Independent Validation of Transgenerational Inheritance of Learned Pathogen Avoidance in *Caenorhabditis elegans*

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Abstract

Transgenerational inheritance of learned behaviors remains a controversial topic in biology. The Murphy lab previously demonstrated that *C. elegans* exposed to pathogenic *Pseudomonas aeruginosa* (PA14) not only learn to avoid this bacterium but also transmit this avoidance behavior to untrained offspring. A recent study has challenged these findings, questioning the robustness of this phenomenon. Here, we independently validate the transgenerational inheritance of PA14 avoidance in *C. elegans*. Using standardized choice assays, we show that worms trained on PA14 develop significant avoidance that persists in F1 and F2 generations, though with decreased strength after P0. Our results provide independent confirmation of transgenerational inheritance of PA14 avoidance through the F2 generation, in agreement with previous findings from the Murphy lab and in contrast to a recent study by the Hunter group that failed to detect this phenomenon beyond the F1 generation.

Introduction

Caenorhabditis elegans inhabits soil environments where it encounters diverse bacteria, including both nutritional and pathogenic species (Schulenburg and Félix, 2017; Neher, 2010). This nematode has evolved sophisticated sensory mechanisms to distinguish beneficial from harmful bacteria (Kim and Flavell, 2020; Guillermin, 2018). Interestingly, naïve *C. elegans* initially prefer the pathogenic bacterium *Pseudomonas aeruginosa* (PA14) over the non-pathogenic laboratory strain *Escherichia coli* (OP50) but rapidly learn to avoid PA14 following exposure (Zhang and Bargmann, 2005).

The Murphy lab first demonstrated that this learned avoidance behavior can be transmitted to progeny, persisting through the F2 generation (Moore et al. Cell 2019; Kaletsky et al. Nature 2020; Moore et al. Cell 2021; Sengupta et al., 2024). This transgenerational epigenetic inheritance (TEI) has been linked to small RNA pathways and the dsRNA transport proteins SID-1 and SID-2. Recently, Sengupta et al. (2024) further identified a specific small RNA responsible for inducing transgenerational inheritance of learned avoidance.

However, Gainey et al. (2025), representing the Hunter group, reported that while parental and F1 avoidance behaviors were evident, transgenerational inheritance was not reliably observed beyond the F1 generation under their experimental conditions. These conflicting findings highlight the importance of methodological details and underscore the need for independent replication. Here, we rigorously examine whether parental exposure to PA14 elicits consistent avoidance in subsequent generations, addressing potential methodological variations that might influence results. Our study specifically focuses on the transmission of learned avoidance through the F2 generation—beyond the intergenerational (F1) effect—because this is where divergence between published

studies begins. Our goal was to evaluate whether the reported transgenerational signal persists across two generations without reinforcement.

Results and Discussion

We investigated whether *C. elegans* exposed to PA14 exhibit learned avoidance behavior that persists across generations. Naïve worms showed a preference for PA14 over OP50, with a negative choice index (approximately -0.4). After 24 hours of exposure to PA14, trained worms developed strong aversion to the pathogen, exhibiting a significantly positive choice index (Figure 1).

Critically, this avoidance behavior persisted in the untrained F1 and F2 progeny of trained worms, with both generations showing significantly higher choice indices compared to naïve controls (Fig. 1, Table S1). However, the strength of avoidance diminished somewhat in F2 relative to F1 progeny, suggesting a gradual dilution of the inherited response over generations.

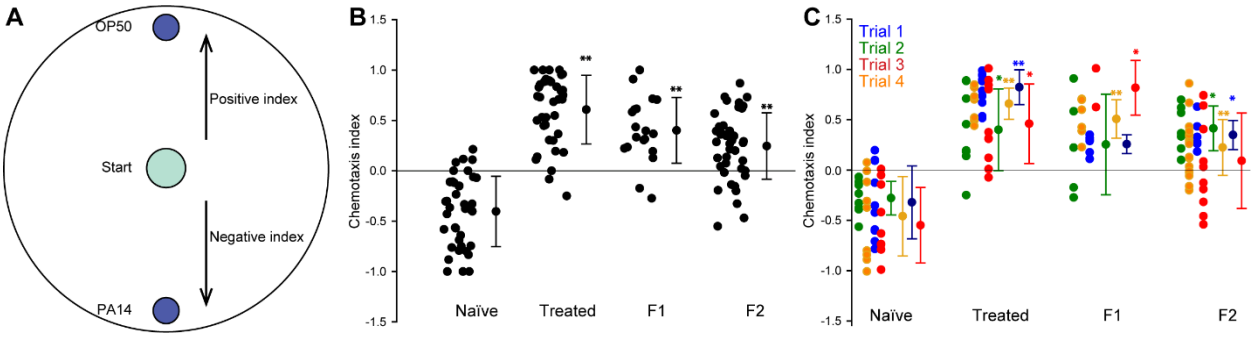

Figure 1. Transgenerational inheritance of PA14 avoidance in *C. elegans*. **A.** Chemotaxis assay setup. A 25 µL drop of OP50 and PA14 bacteria of the same optical density (OD_{600} = 1) plus 1.0 µL of 400 mM NaN3 were placed equidistant from the center of a 10cm chemotaxis plate where worms were placed at the start of the assay (see methods). indices were measured for naïve worms, trained worms, first-generation (F1), and second-generation (F2) offspring. **B.** Pooled results from four independent trials showing that Trained worms, which were exposed to PA14 for 24 hours, exhibited significantly higher choice indices, indicating learned avoidance of PA14. This aversion persisted in F1 and F2 progeny, demonstrating transgenerational inheritance of pathogen avoidance behavior. **C.** Comparison of data separated into individual trials. Ranked summed test, means and SEM shown (see Table S1 for statistics).

Our findings independently validate the transgenerational inheritance of learned pathogen avoidance initially reported by the Murphy lab. In contrast, Gainey et al. (2025) failed to observe such inheritance beyond F1 despite exhaustive attempts and protocol refinements. While we cannot definitively reconcile these differences, our results suggest that under tightly controlled conditions—including bacterial lawn density, OD_{600}=1.0 standardization, and immobilization with sodium azide restricted to one hour (to capture initial preference behaviors)—transgenerational inheritance through F2 is both detectable and statistically robust.

We echo Gainey et al.'s observation that behavioral assays can be highly sensitive to environmental variables. This highlights the importance of consistency across experimental setups and support the view that context-dependent variation may underlie previously reported discrepancies.

These results support a growing body of evidence that *C. elegans* can transmit acquired behavioral adaptations to offspring through non-genetic mechanisms, likely involving small RNAs and epigenetic modifications as suggested by Sengupta et al. (2024). The gradual decrease in avoidance strength by the F2 generation suggests that the inherited signal attenuates over successive generations without reinforcement.

Methods

Nematode Strains and Maintenance

Wild-type *Caenorhabditis elegans* (N2) were maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50 at 20°C under standard conditions. Worm populations were passaged regularly to avoid overcrowding and starvation.

Bacterial Cultures

We inoculated 6 mL of LB medium (pH 7.5) with single colonies of OP50 or *Pseudomonas aeruginosa* PA14 and incubated them overnight at 37°C with shaking at 250 rpm. For PA14, cultures did not exceed 16 hours of incubation or develop visible biofilms. For both OP50 and PA14, the overnight culture was diluted to an OD_{600} of 1.0 in fresh LB media prior to seeding.

Synchronization of Worms

Gravid adults were collected from OP50-seeded NGM plates by washing with NGM buffer. After settling by gravity, a standard bleach solution was added and gently nutated for 5–10 minutes. The embryo pellet was washed 2–3 times with NGM after centrifugation. Approximately 250–350 eggs were plated onto each OP50-seeded NGM plate and incubated at 20°C for 48–52 hours until reaching late L4 stage.

Training and Choice Assay

Training plates (10 cm NGM) were seeded with 1 mL of overnight bacterial cultures and incubated at 25°C for 2 days. Choice assay plates (6 cm NGM) were prepared with 25 µL spots of OP50 and PA14 on opposite sides placed 24 hrs prior to the assays. Late L4 worms were transferred to training plates (OP50 or PA14) for 24 hours at 20°C. For choice assays, 1.0 µL of 400 mM sodium-azide was placed on each bacterial spot before adding worms. After one hour, the number of worms immobilized on each spot was counted to calculate a Choice Index:
Choice Index = (fraction of worms on OP50) - (fraction of worms on PA14).

Transgenerational Testing

Synchronized F1 progeny were obtained by bleaching trained adult worms and allowing embryos to develop on OP50-seeded plates at 20°C. On Day 1 of adulthood, F1 worms were tested using the same choice assay. The F2 generation was derived from untrained F1 adults, synchronized in the same manner, and tested in identical conditions.

Controls and Additional Considerations

The data presented in this study are the result of four independent rounds of experimentations. Each individual assay was performed with animals harvested from

unique culture plates. An average of 55 animals participated in each assay (were immobilized in the NaN3 and tallied at the end of the assay). We conducted an average of 8.5 assays per condition during each of the four replicates. All plates used in the study were prepared at least two days before experiments to ensure consistent bacterial lawn growth. Worms were monitored to prevent starvation or overcrowding, as these conditions could influence their bacterial preferences and behavioral responses. Fresh bacterial stocks were maintained at -80°C and streaked weekly to ensure culture consistency.

Statistical Analysis

Graphs and statistical comparisons were conducted using Sigmaplot 14 (Inpixon). We used the InterQuartile Range (>1.5xIQR) method to evaluate all data. Exclusion of outliers (5/143 data points: 3.5%) did not affect statistical significance or effect. Comparisons involved One Way ANOVAS when dataset passed normality (Shapiro-Wilk), and equal variance (Brown-Forsythe) tests. Failed either of these comparisons involved non-parametric tests (Kruskal-Wallis One Way Analysis of Variance on Ranks). Post hoc comparisons involved Multiple Comparisons versus Control Group (Holm-Sidak method) following ONE Way ANOVAS, or Multiple Comparisons versus Control Group (Dunn's Method) following Kruskal-Wallis One Way Analysis of Variance on Ranks (See Table S1 for statistical data, and Supp. File 1 for measurements).

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Competing Interests

The authors declare no competing interests.

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Supplementary Information

Table S1. Summary of statistical analysis. H: H-statistic; DF: degrees of freedom; Q: standardized difference between ran sums; Trial: Independent trials conducted on different dates; N: independent assays conducted on a trial (each trial derived from individual culture plate); **Naïve**: Day-1 adults without previous exposure to PA14; **Treated**: Day-1 adults with previous exposure to PA14; **F1**: Day-1 adults without previous exposure to PA14 derived from **Treated** group; **F2**: Day-1 adults without previous exposure to PA14 derived from **F1** group.

TRIAL	Naïve	Treated	F1	F2	H	DF	P	Dunn's Naïve - Treated			Dunn's Naïve - F1			Dunn's Naïve - F2		
	N	N	N	N				Diff Rank	Q	P	Diff Rank	Q	P	Diff Rank	Q	P
1	10	12	6	7	29.23	3	<0.001	23.5	5.4	<0.001	9.7	1.8	0.2	12.3	2.4	0.044
2	8	8	5	7	12.8	3	0.005	12.4	3	0.008	10.1	2.2	0.09	13.1	3.1	0.006
3	10	10	2	11	18	3	<0.001	16.6	3.8	<0.001	21.4	2.9	0.013	10	2.4	0.053
					Shapiro-Wilk	Brown-Forsythe	Alpha-Power	Holm-Sidak Naïve - Treated			Holm-Sidak Naïve - F1			Holm-Sidak Naïve - F2		
								Diff Mean	t	P	Diff Mean	t	P	Diff Mean	t	P
4	10	8	6	18	0.48	0.084	0.05/1.00	1.1	8.3	<0.001	0.97	6.6	<0.001	0.68	6.1	<0.001
								Dunn's Naïve - Treated			Dunn's Naïve - F1			Dunn's Naïve - F2		
	Naïve	Treated	F1	F2	H	DF	P	Diff Rank	Q	P	Diff Rank	Q	P	Diff Rank	Q	P
Pooled	38	38	19	43	77.6	3	<0.001	78.4	8.5	<0.001	60.6	5.4	<0.001	46.9	5.3	<0.001