance, and that at least two other wild bacteria, *P. vranovens* and *P. fluorescens* 15, induce this transgenerational avoidance. The avoidance induced by each *Pseudomonas* species functions through a specific, distinct small RNA (Pv1 in *P. vranovens* and Pfs1 in *P. fluorescens* 15, respectively) that either directly or indirectly reduce the levels of the gene *maco-1*, which in turn regulates *daf-7* expression in the ASI neuron and subsequent avoidance behavior. The conservation of multiple components of this small RNA TEI mechanism across *C. elegans* strains and in multiple *Pseudomonas* species suggests that this transgenerational learned avoidance behavior is likely to be functional and physiologically important in wild conditions.

## Introduction

*C. elegans* in the wild is found primarily in rotting fruit, where a range of bacterial species serve as abundant food sources (Félix & Duveau, 2012). About a third of these species are of the *Pseudomonas* family (Samuel *et al*, 2016), and many of those *Pseudomonas* species are pathogenic to *C. elegans*. Therefore, the development of multiple strategies to detect and avoid these pathogens that act at different time scales is critical for *C. elegans*' fitness and survival.

*C. elegans* and a clinical isolate of *Pseudomonas aeruginosa*, PA14, have been studied as a model of host-pathogen interactions for several decades (Tan *et al*, 1999). PA14 kills *C. elegans* through several distinct mechanisms (e.g., slow and fast killing (Mahajan-Miklos *et al*, 1999)) and typically *C. elegans* adults may only survive for a few days on PA14 (Tan *et al*, 1999), cutting its reproductive period short. *C. elegans*' survival depends on the expression of anti- microbial genes that are induced downstream of innate immune signals in response to PA14 exposure (Troemel *et al*, 2006), and changes to developmental rates and decisions that increase the odds of the next generation's survival.

In addition to the activation of pathogen survival genes and effects on reproduction and development, *C. elegans* also changes its behavior in the presence of PA14. Despite its potential to kill *C. elegans*, worms are initially attracted to PA14 (Zhang *et al*, 2005), because *C. elegans* cannot distinguish pathogenic from non-pathogenic *Pseudomonas* species that may serve as nutritious food sources (Sengupta *et al*, 2024). Specific mechanisms to detect, leave, and avoid PA14 are activated via secreted bacterial metabolites (Meisel *et al*, 2014; Prakash *et al*, 2021)

or through internal signals that indicate illness (Melo & Ruvkun, 2012; Singh & Aballay, 2019; Zhang *et al*, 2005). Another mechanism of learned avoidance can be activated upon exposure to pathogenic *Pseudomonas* and is mediated by specific bacterial small RNAs (Moore *et al*, 2019; Kaletsky *et al*, 2020; Sengupta *et al*, 2024; Seto *et al*, 2024). These distinct avoidance pathways are triggered by specific molecular cues, are independently regulated, and act on different time scales. Specifically, PA14 metabolites induce a distinct, ASJ-mediated avoidance pathway in P0s (Meisel *et al*, 2014); 4hrs of PA14 exposure induces avoidance directly in the worms and increased attraction in the F1s, while 8hrs of treatment can induce F1 avoidance but does not persist beyond F1 (Pereira *et al*, 2020; Zhang *et al*, 2005); and specific bacterial small RNAs from PA14 as well as from wild *Pseudomonas* species induce avoidance after 24hrs of treatment that is maintained through four generations of progeny (Kaletsky *et al*, 2020; Moore *et al*, 2019; Sengupta *et al*, 2024; Seto *et al*, 2024). Through extensive mutant analyses, we have found that this transgenerational avoidance mechanism requires a wide range of machinery, including RNA interference components, germline components, the Cer1 Ty3 Gypsy retrotransposon, and neuronal function (Moore *et al*, 2019; Kaletsky *et al*, 2020; Moore *et al*, 2021; Sengupta *et al*, 2024; Seto *et al*, 2024).

Here we discuss the molecular conditions that are necessary to induce the bacterial small RNA-induced pathway of transgenerationally-inherited learned avoidance behavior. Our results suggest that one major difference in execution of the assay itself, the presence or absence of the paralytic sodium azide, may account for some reported discrepancies in naïve and learned chemotaxis results (Gainey *et al*, 2024). Perhaps more importantly, PA14 must be grown under conditions that induce the expression of the small RNA, P11, that is necessary and sufficient for transgenerational inheritance of learned pathogen avoidance; P11 regulates nitrogen fixation in PA14, and these conditions promote biofilm formation, and thus may be important signals for *C. elegans* to detect the pathogen. These two factors, P11 expression and azide presence in the assay, are critical in obtaining consistent naïve and learned avoidance results. Together with the identification of genes and pathways that are involved, we are beginning to understand the molecular requirements for transgenerational epigenetic inheritance of learned avoidance.

## Results

### Choice assays require a paralytic to accurately capture first choice

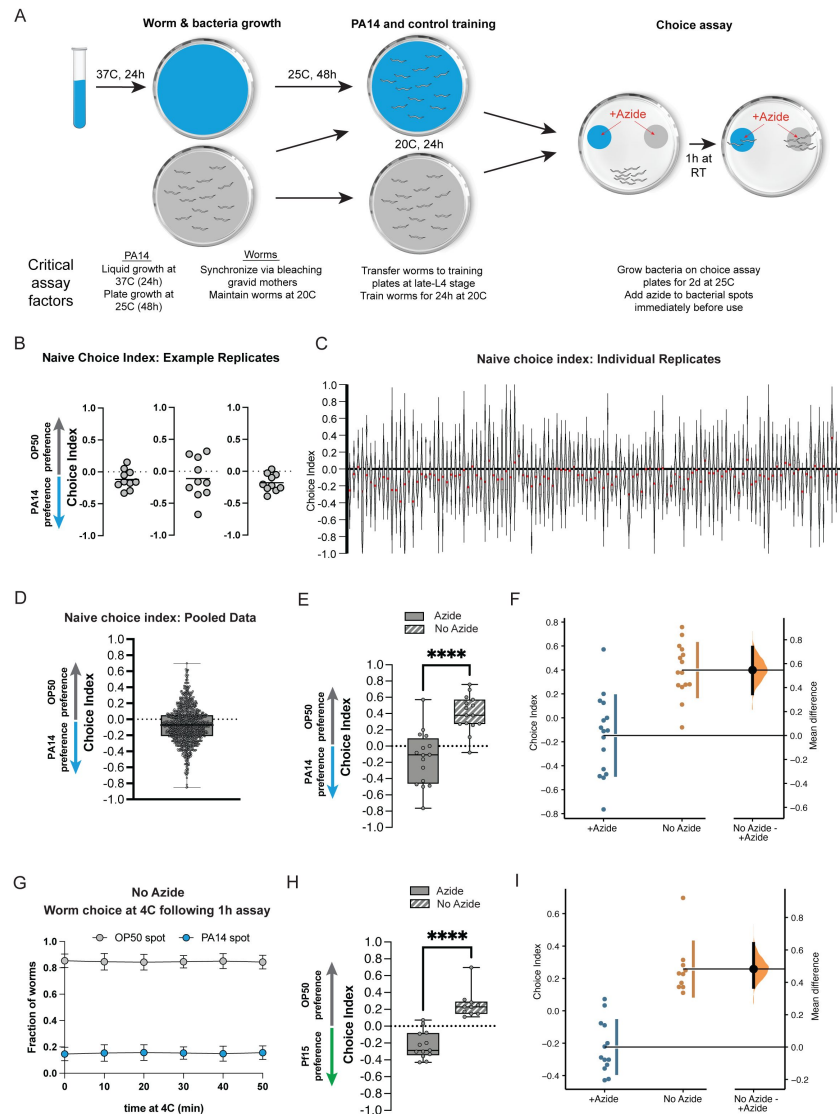
Because *C. elegans* has several different mechanisms of pathogen avoidance that can be detected via different signals (olfactory cues, gustatory cues, illness-induced signals, bacterial metabolites, bacterial RNAs, etc.), pathogen avoidance behavior has been tested using several different assays (as reviewed in (Liu & Zhang, 2020)). Among these are variations on the bacterial choice assay, which is modeled on standard chemotaxis choice assays (two small bacterial spots with azide to trap worms at their first choice (Bargmann *et al*, 1993; Hart, 2006; Zhang *et al*, 2005; Jin *et al*, 2016; Lee & Mylonakis, 2017; Ooi & Prahlad, 2017; Liu *et al*, 2018; Moore *et al*, 2019); variations on single-worm head-turning assays (Ha *et al*, 2010); and the leaving assay (PA14 spot in middle of plate, no other bacteria, no azide, and monitored over long periods for worms leaving the spot e.g., 8-24hrs (Meisel *et al*, 2014; Miller *et al*, 2015; Ma *et al*, 2017; Horspool & Chang, 2017; Hao *et al*, 2018; Singh & Aballay, 2019; Wolfe *et al*, 2019; Filipowicz *et al*, 2022)). Each assay measures responses on a specific time scale; for example, the two-bacteria choice assay measures initial preference of a population of animals over an hour, the head-turning assay measures immediate preference of individual animals, and the leaving assay (which is often performed overnight) measures final preference over many hours of PA14 exposure. Therefore, the conditions used in one assay might not be appropriate for all assays.

The standard bacterial choice assay is performed on plates, with two small spots of bacteria (here, PA14 and OP50) separated by several centimeters that the worms must traverse in response to olfactory cues (**Figure 1A**); monitoring the accumulation of worms as they become paralyzed with the anesthetic sodium azide at each bacterial spot allows the experimenter to accurately assess the worm's first choice. Studies by many groups, including Zhang *et al*, 2005, Meisel *et al*, 2014, Ooi & Prahlad, 2017, Pereira *et al*, 2020, Filipowicz *et al*, 2022, and others over the past two decades have previously demonstrated that *C. elegans* are initially attracted to PA14 without prior exposure to the pathogen ("naïve") but switch their behavior to avoidance of PA14 following exposure to PA14. Our lab has also consistently replicated those naïve attraction and learned avoidance results: in fact, we see that the mild naïve attraction to PA14 is fairly consistent between individual plates and individual replicates over many experiments across several studies (**Figure 1B-D**, **Table S1**) (Moore *et al*, 2019; Kaletsky *et al*, 2020; Moore *et al*, 2021; Sengupta *et al*, 2024; Seto *et al*, 2024). This initial naïve attraction to PA14 serves as a necessary baseline for all further experiments. That is, if no naïve attraction to PA14 is observed and instead the worms already avoid PA14 prior to PA14 exposure, it might be difficult to observe any additional learned avoidance of PA14 after training. Failure to observe naïve attraction to PA14 might also suggest that the experimenters' assay conditions are not the same as those used by previous groups. A recent study failed to consistently observe this initial naïve avoidance of PA14 (Gainey *et al*, 2024); therefore, we investigated possible origins of this deviation in these results from other groups' previously published results.

To determine possible origins of deviations in naïve choice assay results, we carried out the two-bacteria choice assay under standard conditions (1hr of a binary choice of PA14 vs OP50, which includes the paralytic sodium azide added to the bacterial spots) as well as the conditions reported by Gainey *et al.*, which primarily differs by omitting azide and letting the worms choose in the absence of azide for 1 hour at room temperature, and then moving the plates to cold temperature (4°C) prior to counting, as described by Gainey *et al.* (2024). We found that when azide is present at the bacterial spots to trap naïve worms at their first choice, the worms demonstrate attraction to PA14, as has been previously shown, but in the absence of azide, worms show significant avoidance of PA14 even without prior exposure to the pathogen (**Figure 1E-F**); the difference between the +azide and -azide chemotaxis indices is both significant and large in magnitude (a difference of 0.57 in CI and  $p < 0.0001$ ; **Figure 1F**). We note that these no-azide results are almost identical to the results that Gainey *et al.* reported for their naïve P0s, while the +azide results are similar to our previously-published naïve data (**Figure 1B-D**), as well as published data from other labs.

To better understand the source of the discrepancy with the Gainey *et al.* results, we first investigated the worms' behavior at 4°C after the hour of the assay at room temperature; while we do see that the worms continue to move, in fact, their terminal positions were already reached at the end of the room temperature period *before* they were placed in the cold (**Figure 1G**,  $t=0$ ); therefore, the discrepancy in attraction to / avoidance of PA14 seems to have occurred during the initial part of the assay, when the worms were choosing for 1 hour at room temperature without azide. This may be particularly relevant for the Gainey *et al.* study, as they reportedly moved the worms at different times ("*These no-azide assay plates were moved to 4°C after 30-60 minutes*"), which likely induced further variance. We also observed worms still traveling away from the PA14 spot at one hour, further underscoring the point that performing the assay without azide fails to trap the worms at their first choice, even if the worms are transferred to 4°C, because the worms have already made the switch to attraction to avoidance by that point.

The simplest interpretation of the discrepancy between the  $\pm$ azide assay conditions is that the worms are initially attracted to and choose PA14; in the presence of azide, the worms remain at this first-choice spot, but over the course of an hour-long choice assay in the absence of azide, the worms learn to avoid PA14, and migrate to the OP50 spot. This phenomenon was already carefully demonstrated by Ooi and Prahlad (2017) with a time-course assay: in the absence of azide to



**Figure 1**

## The omission of the paralytic sodium azide from choice assays has significant effects on naïve choice assays.

(a) Schematic of PA14 and worm growth conditions, worm training, and choice assay parameters. Critical factors affecting assay performance are highlighted. (b) Scatter dot plots of representative replicate choice assays (OP50 versus PA14 bacteria) from naïve Day 1 adult worms (from Moore et al. 2019 [Kaletsky et al. 2020](#), and Moore et al. 2021). Each dot represents an individual choice assay plate ( $n = 10$ ) containing  $\sim 50$ – $100$  worms per plate (average  $\sim 80$ ). Choice index = (number of worms on OP50 – number of worms on PA14)/(total number of worms). (c) Individual replicates of naïve choice assays from Moore et al., 2019 [Kaletsky et al. 2020](#), and Moore 2021 are shown as violin plots. Each replicate is a separate experiment made up of 5–10 plates. The median is shown in red. (d) Individual replicates from (c) were pooled. Box plots: center line, median; box range, 25–75th percentiles; whiskers denote minimum–maximum values. (e) Naïve worms were placed on choice assay plates with spots of PA14 and OP50 with or without sodium azide on the bacterial spots, then allowed to choose for one hour, followed by one hour at 4°C. No azide: CI =  $0.4 \pm 0.06$ ,  $p < 0.0001$ . (f) Estimation plot of difference in CI between azide and no azide plates; mean difference =  $0.482$  [95.0%CI 0.369, 0.64]. (g) After 1 hr of the choice assay at room temperature, choice assay plates were placed at 4°C. Worms were counted at each spot at the indicated time points. Mean  $\pm$  SD from three choice assay plates. Timepoint 0 = immediately before 4°C incubation. (h) Worms were placed on choice assay plates with spots of PF15 and OP50 with or without sodium azide on the bacterial spots, then allowed to choose for one hour, followed by one hour at 4°C. No azide CI =  $0.26 \pm 0.05$ ,  $p < 0.0001$ . (i) Estimation plot of difference in CI between azide and no azide plates; mean difference =  $0.547$  [95.0%CI 0.348, 0.737].

trap the worms, the worms are initially attracted to PA14, but by 45 min the worms show no preference for PA14, and by one hour the worms instead avoid PA14 – that is, within the time of the hour-long assay, in the absence of azide to trap the worms at their first choice, they learn to avoid PA14. Under the no-azide assay conditions, it would be difficult to observe any additional learned avoidance that is the result of training, since the learned preference for OP50 (PA14) is already very high when the worms have been allowed to change from their first choice to their second choice. Therefore, the simplest interpretation is that one cannot accurately measure naïve preference in the absence of azide, and one cannot subsequently properly assess subsequent learned avoidance after training without azide. It is notable that the overwhelming majority of Gainey et al.'s experiments were done without azide; because a third party (Ooi & Prahlad, 2017 [DOI](#)) already showed that there is a substantial difference when no azide is used, as we have shown here, Gainey et al.'s no-azide data cannot be compared with assays done with azide, including all of our work.

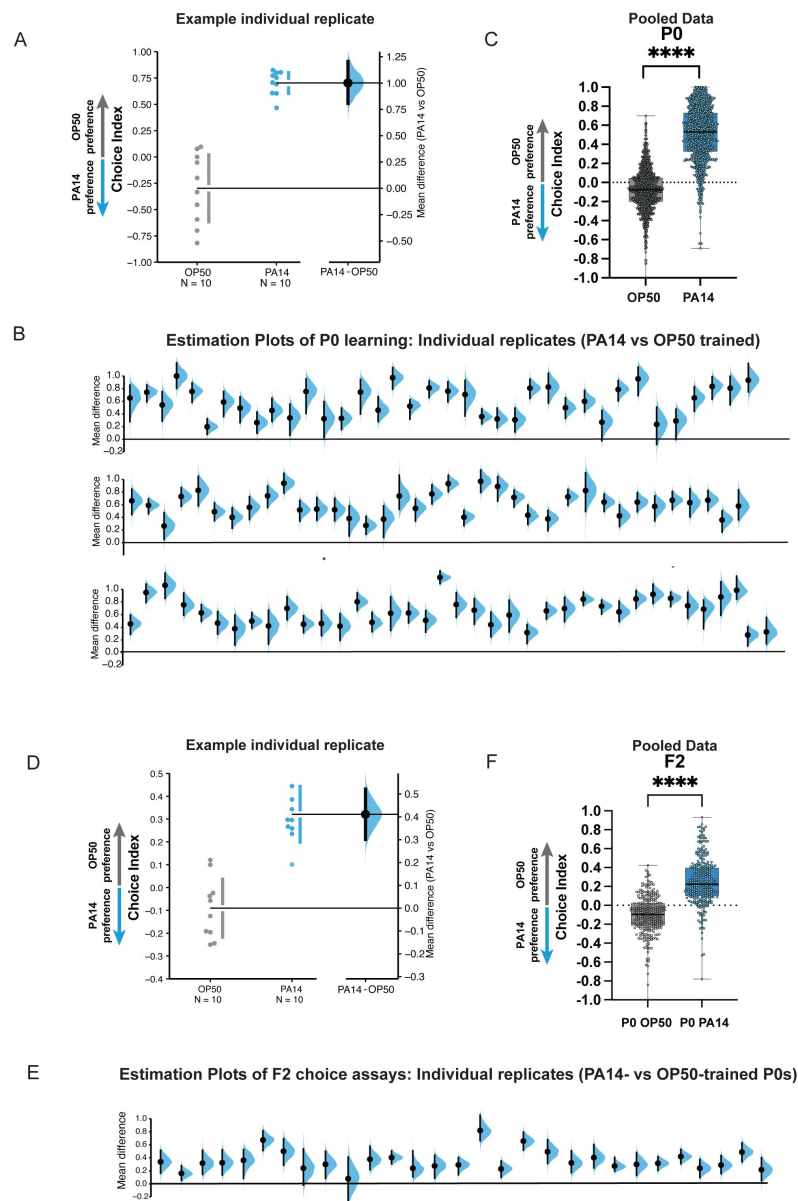
We repeated this ±azide preference assay with another *Pseudomonas* pathogen, *P. fluorescens* 15 (PF15) that we have also found to induce transgenerational avoidance for 5 generations via small RNAs (Seto et al, 2024), and observed similar results (**Figure 1H, I** [DOI](#)). That is, the preference for PF15 is similar to worms' preference for PA14 over OP50, but in the absence of azide, the worms learn to avoid PF15, as they did with PA14. Therefore, we conclude that the inclusion of azide in the choice assay is necessary to properly assess the worms' first choice; in the absence of azide, the worms' naïve choice is not being measured, so it cannot be used to then compare with post-training behavior to assess learned or inherited avoidance.

## Transgenerational inheritance of learned avoidance is consistent and distinct from other avoidance paradigms

*C. elegans* uses many different environmental cues to inform its behavioral decisions; in fact, avoidance of PA14 can be induced by bacterial metabolites, by olfactory cues, by gustatory cues, and by innate immunity pathways. These cues induce avoidance in the P0 generation, and they and other mechanisms can induce avoidance and changes in survival or development in the F1 generation via distinct mechanisms (Pereira et al, 2020 [DOI](#); Hong et al, 2021 [DOI](#); Burton et al, 2020 [DOI](#); Pender et al, 2024 [DOI](#)), but do not extend beyond the F1 generation. Our studies used a single condition for training, PA14 grown on plates at 25°C and training at 20°C for 24hrs (see **Figure 1A** [DOI](#)). Under these conditions, we consistently observe learned avoidance in mothers (P0; **Figure 2A, B, C** [DOI](#)) that is then transgenerationally-inherited (F2; **Figure 2D-F** [DOI](#)). Therefore, our PA14 growth and/or training conditions must be distinct from those growth and training paradigms that only produce P0 or F1 effects.

## P11 expression is condition-specific and critical for TEI of learned avoidance

In searching for the relevant TEI-inducing factor, we tested and eliminated DNA, proteins, and large RNAs as the TEI-inducing component, and found that small RNAs isolated from PA14 grown at 25°C on plates could induce avoidance and TEI (Kaletsky et al, 2020 [DOI](#)). That is, small RNAs isolated from 25°C plate-grown PA14 pipetted onto *E. coli* prior to 24hr *C. elegans* training was able to induce avoidance in those animals and the transgenerational inheritance of that avoidance, just as we had observed from 24hr PA14 lawn-training experiments. We had also noticed that PA14 grown at lower temperatures or in liquid could not induce transgenerational inheritance of learned avoidance. Capitalizing on these observations, we used differential small RNA sequencing to identify the set of small RNAs that are expressed in PA14 grown at 25°C on plates that are not expressed in liquid or at low temperatures; we then cloned each of those six small RNAs into *E. coli* and tested these individual sRNAs for their ability to induce avoidance and TEI (Kaletsky et al, 2020 [DOI](#)). This set of experiments revealed that the small RNA P11 – and only P11 – could induce



**Figure 2**

### P0 and F2 worms trained on PA14 bacteria reproducibly learn to avoid PA14 in choice assays.

**a)** P0 replicates: A representative experiment (replicate) showing the choice index and effect size of 24h PA14-trained worms compared to the OP50-trained control. **(b)** Mean differences of individual replicates from Moore et al. 2019 [\[1\]](#), Kaletsky et al. 2020 [\[2\]](#), and Moore et al. 2021 [\[3\]](#) are shown for PA14- vs OP50-trained mothers. **(c)** Individual replicates from **(b)** were pooled. **(d-e)** F2 animals from P0-control or PA14-trained worms. **(d)** F2 replicates: A representative experiment (replicate) showing the choice index and effect size of F2 worms. **(a, d)** The mean difference is shown as a Gardner-Altman estimation plot. Both groups are plotted on the left axes; the mean difference is plotted on floating axes on the right as a bootstrap sampling distribution. The mean difference is depicted as a dot; the 95% confidence interval is indicated by the ends of the vertical error bar. **(e)** Mean differences of individual replicates from Moore et al. 2019 [\[1\]](#), Kaletsky et al. 2020 [\[2\]](#), and Moore et al. 2021 [\[3\]](#) are shown for F2s from PA14- vs OP50-trained grandmothers. **(b, e)** Box plots: center line, median; box range, 25–75th percentiles; whiskers denote minimum–maximum values. Unpaired, two-tailed Student's *t*-test. \*\*\*\**P* < 0.0001. **(f)** Individual replicates from **(e)** were pooled. **(c, f)** Each replicate is a separate experiment made up of 5–10 plates (average ~80). The mean difference (effect size) is shown as a Cumming estimation plot. Each mean difference is plotted as a bootstrap sampling distribution. Mean differences are depicted as dots; 95% confidence intervals are indicated by the ends of the vertical error bars. Estimation graphics generated as described in Ho et al., 2019.

avoidance in mothers and inheritance of avoidance for four generations (Kaletsky *et al*, 2020 [↗](#)). *C. elegans* trained on *E. coli* expressing P11 sRNA induced the P0-F4 avoidance of PA14 without making the worms sick, and without inducing innate immune pathways, but while also inducing *daf-7::gfp* expression in the ASI (Kaletsky *et al*, 2020 [↗](#)). We observed that *E. coli*-P11 (indicated by the yellow points) consistently induces P0 avoidance (Figure 3A, B [↗](#)) and this learned avoidance is inherited transgenerationally (Figure 3C, D [↗](#)). Therefore, P11 sRNA is both necessary and sufficient to induce the transgenerational inheritance of learned PA14 avoidance.

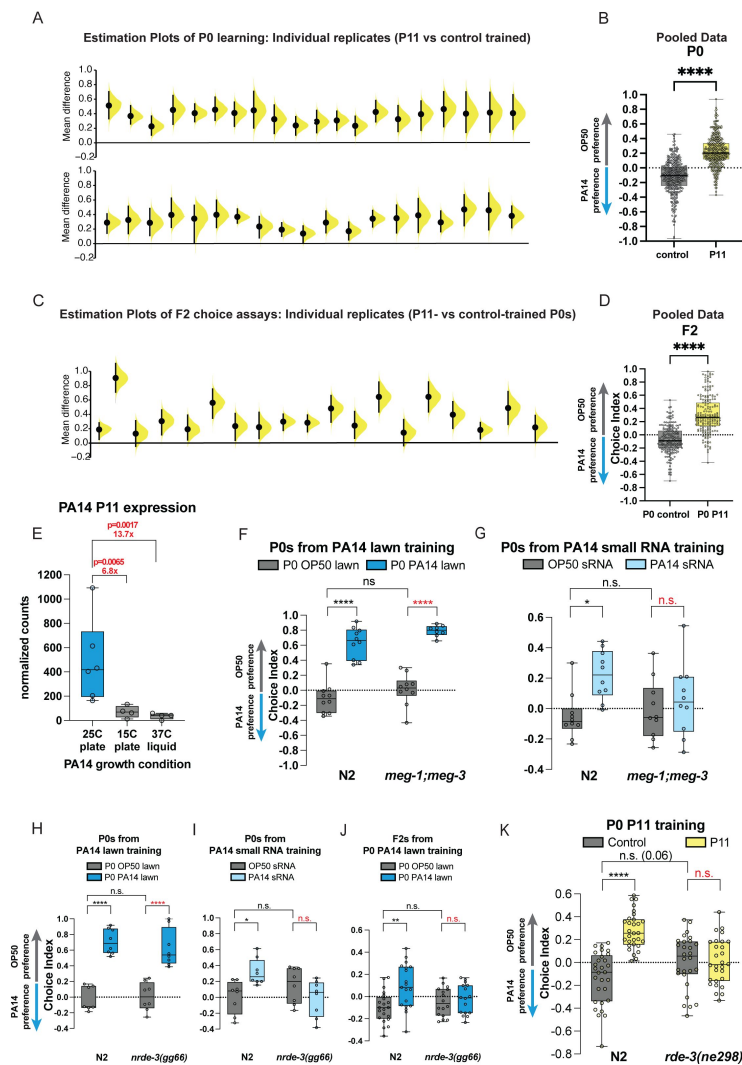
We noted that P0 avoidance induced by *E. coli*-P11 training (Figure 2A [↗](#)) is slightly lower than that induced by training on PA14 bacterial lawns (Figure 3A, B [↗](#)), suggesting that the P0 innate immunity-induced and other avoidance mechanisms are distinct from and additive with the sRNA pathway in mothers. In fact, the average level of avoidance induced by *E. coli*-P11 training is consistent in P0 and F2 (Figure 3C, D [↗](#)) and with the F2 avoidance levels induced by PA14 training (Figure 2E, F [↗](#)), consistent with our previous reports (Kaletsky *et al*, 2020 [↗](#); Moore *et al*, 2019 [↗](#)). In fact, P11-induced avoidance remains consistent from P0 through F4 (Kaletsky *et al*, 2020 [↗](#)), as we later also found for the small RNAs (Pv1, Pfs1) required for *P. vranovensis* (Sengupta *et al*, 2024) and *P. fluorescens* 15 (Seto *et al*, 2024) avoidance. Similarly, while *E. coli*-P11 training induced *daf-7::gfp* expression in the ASI from P0 through F4 (Kaletsky *et al*, 2020 [↗](#)), we did not observe any *daf-7::gfp* expression in the ASJ, consistent with the ASJ response being specifically induced by metabolites produced by PA14 (Meisel *et al*, 2014 [↗](#)).

Therefore, it appears that there are PA14 growth conditions that can induce some mechanisms of P0 and F1 avoidance, but will not induce transgenerational avoidance, because no P11 is expressed in PA14 under those conditions.

To more carefully address this hypothesis, we examined the levels of P11 sRNA from PA14 grown at 25° on plates, 15°C on plates, and 37°C in liquid (Figure 3E [↗](#), adapted from Kaletsky *et al*, 2020 [↗](#)). When comparing 25°C plates with 15°C plates, we observed a 6.8-fold decrease in P11 levels at the lower temperature, and a 13.7-fold decrease in P11 when PA14 is grown in liquid rather than on plates, even at high temperatures. Therefore, it is clear that growth of PA14 in non-25°C/plate-grown conditions may fail to elicit a P11-mediated avoidance response, because no P11 is expressed in PA14 under those conditions. Because Gainey *et al*. (2024) [↗](#) grew PA14 under range of conditions other than on plates at 25°C, it is not surprising that they also failed to observe P11-induced avoidance in the F2 generation in those experiments.

## TEI of learned avoidance requires components of RNA processing pathways

Previously, we showed that many components of small RNA uptake, RNA interference, small RNA processing pathways, and the germline are necessary for transgenerational inheritance of learned avoidance (Moore *et al*, 2019 [↗](#); Kaletsky *et al*, 2020 [↗](#)). We find that the *meg-1;meg-3* double mutants, which perturb P granules and the germline (Wang *et al*, 2014 [↗](#)), can avoid PA14 after lawn training, but are defective for small RNA-induced learning (worms trained on *E. coli* with small RNA purified from PA14 added to the lawn) (Figure 3F [↗](#)). Similarly, NRDE-3, an Argonaute protein that functions in nuclear RNAi (Guang *et al*, 2008 [↗](#)), is also required for small RNA-induced learned avoidance (Figure 3G [↗](#)). Like other mutants required for the small RNA TEI pathway, the P0 generation of *meg-3;meg-4* and *nrde-3* mutants still avoid PA14 after training on intact PA14 lawns (Figure 3F, H [↗](#)) through other avoidance pathways (innate immunity, metabolites, etc.), once again highlighting the independence of these different avoidance-inducing pathways. Only the small RNA-induced avoidance is transgenerational; that is, F2s of wild-type worms continue to avoid PA14 after lawn training, but F2s of *nrde-3* mutants are defective for this inherited small RNA-induced avoidance (Figure 3J [↗](#)).



**Figure 3**

### P11 sRNA and components of germline and RNAi function are required for transgenerational inheritance.

(a) P0 worms trained on *E. coli* expressing the P11 small RNA reproducibly avoid PA14 in choice assays. Individual replicates from Kaletsky et al. 2020 [Kaletsky et al. 2020](#) and Moore et al. 2021 [Moore et al. 2021](#) are shown for P0 worms obtained from P11 vs control training. (b) Individual replicates from (a) were pooled. (c) F2 animals from P0 worms trained on *E. coli* expressing the P11 small RNA reproducibly avoid PA14 in choice assays. Individual replicates from Kaletsky et al. 2020 [Kaletsky et al. 2020](#) and Moore et al. 2021 [Moore et al. 2021](#) are shown for F2 worms obtained from P11- vs control-trained grandmothers. (d) Individual replicates from (a) were pooled. (a, c) Each replicate is a separate experiment made up of 5-10 plates (average ~80). The mean difference (effect size) is shown as a Cumming estimation plot. Each mean difference is plotted as a bootstrap sampling distribution. Mean differences are depicted as dots; 95% confidence intervals are indicated by the ends of the vertical error bars. (e) Normalized RNA counts for P11 expression from PA14 under different bacterial growth conditions. Sequencing data and figure adapted from Kaletsky et al., 2020 [Kaletsky et al. 2020](#). Adjusted p-values from DESeq2. (f) P0 *meg-1; meg-3* mutants exhibit normal learned PA14 avoidance when trained on PA14 lawns, but (g) P0 *meg-1; meg-3* mutants do not exhibit learned PA14 avoidance when trained with PA14 sRNA. (h) P0 *nrde-1(gg66)* mutants exhibit normal learned PA14 avoidance when trained on PA14 lawns, (i) but are defective for PA14 avoidance learning when exposed to PA14 sRNA. (j) F2 *nrde-3* mutants from P0-PA14 lawn-trained grandmothers do not avoid PA14 compared to wild type controls. (k) Wild-type P0 worms exposed to *E. coli*-expressing P11 avoid PA14, but *rde-3* mutants do not. (b, d-k) Box plots: center line, median; box range, 25-75th percentiles; whiskers denote minimum-maximum values. (b, d) Unpaired, two-tailed Student's *t*-test. (f-k) Two-way ANOVA with Tukey's multiple comparison's test. \* $P \leq 0.05$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P < 0.0001$ , NS, not significant. Estimation graphics generated as described in Ho et al., 2019.

We also find that *rde-3/mut-2*, which encodes a ribonucleotidyltransferase that is involved in siRNA accumulation, piRNA-mediated silencing, and p(UG)ylation of small RNAs (Chen *et al*, 2005 [↗](#); Shukla *et al*, 2020 [↗](#); Priyadarshini *et al*, 2022 [↗](#)) in epigenetic inheritance, is also required for *E. coli* P11-induced learned avoidance (**Figure 3K** [↗](#)). Thus, components of small RNA transport, small RNA processing, RNAi function, germline components, chromatin factors, neuronal function, and the Cer1 retrotransposon (Moore *et al*, 2021 [↗](#)) are required for the transgenerational inheritance of learned pathogen avoidance (**Figure 4A** [↗](#)).

## Discussion

*C. elegans*' physiological responses to PA14 and other pathogenic bacteria may allow the worms to increase their chances of survival. Intergenerational responses, such as increased survival (Burton *et al*, 2020 [↗](#); Pender *et al*, 2024 [↗](#)), changes to development (Palominos *et al*, 2017 [↗](#)), and internal physical responses (Singh & Aballay, 2019 [↗](#); Das *et al*, 2024 [↗](#)) likely also increase the possibility of the survival of their offspring. Switching behavioral responses from attraction to avoidance for a few generations may also increase the survival of the species (Moore *et al*, 2019 [↗](#)) by reducing the chances of interactions with the pathogens. Of the many cues that induce avoidance, only the bacterial small RNA pathway induces transgenerational inheritance of learned pathogen avoidance, and this pathway is molecularly distinct from other pathways that induce avoidance only in the P0 or F1 generations (Kaletsky *et al*, 2020 [↗](#)). That is, if PA14 is grown under conditions in which P11 is *not* expressed, the bacteria may still induce P0 and even F1 avoidance due to the presence of olfactory, metabolite, and other cues, but avoidance in the F2 generation and beyond will be absent. Therefore, if F2 avoidance is not observed, it might be due to the lack of P11 expression in those conditions, and conversely, avoidance in the P0 and F1 conditions should not be misconstrued as positive evidence of P11 expression.

P11 sRNA is a regulator of PA14's nitrogen assimilation operon, which includes nitrite and nitrate reductases (NirB, NirD, and NasC) that control the production of ammonium (Marogi *et al*, 2024 [↗](#)). Nitrogen assimilation appears to be critical for growth on surfaces at specific temperatures, including inside its host, *C. elegans* (Marogi *et al*, 2024 [↗](#)). Nitrogen assimilation is an energetically costly process, and PA14 tightly regulates this operon via control of the P11 expression. Interestingly, loss of P11 reduces ammonia production and thus reduces *C. elegans*' chemoattraction to PA14 (Marogi *et al*, 2024 [↗](#)); therefore, differences in *C. elegans* behavior when treated with PA14 vs. ΔP11-PA14 mutants can be due to both the loss of the P11 small RNA itself, and also due to the loss of ammonia production. Therefore, any growth conditions that result in disruption of P11 expression might also contribute to decreases in naïve attraction that Gainey *et al*. observed; that is, if PA14 is grown under conditions where P11 sRNA is not expressed, not only would training on this bacteria not induce transgenerational inheritance of avoidance due to the lack of P11 signal, but these conditions might also decrease *C. elegans*' naïve attraction to this PA14, further reducing behavioral differences between naïve and trained chemotaxis indices. Thus, growth conditions that decrease P11 expression might have a large effect on both naïve and trained chemotaxis choice indices, and thus on interpretation of these effects. Because P11 sRNA is both necessary and sufficient for the transgenerational inheritance of pathogen avoidance, and its expression level is acutely sensitive to both temperature and physical environmental conditions, it would be important to confirm P11 expression levels (e.g., via qPCR) in PA14 rather than assuming that P11 is constitutively expressed, particularly when attempting to report the lack of an F2 effect. Unfortunately, Gainey *et al*. (2024) [↗](#) never carried out any tests of P11 sRNA in PA14, so it is impossible to now know how the PA14 growth conditions in any of their experiments might have affected P11 levels and subsequent *C. elegans* responses. Additionally, learned avoidance in the P0 and F1 generations has no relationship to the P11 pathway, and therefore cannot be used as confirmation of P11 expression.

**A**

| Category                             | Gene                                                                                                                         | Normal<br>naïve preference | Small-RNA<br>learning | References                                                                                                                                                                                                                  |
|--------------------------------------|------------------------------------------------------------------------------------------------------------------------------|----------------------------|-----------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>wild type</b>                     |                                                                                                                              | +                          | +                     | Moore et al. 2019; Kaletsky et al. 2020; Moore et al. 2021; Sengupta et al. 2024; Seto et al. 2024                                                                                                                          |
| imprinting/memory                    | <i>crh-1</i>                                                                                                                 | +                          | +                     | Moore et al. 2019                                                                                                                                                                                                           |
| immunity                             | <i>pmk-1</i><br><i>vhp-1</i>                                                                                                 | +                          | +                     | Moore et al. 2019; Kaletsky et al. 2020<br>Sengupta et al. 2024; Seto et al. 2024                                                                                                                                           |
| miRNA                                | <i>alg-1</i>                                                                                                                 | +                          | +                     | Kaletsky et al. 2020                                                                                                                                                                                                        |
| viral RNAi                           | <i>drh-1</i>                                                                                                                 | +                          | +                     | Kaletsky et al. 2020                                                                                                                                                                                                        |
| LTR TE                               | <i>cer-4</i>                                                                                                                 | +                          | +                     | Kaletsky et al. 2020                                                                                                                                                                                                        |
| p granules                           | <i>meg-3*</i><br><i>dcr-1</i> int. rescue<br><i>prg-1</i> GL rescue                                                          | +                          | +                     | Kaletsky et al. 2020<br>Kaletsky et al. 2020                                                                                                                                                                                |
| RNAi                                 | <i>sid-2</i>                                                                                                                 | +                          | -                     | Moore et al. 2019; Kaletsky et al. 2020; Moore et al. 2021; Sengupta et al. 2024; Seto et al. 2024                                                                                                                          |
| transport                            | <i>sid-1</i>                                                                                                                 | +/-**                      | -                     | Moore et al. 2019; Kaletsky et al. 2020; Moore et al. 2021; Sengupta et al. 2024                                                                                                                                            |
| RNAi/sRNA processing & amplification | <i>dcr-1</i><br><i>rde-3</i><br><i>rde-2</i><br><i>rde-4</i><br><i>mut-7</i><br><i>rff-1</i><br><i>rff-3</i><br><i>glp-1</i> | +                          | -                     | Kaletsky et al. 2020; Sengupta et al. 2024<br>this paper<br>Kaletsky et al. 2020<br>Kaletsky et al. 2020<br>Kaletsky et al. 2020<br>Kaletsky et al. 2020<br>Kaletsky et al. 2020<br>Kaletsky et al. 2020; Moore et al. 2021 |
| germline p granules                  | <i>meg-1; meg-3</i><br><i>meg-3; meg-4</i>                                                                                   | +                          | -                     | this paper<br>Kaletsky et al. 2020                                                                                                                                                                                          |
| piRNAs                               | <i>prg-1</i>                                                                                                                 | +                          | -                     | Moore et al. 2019; Kaletsky et al. 2020                                                                                                                                                                                     |
| argonautes                           | <i>hrde-1</i><br><i>nrde-3</i><br><i>rde-1</i><br><i>set-25</i><br><i>hpl-2</i>                                              | +                          | -                     | Moore et al. 2019; Sengupta et al. 2024<br>this paper<br>Kaletsky et al. 2020<br>Moore et al. 2019                                                                                                                          |
| chromatin modifiers                  | <i>Cer1</i>                                                                                                                  | +/-                        | -                     | Moore et al. 2019; Kaletsky et al. 2020<br>Kaletsky et al. 2020; Moore et al. 2021; Sengupta et al. 2024; Seto et al. 2024                                                                                                  |
| LTR TE                               | <i>daf-7</i>                                                                                                                 | +                          | -                     | Moore et al. 2019; Kaletsky et al. 2020; Moore et al. 2021; Sengupta et al. 2024; Seto et al. 2024                                                                                                                          |
| neuron function                      |                                                                                                                              | -                          | -                     |                                                                                                                                                                                                                             |
| nuclear RNAi                         | <i>nrde-4</i>                                                                                                                | -                          | -                     | Moore et al. 2019                                                                                                                                                                                                           |
| chromatin modifiers                  | <i>set-2</i><br><i>rbr-2</i><br><i>set-32</i><br><i>wdr-5.1</i><br><i>maco-1</i>                                             | -                          | -                     | Moore et al. 2019<br>Moore et al. 2019<br>Moore et al. 2019<br>Moore et al. 2019<br>Moore et al. 2019                                                                                                                       |
| neuron function                      | ASI ablation                                                                                                                 | -                          | -                     | Kaletsky et al. 2020; Moore et al. 2021; Sengupta et al. 2024; Seto et al. 2024                                                                                                                                             |
| ephrin signaling                     | <i>vab-1</i><br><i>vab-2</i>                                                                                                 | -                          | -                     | Moore et al. 2019<br>Seto et al. 2024<br>Seto et al. 2024                                                                                                                                                                   |

**B**

| Pathogen exposure               | Exposure conditions                   | Bacterial molecular signal           | <i>daf-7</i> change? | Worm behavior assay                            | P0             | F1             | F2-F4              |
|---------------------------------|---------------------------------------|--------------------------------------|----------------------|------------------------------------------------|----------------|----------------|--------------------|
| <i>P. aeruginosa</i> (PA14)     | lawn-leaving assay, 15h @ 25C         | metabolites                          | ASJ                  | lawn-leaving assay, 15h @ 25C, no azide        | ✓ <sup>1</sup> |                |                    |
|                                 | lawn-leaving assay, up to 24h @ 25C   | innate immunity/ intestinal bloating | <i>daf-7</i> req'd   | lawn-leaving assay, up to 24h @ 25C, no azide  | ✓              | ✓ <sup>2</sup> |                    |
|                                 | 4h on PA14 lawns grown at 27C for 2d  | innate immunity/ olfactory cues?     | n/a                  | choice assay w/bacterial supernatant +/- azide | ✓              | ✓              | ✓ <sup>3,4</sup>   |
|                                 | 8h on PA14 lawns grown at 27C for 2d  | innate immunity/ olfactory cues?     | n/a                  | choice assay w/bacterial supernatant no azide  | ✓              | ✓              | ✓ <sup>4</sup>     |
| <i>P. vranovensis</i> (GRb0427) | 24h on PA14 lawns grown at 25C for 2d | P11 small RNA                        | ASI                  | choice assay w/bacteria spots +azide           | ✓              | ✓              | ✓ <sup>5,6,7</sup> |
|                                 | 24h on PA14 lawns grown at 25C for 2d | Pv1 small RNA                        | ASI                  | choice assay w/bacteria spots +azide           | ✓              | ✓              | ✓ <sup>8</sup>     |
| <i>P. fluorescens</i> (PF15)    | 24h on PA14 lawns grown at 25C for 2d | Pf1s small RNA                       | ASI                  | choice assay w/bacteria spots +azide           | ✓              | ✓              | ✓ <sup>9</sup>     |

Transgenerational Responses

**Figure 4**

### Summary of genes tested for small RNA-mediated pathogen avoidance and characterization of PA14 avoidance-based assays.

(a) Summary of genes tested for naïve *Pseudomonas* preference behavior and small-RNA based learning and inheritance. (b) Table describing different assays measuring *Pseudomonas* learning and avoidance. *Pseudomonas* avoidance learning in the parental generation (P0), intergenerationally (F1), and transgenerationally (F2 through F4) is noted. References: 1, Meisel et al. 2014 [1](#). 2, Singh & Aballay, 2019 [2](#). 3, Zhang et al. 2005 [3](#). 4, Pereira et al. 2020 [4](#). 5, Moore et al. 2019 [5](#). 6, Kaletsky et al. 2020 [6](#). 7, Moore et al. 2021 [7](#). 8, Sengupta et al. 2024. 9, Seto et al. 2024.

In addition to P11, we also previously found that *C. elegans* ingestion and processing of specific bacterial small RNAs produced by *P. vranovensis* (Sengupta *et al.*, 2024) and *P. fluorescens* 15 (Seto *et al.*, 2024) induce pathogen avoidance that lasts through the F4 generation. These specific small RNAs are Pv1 and Pfs1 (from *P. vranovensis* and PF15, respectively), and they each contain a stretch of either 17 (P11) or 16 (Pv1, Pfs1) nucleotides in predicted stem loops that are a perfect match to specific exons of the gene *maco-1* (P11, Pv1) or *vab-1* (Pfs1), which are then downregulated upon treatment with these pathogenic bacteria or these small RNAs. In fact, editing the small RNAs to disrupt the perfect match to *maco-1* or *vab-1* abrogates the learned avoidance (Sengupta *et al.*, 2024; Seto *et al.*, 2024), underscoring the requirement for the perfect match sequence between the bacterial small RNAs and their target *C. elegans* genes. MACO-1 is an ER resident protein that has been previously implicated in regulation of chemotaxis, the dauer developmental decision, and thermotaxis (Arellano-Carbajal *et al.*, 2011 [↗](#); Miyara *et al.*, 2011 [↗](#); Neal *et al.*, 2016 [↗](#)). *maco-1* downregulation upon PA14 treatment (25°C on plates) or upon treatment with *E. coli* expressing P11 (*E. coli*-P11) results in upregulation of *daf-7::gfp* in the ASI neurons (Kaletsky *et al.*, 2020 [↗](#)). Using qPCR, we confirmed the downregulation of *maco-1* in P0 through the F4 generations and observed its return to normal levels in the F5 generation (Sengupta *et al.*, 2024), corresponding with avoidance behavior. Furthermore, mutants of *maco-1* exhibit high naïve avoidance of PA14 and high levels of *daf-7::gfp* expression in the ASI prior to training, and because of this high naïve avoidance, they are unable to learn further avoidance upon training on PA14 (Kaletsky *et al.*, 2020 [↗](#)). P0 worms treated with *maco-1* RNAi exhibit transgenerational inheritance of learned avoidance, as well (Kaletsky *et al.*, 2020 [↗](#)). The downregulation of *vab-1* upon PF15 or Pfs1 sRNA treatment also results in the downregulation of *maco-1* expression levels (as shown by RNA-seq in P0 mothers), suggesting that *vab-1* is upstream of *maco-1*; qPCR confirmed that *maco-1* is downregulated in the F2 generation of *E. coli*-Pfs1-trained mothers as well (Seto *et al.*, 2024). Therefore, the transgenerational bacterial small RNA-*C. elegans* avoidance mechanism induced by at least three different *Pseudomonas* species through three different small RNAs requires the downregulation of *maco-1*, which results in *daf-7* changes in the ASI; perfect matches between bacterial small RNAs and their *C. elegans* target genes are required for the F2-F5 heritable shift from attraction to avoidance. Thus, the role of *maco-1* in this pathway has been confirmed through multiple avenues of experimentation.

Through extensive mutant analyses (testing of almost 40 mutants over the course of six studies), we have found that this sRNA mechanism of transgenerational inheritance of learned pathogen avoidance requires a wide range of machinery involved in bacterial small RNA uptake, RNA processing, signaling, and neuronal behavioral execution (Figure 4A [↗](#)). These genes include RNA interference components, Argonautes, and RNA-dependent polymerases; germline components such as P-granule, piRNA components, COMPASS and chromatin modifiers; the Cer1 Ty3 Gypsy retrotransposon; and neuronal genes (*maco-1*/macoilin, *vab-1*/Ephrin receptor, *vab-2*/Ephrin) as well as the *daf-7*/TGF-beta ligand in the ASI neuron (Figure 4A [↗](#)). We also ruled out several genes that function in other forms of pathogen avoidance, such as imprinting, innate immunity signaling, microRNA and viral RNAi processors, and other transposable elements, further distinguishing the bacterial small RNA TEI pathway from other avoidance mechanisms that may only function in the P0 or F1 generations (Figure 4A, B [↗](#)). Through tissue-specific rescue experiments we also showed that *dcr-1* functions in the intestine and *prg-1* is necessary in the germline for small RNA-mediated learned pathogen avoidance. Our studies of other bacterial species have identified not only additional bacterial small RNAs that are “read” by *C. elegans* (Sengupta *et al.*, 2024; Seto *et al.*, 2024), but also have identified additional *C. elegans* gene targets that function in the pathway (e.g., *vab-1*, which regulates *maco-1*; Seto *et al.*, 2024). It should be noted that in each of these mutant experiments, wild-type worms were tested as controls; therefore, our wild-type naïve attraction and learned avoidance results have been replicated many times over the course of these six studies.

## Conclusions

Here we have investigated two experimental factors that might affect investigators' interpretation of PA14 training-induced avoidance choice assays: the expression of P11 sRNA in PA14 and proper execution of the choice assay itself. It would be incorrect to assume that because F2 avoidance is not demonstrated under all PA14 growth conditions that “*the published model is unlikely to be relevant in a natural environment*” (Gainey *et al*, 2024 [🔗](#)). Instead, our results suggest the opposite: that the worms' ability to interpret P11 expression is due to P11's importance in regulating nitrogen fixation under biofilm-inducing conditions that also induce a pathogenic state that is important for *C. elegans* to remember over several generations. *C. elegans* appears to have evolved a mechanism to use P11 and other specific small RNAs as biomarkers of future infection, as the presence of those specific small RNAs correlates well with pathogenesis – and the specific small RNA detected by *C. elegans* in each case might be tightly correlated with pathogenic potential of that particular *Pseudomonas* species. Furthermore, the fact that we found that other *Pseudomonas* species, including *Pseudomonas* from the worm's microbiota, use the same bacterial sRNA/C. *elegans* target gene mechanism indicates that this natural example of trans-kingdom signaling and transgenerational avoidance is highly conserved. In those bacteria, the key small RNAs appear to be constitutively expressed, and thus even more likely to be encountered by *C. elegans* upon ingestion. Together, these data suggest that the transgenerational inheritance of small RNA-induced learned bacterial avoidance is likely to be widespread in the wild, indicating its likely importance as a mechanism to promote long-term survival of *C. elegans* in its natural habitat.

## Methods

### General worm maintenance

Worm strains were maintained at 20°C on high growth medium (HG) plates (3 g/l NaCl, 20 g/l bacto-peptone, 30 g/l bacto-agar in distilled water, with 4 ml/l cholesterol (5 mg/ml in ethanol), 1 ml/l 1 M CaCl<sub>2</sub>, 1 ml/l 1 M MgSO<sub>4</sub> and 25 ml/l 1 M potassium phosphate buffer (pH 6.0) added to molten agar after autoclaving) on OP50 using standard methods. The following worm strains were provided by the *C. elegans* Genetics Center (CGC): JH3229, *meg-1(vr10);meg-3(tm4259)* X; YY158, *nrde-3(gg66)* X; WM30, *mut-2/rde-3(ne298)* I.

### *E. coli* and PA14 culture conditions

OP50 was provided by the CGC. PA14 was a gift from Z. Gitai. OP50 and PA14 were grown in liquid cultures in LB at 37°C with shaking. *Escherichia coli* strains expressing PA14 sRNA were cultured overnight in Luria broth supplemented with 0.02% arabinose w/v and 100 µg/ml carbenicillin. Overnight cultures of bacteria (prepared as described in ‘General bacteria cultivation’) were diluted in LB to an optical density at 600 nm (OD<sub>600</sub>) = 1 and used to fully cover nematode growth medium (NGM) (3 g/l NaCl, 2.5 g/l bacto-peptone, 17g/l bactoagar in distilled water, with 1 ml/l cholesterol (5 mg/ml in ethanol), 1 ml/l 1 M CaCl<sub>2</sub>, 1 ml/l 1 M MgSO<sub>4</sub> and 25 ml/l 1 M potassium phosphate buffer (pH 6.0) added to molten agar after autoclaving) plates. For preparation of control *E. coli* and *E. coli* expressing the PA14 P11 small RNA, bacteria were seeded on NGM plates supplemented with 0.02% arabinose and 100 µg/ml carbenicillin. All plates were incubated for 2 days at 25 °C, unless specified otherwise (in separate incubators for control and pathogen-seeded plates). OP50 and PA14 small RNAs were collected as described in [Kaletsky et al. 2020 \[🔗\]\(#\)](#).

### Aversive learning assay

Worm preparation for training: synchronized L4 worms were washed off plates using M9 and left to pellet on the bench top for approximately 5 min. Then, 5 µl of worms were placed onto sRNA-spotted training plates, and 10 µl or 40 µl of worms were plated onto OP50 or *E. coli* expressing

control/P11, or PA14-seeded training plates, respectively. Worms were incubated on training plates at 20 °C in separate containers for 24 h. After 24 h, worms were washed off plates using M9 and washed an additional 3 times to remove excess bacteria.

Choice plates and naïve day 1 worms were prepared as in [Moore et al. 2019](#) and [Seto et al. 2024](#). On the day of the assay, choice assay plates were left at room temperature for 1 h before use. For standard (sodium azide added) experiments, 1 µl of 1 M sodium azide was spotted onto each respective bacteria spot to be used as a paralyzing agent during choice assay and preserve first bacterial choice. For conditions without azide, no azide was added.

Worms were washed off training plates in 2 mL M9 into 1.5 mL tubes and allowed to pellet by gravity. Worms were washed 2-3 additional times in M9. Using a wide orifice pipet tip, 5 µl of worms (approximately 50-200 worms) were spotted at the bottom of the assay plate, midway between the bacterial spots. Plates were incubated at room temperature for 1 h before moving plates to 4C. Plates were left at 4C for ~1h before manually counting the number of worms on each bacterial spot.

## Meta-analysis

We collected all examples of N2 naïve chemotaxis (PA14 vs OP50 choice assays) prior to training, N2 P0 and F2 choice assays after P0 training on PA14 vs OP50, and P0 and F2 choice assays after P0 training on *E. coli*-P11 vs control *E. coli* from [Moore et al. 2019](#), [Kaletsky et al. 2020](#), and [Moore et al. 2021](#). These data are collected in Table S2. Multiple two-group estimation plots were generated for side-by-side comparison of multiple mean differences across independent experiments. Each mean difference is plotted as a bootstrap sampling distribution.

## Additional files

**Table S1.** Summary data compiled from Moore et al. 2019, Kaletsky et al. 2020, and Moore et al. 2021. [↗](#)

**Table S2.** Figure 3 worms counts from choice assays. [↗](#)

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**Reviewer #1 (Public review):**

**Summary:**

The manuscript from Kaletsky et al is a response to a paper recently published by Craig Hunter's group (Gainey et al 2024). The Murphy lab has previously shown that learned avoidance of *C. elegans* to PA14 can be transmitted through four generations. In a series of detailed studies, they defined the mechanism of this transgenerational epigenetic inheritance (TEI), identifying both PA14 and *C. elegans* factors required for this effect (Moore et al., 2019, Kaletsky et al., 2020; Moore et al., 2021). PA14 produces a small RNA, P11, that is necessary and sufficient for transgenerational epigenetic inheritance of avoidance behaviour in *C. elegans*. In the worm, P11 decreases *maco-1* expression, which in turn regulates *daf-7*.

In the study by Gainey et al (eLife 2024), the authors report their attempt at replicating the original findings of the Murphy lab using a modified experimental setup. The Gainey study observed avoidance of PA14 and upregulation of *daf-7::GFP* in the F1 progeny of trained parents, but not in subsequent generations. Importantly, although they examined a number of different deviations of the protocol, they did not repeat the original experiment using the exact protocol outlined in the Moore or Kaletsky papers. Nevertheless, the authors concluded that "this example of TEI is insufficiently robust for experimental investigations".

The manuscript by Kaletsky et al. attempts to provide an explanation as to why Gainey et al., were unable to observe transgenerational avoidance of PA14. They identify two discrepancies in the methodology used between the two studies and examine the possible impacts of these.

One of the primary differences in protocols between the two papers is how avoidance is measured. The Murphy group uses the traditional method of adding azide to bacterial spots on the choice plates to trap worms once they have come close to the food spot. The animals are on the plate for 1 hour but most have likely been immobilized before this time point. Gainey et al. omit the azide and instead shift animals to 4C after 30-60 minutes of exposure to immobilize the worms for counting. Kaletsky et al show that the choice of assay has a significant impact on measuring attraction and avoidance.

While Gainey et al., assert that the addition of azide had no discernable effect on the choice assay results, these data are not shown in their paper. Kaletsky et al. test these conditions head-to-head with the same 1 hour exposure time, showing that with azide, the initial response to PA14 in untrained worms is attraction. By contrast, in the absence of azide, when cold temperature is used to immobilize the worms, the response recorded is aversion to PA14. The choice assay generated by Kaletsky et al without azide is consistent with the choice assays in untrained worms shown in the Gainey paper, demonstrating that this is likely one factor that contributed to the different outcomes reported in the Gainey paper.

Kaletsky et al. propose that learned aversion to PA14 may be occurring within the 1-hour exposure time when worms are not trapped in their initial decision with the use of azide. This is consistent with previous findings from another group (Ooi and Prahlaad 2017), showing that 45 minutes of exposure is sufficient to overcome the attraction to PA14 and shift to avoidance of PA14. Importantly, the Gainey paper notes exposure times between 30 and 60 minutes before shifting worms to 4C to count, this window may have generated additional variability between assays.

The second possibility explored by Kaletsky et al. is that the expression of P11 differed between the studies. Because P11 is required for TEI, differences in P11 expression is a reasonable explanation for different observations between studies. Unfortunately, in the

Gainey study, P11 levels were not measured; it is therefore not possible to know whether low or absent levels of P11 explain the inability to observe TEI. Nevertheless, Kaletsky et al. test the potential for changes in one growth condition, temperature, to influence the production of P11. Indeed, the expression of P11 differs in PA14 grown at different growth temperatures, providing an additional explanation for the discrepancies.

While it is possible that temperature is the culprit, it may be another culture condition or media component suppressing P11 expression. Nevertheless, the fact that expression of P11 can so easily be modified demonstrates that P11 expression is not immune to differences in culture conditions. Given its role in nitrogen fixation, I would be surprised if it was not regulated by environmental conditions. Differences in iron content between media batches are notorious for altering bacteria phenotypes. Although outside the scope of this study, with the connection to biofilm formation, I would be curious if iron levels had an impact on P11 expression. All in all, the data highlight the fact that P11 levels should be measured if TEI is not seen.

#### Strengths:

Overall, this is an excellent study that has provided additional understanding of the difference between naïve preference and TEI and provides guidance for investigators in replicating TEI experiments. The manuscript is very well written and provides additional understanding regarding the replication of TEI in response to *P. aeruginosa*.

The manuscript provides an important discussion about differences in methodology and how they might reflect specific biology. Many examples of experimental deviations that have large impacts have simple biological explanations. I believe the authors have done an excellent job making this point.

#### Weaknesses:

None noted.

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#### Reviewer #2 (Public review):

In addition to the study by Kaletsky et al. (2025), I read the bioRxiv and eLife versions, as well as the eLife reviewer comments, for Gainey et al. (2024), to which Kaletsky et al. respond.

Kaletsky et al. provide detailed, rigorous, and reproducible protocols and results. The authors point out the critical methods that the Hunter group failed to follow/confirm (e.g. azide to paralyze animals during pathogenic learning/memory assays; the expression of the P11 small RNA that is both necessary and sufficient for TEI of avoidance behavior; a single condition for training - PA14 grown on plates at 25°C and training at 20°C for 24 hr - that the Hunter lab did not follow and could not reproduce). The Kaletsky et al. response is evidence-based, fair, level-headed and unbiased, which is in contrast to the Gainey et al. paper.

Reading the eLife review of Gainey et al., I note that the reviewers repeatedly pointed out that authors did not follow published protocols by the Murphy lab.

Public response by Gainey et al. to Reviewer 2: "It remains possible that we misunderstood the published Murphy lab protocols, but we were highly motivated to replicate the results so we could use these assays to investigate the reported RNAi-pathway dependent steps, thus we read every published version with extreme care."

Public response by Gainey et al. to Reviewer 3: "We agree that our study was not exhaustive in our exploration of variables that might be interfering with our ability to detect F2

avoidance."

Gainey et al. provide reasons/excuses for why they did not follow published methods - notably their subjective decision to exclude the paralyzing agent sodium azide from their choice assays, but their abstract reads "We conclude that this example of transgenerational inheritance lacks robustness." I strongly disagree with this conclusion.

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### Reviewer #3 (Public review):

A recent bioRxiv paper from Craig Hunter's lab (Gainey et al. 2024) puts into question several manuscripts that report that pathogen avoidance by the nematode *C. elegans* to the pathogenic bacteria, *Pseudomonas aeruginosa*, for several generations after initial exposure is not robust nor repeatable. From the Hunter lab publication, the authors tried to eliminate genetic drift of the pathogenic bacterial strains and *C. elegans*, as well as several experimental conditions, including assay temperature conditions and the effect of light.

The papers (Moore et al. 2019, Kaletsky et al. 2020, Moore et al. 2021 and Sengupta et al. 2024) that the Gainey et al. manuscript brings into question discovered that *Pseudomonas aeruginosa* can produce a small RNA (sRNA), P11, that is necessary and sufficient for pathogen avoidance of the future generation of *C. elegans* (up to F4 generation). The Gainey et al. manuscript does not assess the status of P11 production in their work.

Here, the Murphy group has made several new discoveries that highlight the differences with the work performed in the Hunter lab. One, the assay used to test attraction and avoidance of *C. elegans* for pathogenic bacteria differs amongst the two groups. In the Murphy lab papers, and many others in this field, the assay is established whereby worms can decide between spots of non-pathogenic bacteria (*E. coli*) or pathogenic (*P. aeruginosa*) on a single plate separated by a few centimeters. Also included in each spot is an aliquot of NaN<sub>3</sub> to freeze the animals upon entry into their first bacterial choice. *C. elegans* will initially choose the pathogenic bacteria as its first choice and then learn to avoid the pathogenic spot thereafter. Therefore, establishing this first baseline attraction point is essential for determining future avoidance events. The Hunter lab did not use NaN<sub>3</sub> and instead relied upon moving plates to 4°C to slow the worm's movements to count the population. Furthermore, the Hunter lab allowed the "choice" to proceed for an hour before moving to 4°C, making capture of the initial attraction phase of the choice assay difficult to discern since the worms could move freely from their initial choice due to the lack of the paralyzing NaN<sub>3</sub>.

The second major advance that the Murphy group has found is that the growth of *P. aeruginosa* prior to being used for the choice assay is critical. Growth on plates at 25°C, but not 20°C on plates or in liquid at 37°C, can produce the transgenerational inheritance of pathogen avoidance. Interestingly, P11 is only produced by *P. aeruginosa* at 25°C grown on plates. The Hunter group grew the *Pseudomonas* bacteria at 37°C in liquid with gentle shaking and then spotted onto assay plates followed by growth for 2 days at 25°C and then equilibrated to room temperature before the choice assay. The Hunter lab did not check the status of P11 production in any of their experiments.

The results from the Murphy group are solid and they go on to find genetic requirements in *C. elegans* required for the transgenerational response to *P. aeruginosa* and P11. Furthermore, they repeat their results with additional members of the *Pseudomonas* clade and find the same transgenerational avoidance response and new sRNAs responsible for the avoidance response to the newly tested *Pseudomonas* members.

Overall, the discrepancies between the Hunter work and the numerous papers for the Murphy group would tend to complicate this area of research. However, this eLife paper plainly illustrates the straightforward nature of the experimental setup and reconfirms the necessary and sufficient nature of P11 in orchestrating the multigenerational response to pathogenic *Pseudomonas*. It appears that ensuring the production of P11 from the *Pseudomonas* culture and ensuring that the assay captures the initial bacterial choice are essential to observe the transgenerational inheritance of the avoidance phenotype.

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