

Evolution Beyond DNA: Long-Term Experimental Evolution in *Pristionchus pacificus*

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät
der Eberhard Karls Universität Tübingen
zur Erlangung des Grades eines
Doktors der Naturwissenschaften
(Dr. rer. nat.)

vorgelegt von
Shiela Pearl Quiobe
aus Iligan City/Philippinen

Tübingen
2026

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der
Eberhard Karls Universität Tübingen.

Tag der mündlichen Qualifikation:

26.05.2026

Dekan:

Prof. Dr. Thilo Stehle

1. Berichterstatter:

Prof. Dr. Ralf J. Sommer

2. Berichterstatter:

Prof. Dr. Heinz-R. Köhler

3. Berichterstatter:

Prof. Dr. Hinrich Schulenburg

Table of Contents

I. ACKNOWLEDGEMENTS.....	5
II. SUMMARY.....	6
III. DEUTSCHE ZUSAMMENFASSUNG	7
IV. LIST OF PUBLICATIONS.....	8
1. INTRODUCTION.....	9
1.1. Conceptual and historical foundations of adaptation and inheritance.....	9
1.2. From Plastic Responses to Heritable Outcomes: Dissecting Nongenetic Inheritance	10
1.3. Epigenetics- we are more than just the sum of our genes.....	10
1.4. Major caveats in studying Transgenerational epigenetic inheritance (TEI)...	11
1.5. Beyond the human perspective: Well-characterized pathways of TEI across species	12
1.6. Molecular signals of TEI	13
1.6.1. DNA methylation.....	13
1.6.2. Histone modifications	14
1.6.3. Non-coding RNAs.....	14
1.7. Mechanisms of TEI.....	15
1.8. Plasticity in Evolutionary Theory.....	17
1.9. <i>Pristionchus pacificus</i> as a model system in plasticity research	18
1.10. TEI in the light of evolution	22
2. RESEARCH OBJECTIVES	23
3. RESULTS.....	25
3.1 Application of ALFA-Tagging in the Nematode Model Organisms <i>Caenorhabditis elegans</i> and <i>Pristionchus pacificus</i>	25
3.1.1 Synopsis	25
3.1.2 Own contribution.....	25
3.2 EBAX-1/ZSWIM8 destabilizes miRNAs, resulting in transgenerational inheritance of a predatory trait.....	26
3.2.1 Synopsis	26
3.2.2 Own contribution.....	26
3.3 Vitamin B12 induces memory of predation through vitellogenin provisioning ..	27
3.3.1 Synopsis	27
3.3.2 Own contribution.....	27
3.4 Lessons learned from manual curation of thousands of gene models in the nematode <i>Pristionchus pacificus</i>	28
3.4.1 Synopsis	28
3.4.2 Own contribution.....	28

3.5 Bacterial diet and genetic background influence mutation rate in <i>Pristionchus pacificus</i>	29
3.5.1 Synopsis	29
3.5.2 Own contribution	29
3.6 The logic of transgenerational inheritance of cannibalism	30
3.6.1 Synopsis	30
3.6.2 Own contribution	30
3.7 Darwinian games in a cannibalistic polyphenism and its transgenerational inheritance	31
3.7.1 Synopsis	31
3.7.2 Own contribution	31
3.8 A phenotypically anchored map of endogenous triggers controlling miRNA destabilization in <i>Pristionchus pacificus</i>	32
3.8.1 Synopsis	32
3.8.2 Own contribution	32
3.9 Genetic analysis of the Weismann Barrier in <i>Pristionchus pacificus</i>	33
3.9.1 Synopsis	33
3.9.2 Own contribution	33
4. DISCUSSION	34
5. REFERENCES	38
6. APPENDIX	47

I. ACKNOWLEDGEMENTS

My PhD journey has been shaped by the support of many, but I am sincerely grateful to my supervisor, Dr. Ralf Sommer, whose mentorship has profoundly shaped not only my scientific career but also changed the course of my life. Your insight, rigor, and encouragement have fundamentally influenced my development as a scientist. Thank you for taking a chance on my scientific capabilities.

My special thanks go to our beloved technicians in the lab, who were always ready to help, no matter how big or small the queries. Thanks to Hanh for all the CRISPR injections -without her, this progress would not have been possible. I would also like to thank Walli for all the assistance with all downstream technical work. Most especially, I want to acknowledge Raphaela, my closely supervised technician for two years, who has supported me through the “thick-and-thin” moments of my projects. Thank you to Heike for freezing all my generated mutant lines. Previous lab members also provided invaluable professional guidance, especially Mohannad, who introduced me to the system and mentored me during the first two years of my PhD. I am grateful to all the Sommer lab members who never complained about the many boxes I left in the cold room (almost every year!). I thank my labmates Hao and Neha, who have been my go-to persons for both professional and personal support throughout this journey. Thanks to other lab members who contributed in small but meaningful ways, making each day better: Tugba, Merve, Yinan, and Pietro- for the daily doses of chocolate during late nights in the lab. I am deeply grateful to Dr. Ata Kalirad, Dr. Christian Rödelberger, Dr. Adrian Streit, and Dr. Catia Igreja for their valuable discussions and insightful input, which greatly enriched this work.

I would like to especially thank Dominic for his patience with me, for understanding my work ethic, for encouraging me to take occasional breaks, and for making sure that I reached this milestone with sanity. I also feel very fortunate to have met such wonderful people outside of work. Thanks also to Timo and Issi for introducing me to various card and board games in German, which I have come to truly enjoy.

Lastly, I dedicate this hard work to my parents, Eduardo and Jovelyn, for shaping who I am today. To my late mother, you saw the start of this journey but left too soon to witness me achieve this milestone. I know you are with me, your love and guidance continue to inspire everything I do.

II. SUMMARY

Evolution is a continuous and historical process that can be studied experimentally in organisms with rapid generation times. Because evolution ultimately depends on the generation and inheritance of phenotypic variation, a key unresolved question is the extent to which environmental factors can produce heritable changes. Therefore, environmental effects on trait inheritance remain debated because it is often difficult to distinguish true transgenerational inheritance from short-term parental influences. While mammalian systems face technical limitations, nematodes provide powerful experimental models. In this work, I investigated transgenerational epigenetic inheritance (TEI) of environmentally induced traits using *Pristionchus pacificus*, a nematode that exhibits mouth-form plasticity, including predation. I established long-term environmental induction experiments with alternative diets by propagating 110 isogenic lines over 101 generations, together with complementary food-reversal assays. I found that a *Novosphingobium* diet enriched in vitamin B12 induces the predatory mouth morph, which is subsequently transmitted across generations and occasionally becomes canalized at low frequency. To uncover the underlying mechanisms, I performed large-scale genetic screening and identified the target-directed microRNA degradation (TDMD) factor EBAX-1/ZSWIM8. I showed that *Ppa*-EBAX-1 specifically destabilizes the expanded *miR-35/miR-2235a* microRNA cluster in *P. pacificus*, revealing an unexpected repressive role of microRNAs in regulating TEI of the predatory morph. By defining the dietary trigger landscape, I further demonstrated that vitamin B12 is sufficient to induce TEI in a concentration-dependent manner and uncovered a complex regulatory network of potential RNA triggers linked to TDMD-like processes. Overall, my work over the past five years provides conceptual and experimental advances on nematode TEI. This experimental framework leverages the natural organismal readout of plasticity in *P. pacificus* and offers key insights into developmental plasticity, its transgenerational inheritance, and low-frequency canalization of traits. Together, these findings establish a foundation for addressing broader evolutionary questions and provide a mechanistic link between environmental responsiveness and heritable phenotypic change.

III. DEUTSCHE ZUSAMMENFASSUNG

Evolution ist ein kontinuierlicher und historischer Prozess, der experimentell in Organismen mit kurzen Generationszeiten untersucht werden kann. Ein zentrales ungelöstes Thema ist, inwieweit Umweltfaktoren vererbare phänotypische Veränderungen hervorrufen können, da echte transgenerationale Vererbung oft schwer von kurzfristigen elterlichen Effekten zu unterscheiden sind. Um dies zu untersuchen, habe ich die transgenerationale epigenetische Vererbung (TEI) umweltbedingt induzierte Merkmale in *Pristionchus pacificus*, einem Nematoden mit Mundformplastizität einschließlich räuberischem Fressverhalten, analysiert. Ich etablierte langfristige Umweltinduktions-Experimente mit alternativen Futterquellen, indem ich 110 isogene Linien über 101 Generationen propagierte und ergänzende Futterwechsel-Experimente durchführte. Ich konnte zeigen, dass eine *Novosphingobium*-Diät, angereichert mit Vitamin B12, die räuberische Mundform induziert, die über mehrere Generationen weitergegeben wird und gelegentlich kanalisiert wird. Mittels groß angelegter Mutagenese-Experimente identifizierte ich den 'target-directed miRNA degradation (TDMD)-Faktor EBAX-1/ZSWIM8 als zentralen Regulator, der das *miR-35/miR-2235a*-MicroRNA-Cluster destabilisiert und damit eine unerwartete repressive Rolle von MicroRNAs bei der TEI der räuberischen Mundform aufzeigt. Supplementationsexperimente haben gezeigt, dass Vitamin B12 ausreicht, um TEI konzentrationsabhängig auszulösen. Des Weiteren habe ich potentielle Auslöser des miRNA Abbaus identifiziert und dabei ein komplexes regulatorisches Netzwerk potenzieller RNA-Auslöser aufdecken können, die mit TDMD-ähnlichen Prozessen verknüpft sind. Insgesamt liefert meine Arbeit neue konzeptionelle und experimentelle Erkenntnisse über Entwicklungsplastizität, deren transgenerationale Vererbung und niedrige Frequenz von phänotypischer Kanalisation. Zusammen bilden diese Erkenntnisse die Grundlage für die Beantwortung weitergehender evolutionärer Fragen und liefern einen mechanistischen Zusammenhang zwischen der Reaktion auf Umwelteinflüsse und vererbbarer phänotypischer Veränderungen.

IV. LIST OF PUBLICATIONS

1. Igreja, C., Loschko, T., Schäfer, A., Sharma, R., **Quiobe, S. P.**, Aloshy, E., Witte, H., & Sommer, R. J. (2022). Application of ALFA-Tagging in the Nematode Model Organisms *Caenorhabditis elegans* and *Pristionchus pacificus*. *Cells*, 11(23), 3875. <https://doi.org/10.3390/cells11233875>
2. **Quiobe, S. P.**, Kalirad, A., Röseler, W., Witte, H., Wang, Y., Rödelsperger, C., & Sommer, R. J. (2025). EBAX-1/ZSWIM8 destabilizes miRNAs, resulting in transgenerational inheritance of a predatory trait. *Science Advances*, 11(11), eadu0875. <https://doi.org/10.1126/sciadv.adu0875>.
3. **Quiobe, S. P.**, Kalirad, A., Zurheide, R., Witte, H., Rödelsperger, C., & Sommer, R. J. (2026). Vitamin B12 induces memory of predation through vitellogenin provisioning. *Nature Communications*, 17:3408. <https://doi.org/10.1038/s41467-026-71494-w>
4. **Quiobe, S. P.**, Wang, Y., Theam, P., Röseler, W., Witte, H., Rödelsperger, C., & Sommer, R. J. (2026). Bacterial diet influences mutation rate in *Pristionchus pacificus*. *G3: Genes|Genomes|Genetics*. Advance online publication. <https://doi.org/10.1093/g3journal/jkag038>.
5. Rödelsperger, C., Agyal, N., **Quiobe, S. P.**, Wu, H., Röseler, W., Ibarra-Morales, D., & Sommer, R. J. (2026). Lessons learned from manual curation of thousands of gene models in the nematode *Pristionchus pacificus*. *Plos one*, 21(3), e0344513. <https://doi.org/10.1371/journal.pone.0344513>
6. **Quiobe, S. P.**, Kalirad, A., & Sommer, R. J. (2026). Under Review in *iScience*
7. Kalirad, A., **Quiobe, S. P.**, & Sommer, R. J. (2026). Under Review in *Nature Communications*

1. INTRODUCTION

The development of living beings arises from the interplay between inherent form and environmental context (Aristotle, *Generation of Animals*). In a modern echo of this ancient insight, the phrase “we are not only what we inherit from our ancestors, but also what our ancestors experienced” captures how epigenetic mechanisms encode environmental and life experiences, transmitting them across generations to shape phenotypes and organismal biology ¹.

1.1. Conceptual and historical foundations of adaptation and inheritance

The most striking biological feature is the dynamic organismal interaction with their environments, leading to eventual existence of adaptation - the fit of organisms to their surroundings. Evolutionary biology has traditionally assumed that the genetic system serves as the sole provider of heritable variation. Historically, explanations of adaptation fall within the dichotomy of Darwinism and Lamarckism. Darwinian theory considers organisms to remain static throughout their lifetime with only those that are best suited to their environment survive and reproduce which allows passing their attributes to their progeny. In contrast, Lamarckian theory posits that organisms tend to change over time which allows them to adapt to environmental change, and these acquired traits are transmitted to their offspring. When discussing the heritability of environmental information, a controversy often invokes the contrast between these two models, with Lamarckian ideas having been largely dismissed. However, the history of heredity is more complex, as detailed in previous reviews ². For instance, Darwin’s theory of pangenesis proposed the existence of “gemmules”, nongenetic particles transmitted through the germline that could mediate the inheritance of acquired traits³.

In the late half of the 20th century, unification of Darwin’s theory of evolution with Mendel’s laws of inheritance marks major advancement in molecular biology⁴. The main assumptions consider phenotypes as subjects of natural selection, but only genetic changes are inherited. This strict division between genotype and phenotype led to the dismissal of the inheritance of acquired traits, first described in Weismann’s germ-plasm theory⁵, mainly known as the “Weismann barrier”. The barrier emphasizes

a strict one-way flow of information from genotype to phenotype, highlighting the germline as the major source of inheritance insulated from any somatic influence.

Nevertheless, accumulating evidence from diverse model organisms reveals numerous cases of nongenetic, non-DNA sequence-based inheritance, challenging the canonical view of a unidirectional relationship between genotype and phenotype.

1.2. From Plastic Responses to Heritable Outcomes: Dissecting Nongenetic Inheritance

Inheritance patterns manifest in diverse forms and mechanisms extend beyond DNA sequence changes, reflecting its biological complexity. Maternal inheritance, the most well-documented forms of nongenetic inheritance, occurs when a mother's environmental conditions directly influence the offspring's phenotype^{6–8}. Such effects arise as a result of cytoplasmic provisioning to the oocyte, but also because many species engage in parental care that can affect offspring development.

A distinction is further drawn between intergenerational and transgenerational types of inheritance⁹. Intergenerational inheritance refers to heritable effects that persist for two generations beyond the directly exposed parental generation, encompassing maternal effects but limited to the immediate descendants. In contrast, transgenerational inheritance applies to effects extending for at least three generations, reflecting a more persistent and autonomous form other than that caused by direct environmental exposure of the germline. Given that these effects arise directly as part of the offspring's environment, inheritance studies should therefore try to eliminate short-term parental influences to focus solely on inherited effects by rearing organisms in a standardized environment for multiple generations before experimentation.

1.3. Epigenetics- we are more than just the sum of our genes

The exploration of inheritance naturally intersects with the field of epigenetics- the study of heritable changes in gene expression without alterations in the underlying DNA sequence¹⁰. The actual term “epigenetics” was first conceptualized by Conrad Hal Waddington which strictly defines the intricate interplay between the genome with

its environment that shape developmental processes¹⁰. Since then, the concept of epigenetics has dramatically evolved, challenging the mainstream reductionist ideas of classical genetics and Darwinism/Neo-Darwinism^{11–13}.

Epigenetic mechanisms include diverse molecular processes, including regulatory RNAs, chromatin modifications, and self-perpetuating structural elements like prions that can influence gene expression and epigenetic states^{14–16}. However, environmentally triggered epigenetic modifications that are functionally analogous to genetic mutations can create phenotypic variation subject to natural selection. Nonetheless, skepticism still remains largely from our limited mechanistic understanding on how such epigenetic changes are maintained and transmitted over multiple generations¹⁷.

1.4. Major caveats in studying Transgenerational epigenetic inheritance (TEI)

Despite growing evidence that epigenetics states can be transmitted across generations, the study of TEI remains fraught accompanied with biological complexity and technical limitations. By definition, the term ‘epigenetic inheritance’ refers to any heritable characteristics transmitted through the germline that are independent of any changes in the DNA sequence. However, disentangling true TEI from parental or any transient environmental carryover requires consideration of several research caveats.

Notably, three key processes that are known barriers in TEI - the Weismann barrier, germline epigenetic reprogramming, and the recently described somatic epigenetic resetting¹⁸. One of the most prominent is the Weismann barrier which posits the irreversible transfer of information from somatic cells to the germline. According to this view, germ cells are devoid of influence from somatic cells therefore acquired traits cannot influence hereditary germline transmission. Although modern research from multiple systems has challenged the rigidity of this barrier, it remains a critical conceptual hurdle. A second challenge is the concept of germline epigenetic reprogramming- a fundamental principle of developmental biology even before the articulation of ‘epigenesis’ proposed by Aristotle in ‘*On the Generation of Animals*’^{19,20}. This process, essential for restoring zygote totipotency, removes epigenetic signatures that arise during development or induced by the environment. While this

reprogramming process ensures that the later embryonic development is fully dictated by the species-specific blueprint, incomplete or failed resetting can allow such epigenetic marks to escape and persist across generations. In mammals, two rounds of epigenetic reprogramming events take place in the early embryo and within the germline. This process effectively erases most epigenetic marks, whether programmed, accidental or environmentally induced, though rare exceptions have been reported ²¹. The third barrier in animals is the somatic epigenetic reprogramming, a process that acts as an additional safeguard to block inherited effects ¹⁸. Even if some epigenetic marks escape reprogramming in the germline or zygote, these marks may still be removed during the formation of somatic tissues, thereby limiting their phenotypic impact in descendants. Together, these processes illustrate why demonstrating TEI is inherently difficult.

While some robust examples exist in plants, nematodes, and fruit flies, evidence in mammals remains limited and controversial ²². Confounding factors which include species-specific differences in epigenetic machinery, variations in environmental exposures and the difficulty of eliminating short-term parental effects add further complexity. Moreover, the field has been hindered by the lack of reproducibility, standardized criteria, and methodologies for validating TEI events^{1,23,24}.

Nonetheless, in animals, specifically nematodes have been instrumental in showing non-DNA-based inheritance, from RNA interference-mediated heritable silencing^{25–27} to environmentally-induced^{28–30}, multigenerational phenotypic changes^{31–35}. These events provide compelling evidence that certain epigenetic states can bypass both the Weismann barrier and germline epigenetic reprogramming, establishing proof-of-principle that environmentally-induced epigenetic states can persist across multiple generations.

1.5. Beyond the human perspective: Well-characterized pathways of TEI across species

Nongenetic inheritance covers a much broader form of inheritance patterns including epigenetic inheritance that are not necessarily classified as “epigenetic”. Such nongenetic forms of inheritance include cytoplasmic, somatic, environmental, behavioral variation and parental effects on vitellogenin provisioning,

mitochondrial inheritance^{9,36}. Examples of inheritance beyond DNA sequence spans kingdoms. The most convincing demonstrations of DNA-independent inheritance often arise in systems where the genome can be experimentally controlled. This is specifically true for parthenogenetic, self-fertilizing (hermaphroditic), or isogenic organisms like the nematode models *Caenorhabditis elegans* or *Pristionchus pacificus*. Even so, alternative factors must be considered, including parental care³⁷, microbiome contributions, maternal milk in mammals³⁸, or even cultural transmission³⁹. Therefore, a critical distinction must be made between intergenerational inheritance, where parental environments directly affect offspring, and true transgenerational inheritance, in which effects persist into the F3 generation or beyond.

1.6. Molecular signals of TEI

Before addressing the mechanisms through which epigenetic information is transmitted, it is necessary to first define the molecular signals that constitute this information. I will introduce these *signals* separately for clarity; however, they rarely act in isolation. It is important to note that these molecular signals often function in concert with each other within the context of the cell and environmental constraints.

1.6.1. DNA methylation

DNA methylation is the best studied epigenetic modification that allows many rapid local adaptations to new environments^{14,40}. This process occurs in several forms in most organisms but mainly through the covalent addition of a methyl group on the cytosines of CpG dinucleotides. While such modifications are present in invertebrates and some fungal species, DNA methylation has its most notable role in transgenerationally inherited phenotypes among plants and vertebrates^{41,42}. In plants, the altered floral morphology of naturally occurring *Linaria vulgaris* mutant, described by Linneaus over 250 years ago, was later attributed to methylation of the *Lcyc* gene instead of the DNA sequence itself⁴³. Similarly, coat color in mice and metabolic outcomes vary due to DNA methylation states caused by the insertion of a transposable element at the *Agouti viable yellow (Avy)* locus⁴⁴.

In eukaryotes, DNA methylation occurs in two main forms: 5-methylcytosine (5mC) and N6-methyladenine (6mA). On the other hand, the nematode *C. elegans* lacks 5

mC since it has no homologs of any DNA methyltransferases (DNMT)⁴⁵. The overall presence of DNA modification in nematodes has been established in phylogenetically basal genera (Clade 1), but still remains controversial^{46,47}. It is important to note that any reports about the presence of 6mA DNA methylation in some higher eukaryotes including nematodes have been questioned^{48–51}, suggesting that several confounding factors of 6mA detection might be due to several inconsistencies involving pitfalls in mapping methods, antibody-biases and bacterial contamination^{52–55}.

1.6.2. Histone modifications

Histone proteins form the core of nucleosomes, the fundamental units of chromatin around which DNA is wrapped. These histones are subject to a variety of chemical modifications, including methylation, acetylation, phosphorylation, and ubiquitination, each of which can influence chromatin structure and gene accessibility. Beyond their central role in regulating gene expression, many histone modifications are well-suited to TEI events, as certain marks can self-propagate and spread across chromatin domains through the coordinated action of the ‘reader’, ‘writer’ and ‘eraser’ proteins^{16,56,57}.

1.6.3. Non-coding RNAs

Although the definition of “epigenetics” is deliberately broad, it is still largely associated with chromatin modifications. Yet the example of non-coding RNAs (like small RNAs)- which can mediate gene silencing both transcriptionally and post-transcriptionally- demonstrates that chromatin changes alone are not the only factors involved in epigenetic inheritance. Several studies in *C. elegans* established a paradigm in which small RNAs act as heritable carriers. The discovery of RNA interference revealed that double-stranded RNA can induce gene-specific silencing across multiple generations through stable small interfering RNAs (siRNAs)^{25–27}.

Beyond RNAi, nematodes have provided striking examples of environmentally induced inheritance. For instance, environmental perturbations such as starvation, viral infection or temperature fluctuations were shown to generate stable heritable responses mediated in part by sRNAs and chromatin modifications^{28–30}. Several other instances like stress resistance, fertility, developmental plasticity and metabolic regulation are supported by feedback loops that maintain sRNA population, histone

modification pathways and nuclear RNAi components which sustain heritable epigenetic changes^{31–35,58}.

1.7. Mechanisms of TEI

Having outlined the primary carriers of epigenetic information and their roles in regulating gene expression, I now turn to the processes that allows their transmission across generations, thereby establishing a transgenerational signal. It is important to note that the nature of these processes depends on the type of epigenetic signal involved which are often interconnected. Specifically, inheritance can occur through either “replicative” or “reconstructive” modes⁵⁹. In replicative transmission, epigenetic information is directly passed across generations via mitosis which then extends to meiotic signal transmission. These signals are maintained through gametogenesis and fertilization, surviving any potential reprogramming events⁵⁹.

However, information should not only be replicated but may also be reconstructed in each generation¹³. In contrast to being replicated, the reconstructive mode of inheritance refers to the erasure of epigenetic marks which are then re-established in the offspring via secondary cues¹³. These modes of transmission illustrate the dynamic and often cooperative nature of epigenetic inheritance, reflecting the complex interplay between molecular signals and developmental processes.

1.7.1. Templates for reconstructive mode of inheritance

1.7.1.1. Non-coding RNAs (ncRNAs)

ncRNAs are key candidates for secondary signals from which primary chromatin-based epigenetic information can be reconstructed¹⁷. In *C. elegans*, self-assembling germline condensates or germ granules play a critical role in RNA-directed epigenetic regulation and inheritance. Germ granules are essential for sRNA inheritance, as their disruption leads to aberrant siRNA expression and progressive silencing of key germline genes across generations⁶⁰. Similar germline structures, such as the piRNA-associated “nuage,” are found in other species including *Drosophila melanogaster*, *Xenopus laevis*, and mice⁶¹, although, whether their role in ncRNA-mediated inheritance is conserved remains to be determined.

1.7.1.2. Biophysical properties

Leading hypothesis on how environmental signals might be translated into epigenetic changes include changes in the biophysical properties of biomolecules within epigenetic pathways^{62,63}. Germ granules represent key factors for translation of heritable epigenetic changes, as their formation via phase separation makes their organization highly sensitive to environmental conditions (pH, temperature, etc.)^{62,63}. In *C. elegans*, the notable role of germ granules in TEI has been suggested through which environmental conditions are translated to heritable outcomes via RNAi intermediates⁶⁰. Moreover, similar temperature-dependent effects on Polycomb-mediated silencing in *Drosophila* highlight the role of biophysical properties of molecules in TEI⁶⁴.

1.7.1.3. 3D genome organization

Studies have shown the critical role of nuclear genome organization in gene regulation, cell identity and development⁶⁵. This complex chromatin landscape is shaped by both genetic and epigenetic elements that may contribute to TEI. Notably, the nuclear positioning of genes influences their activity: loci at the periphery tend to be heterochromatic and silenced, while those in the interior are generally euchromatic and transcriptionally active⁶⁶. Examples found in flies where Polycomb-dependent TEI of the Fab2L epiallele depends on its physical association with another Polycomb-targeted locus⁶⁴. Deletion of Fab7 resets Fab2L chromatin, implying that spatial clustering in the nucleus provides a secondary signal for reconstructing histone 3 lysine 27 tri-methyl (H3K27me3) marks⁶⁴. Also in *Saccharomyces*, maintenance of H3K9me marks requires nuclear positioning⁶⁷.

1.7.1.4. Transcription factor binding activity

The binding of transcription factors (TFs) to their respective target loci is influenced by several genetic and epigenetic factors⁶⁸. Specifically, DNA regions with low cytosine methylation have been correlated with TF binding activity during epigenetic reprogramming events⁶⁹. This suggest that TFs can act as carriers of epigenetic information by protecting their target sites from methylation events in subsequent generations. An example through which TEI-mediated effects induced by prenatal bisphenol A (BPA) exposure in mice alters chromatin accessibility and enhances TF binding activity in the obesity-associated *Fto* locus. Both the enhancer activity and the obesity phenotype persist for 5-6 generations⁷⁰. This example highlights how multiple

factors including biophysical properties, 3D genome organization and TFs can modulate a single case of TEI.

1.7.2. Para-mutational events

All of the mechanisms described above show the vertical transmission of epigenetic states across generations, akin to the Mendelian inheritance of genetic mutations. It is important to note that the possibility of non-Mendelian inheritance in TEI also exists. In some cases, epigenetic transmission is observed through the process known as paramutation. Paramutation is a well-established, non-Mendelian epigenetic phenomenon in which one allele heritably alters the expression state of a homologous allele without changes to the DNA sequence. First described in plants⁷¹, paramutation has since been reported in *C. elegans*⁷², *D. melanogaster*⁷³ and more controversially in mice⁷⁴, all of which involve the inter-allelic transfer of DNA methylation and histone modifications.

1.8. Plasticity in Evolutionary Theory

Understanding the mechanisms of TEI raises important questions about how environmentally induced phenotypes can persist across generations. This links directly to the concept of phenotypic plasticity, where a single genotype produces multiple phenotypes in response to distinct environmental conditions^{75–80}. This phenomenon further adds complexity to the traditional Darwinian-Lamarckian dichotomy.

The concept of plasticity has been recognized for more than a century and encompasses any type of environmentally-induced phenotypic variation that can affect different aspects of an organism's phenotype including its morphology, physiology, behavior and life's history^{75,81}. Since all organisms are shaped by both genetic and environmental factors, plasticity therefore represents a universal feature of life observed across all domains of biology. Its pervasive nature has been demonstrated through several examples^{82–91}, leading to a growing recognition that stands in contrast to the long neo-Darwinian phase that downplayed the role of environmental responsiveness in evolution^{92–94}.

Despite its recognized importance, the role of plasticity in evolutionary novelty remains a highly debated topic. Predictions have been outlined in a four-step model to underpin the role of plasticity in evolutionary novelty ⁹⁵. First, novel traits originate from environmentally responsive and developmentally plastic traits. Second, such responsiveness involves ‘switch’ genes. Third, plasticity occurs in restricted evolutionary pulses. Lastly, plasticity becomes genetically encoded through genetic accommodation and assimilation ⁹⁵. Recent molecular advancements in this field have shed light on mechanisms that allows survival of organisms in fluctuating conditions. Yet, studying phenotypic plasticity remains challenging. Particularly, identifying the molecular pathways involved, assessing to what extent these plastic responses are adaptive and determining how it influences evolutionary trajectories are all areas of active research ⁹⁵. In fact, some plastic responses can be remarkably stable and even heritable across generations ⁹⁶.

For much of evolutionary biology’s history, phenotypic plasticity has been overlooked and undervalued as a driver of evolutionary change ⁷⁷. This dissertation advances our current understanding on the pivotal role by which plasticity fosters novelty of traits. In brief, I used long-term experimental evolution involving a change in dietary exposure of nematodes. This thesis specifically addresses a major gap in plasticity research by focusing on diet-induced, transgenerational inheritance of plastic responses and its underlying molecular mechanisms. Importantly, the observed canalization of traits after TEI provides a crucial step toward understanding how environmentally induced trait transitions to genetically encoded states.

1.9. *Pristionchus pacificus* as a model system in plasticity research

The nematode *Pristionchus pacificus* has been developed across diverse research disciplines, including developmental and population genetics, evolutionary and behavioral ecology, and the neuroscience of predation. *Pristionchus* was initially described in 1996 from a soil sample collected in Pasadena ⁹⁷. Early research into the ecological niche of *Pristionchus* nematodes showed that, despite their global presence in soil habitats, these species consistently exhibit specific association with scarab beetles ⁹⁸. This beetle-nematode association was first identified in Europe and later led to the identification of the Oriental beetle as a prevalent host of *P. pacificus* across

Japan and North America ^{99,100}. More than 50 *Pristionchus* species have been established as living cultures and ongoing sampling efforts have yielded over two thousand natural isolates, hundreds of which now have available whole genome resequencing data, which are largely publically available at <http://www.pristionchus.org/cgi-bin/news.cgi>.

The past decade has seen the rise of *Pristionchus pacificus* mouth-form plasticity in developmental plasticity research ⁹⁵. This nematode offers several advantages that make it a highly tractable model system for experimental evolution and mechanistic studies. Like *C. elegans*, *P. pacificus* has a short generation time that allows rapid propagation across multiple generations and the establishment of several independent parallel lines. *P. pacificus* grows readily under standard laboratory conditions, using *E. coli* OP50 as food source on conventional agar plates. At 20°C, *P. pacificus* completes its life cycle in roughly four days and typically produces about 150-180 self-progenies. It completes the J1 larval stage in the egg, undergoing J2 molt before hatching and consequently begins feeding. Major techniques such as forward genetics, DNA-mediated transformation and several reversal genetic approaches like CRISPR/Cas9 were implemented ^{101–104}. Genome sequencing and resequencing efforts yielded in a rich genomic framework for *P. pacificus*, which range from chromosome-scale assembly ^{105,106}, complemented with RNA-seq-based transcriptomics ¹⁰⁷ and epigenetic datasets ^{108,109}.

Pristionchus nematodes have evolved teeth-like denticles, a major morphological novelty that enables omnivorous feeding and nematode predation ¹¹⁰. In its post-embryonic development, the plasticity of the *P. pacificus* mouth (stoma) develops into two alternative mouth forms in response to environmental conditions: the stenostomatous (St) morph with a single dorsal tooth, specialized for bacterial feeding morph, and the eury stomastous (Eu) morph, a facultative predator with a claw-like dorsal tooth and opposing sub-ventral tooth. Comparative analyses of the feeding structures have elucidated the cellular mechanisms underlying the development of teeth and its complex buccal cavity ¹¹¹. Over the past two decades, research has highlighted developmental plasticity as a major driver of evolutionary novelty. Consistently, in Diplogastridae nematodes, the evolution of teeth coincided with the emergence of polyphenism, illustrating compelling evidence of plasticity-first evolution¹¹². Its mouth-form decision has major behavioral and ecological

consequences which best represents a bi-stable developmental switch, where environmental inputs bias development toward one or two distinct morphs ¹¹³. This type of phenotypic plasticity is distinct from deterministic cases such as in butterfly wing polyphenism or plant leaf morphology ^{114–116}. *P. pacificus* has a well-established gene regulatory network of polyphenism decision, which comprises both deeply conserved and rapidly evolving genes, that governs strain-specific mouth-form ratios. Environmental factors including culture conditions ¹¹⁷, temperature ¹¹⁸, and small-molecule metabolites ¹¹⁹ further influence the Eu-St ratio in isogenic *P. pacificus* strains. For instance, the *P. pacificus* wild-type reference strain PS312 forms Eu morphs in solid agar culture conditions, but becomes St in liquid cultures. This type of feature indicates that *P. pacificus* mouth dimorphism operates as a bi-stable switch, analogous to bacteriophage lambda ¹²⁰. The stable mouth-form observed on agar plates allows unbiased screening for mutants. On the other hand, the RSC011 isolate studied in the work to follow, preferentially forms St morphs on standard *E. coli* OP50 diet and becomes predominantly Eu on certain diets.

P. pacificus offers the ease of obtaining isogenic lines through hermaphroditism (self-fertilization), making it a tractable system for studying molecular mechanisms of developmental plasticity. This mode of reproduction ensures precise genetic control and reproducibility while still permitting outcrossing through mating with males when desired. Unlike obligately outcrossing organisms, in which genetic variability can obscure the identification of loci underlying plastic trait formation, the hermaphroditic reproduction of *P. pacificus* yields genetically identical organisms, thereby eliminating genetic polymorphisms. Notably, studies of nematode evolution revealed that *Pristionchus* species have independently evolved hermaphroditism at least seven times, offering unique insights into genome evolution, karyotype diversification and shifts in sex determination mechanisms ^{107,121,122}.

In natural settings, dauer larvae of *P. pacificus* and other nematodes inhabit adult beetles. Upon the natural death of the beetle in the soil, dauers resume development into adults, giving rise to a short-lived community on decomposing insect carcass, sustained by bacteria and other microbes. As resources are exhausted, newly formed dauers disperse in search of hosts. Within this ephemeral ecological setting, mouth-

form polyphenism, which enables facultative predation, is predicted to stringly influence these competitive dynamics ¹²³.

Recent studies have further shown that dietary changes can strongly influence mouth-form development, making *P. pacificus* a feasible system for easy manipulation under laboratory conditions ¹²⁴. Using the natural binary read-out of environmental influence that exists in *P. pacificus*, long-term environmental induction (LTEI) was performed to systematically track the dynamic mouth-form changes upon novel dietary exposure. Utilizing the previously identified *Novosphingobium* L76 isolate from figs that naturally co-occurs with *Pristionchus* species and is known to affect various predatory-related traits ^{125,126}, I investigated the bacterial influence on its polyphenic response. LTEI approach in *P. pacificus*, as a practically and conceptually simple form of directed evolution, was performed for 101 generations. By integrating diet-switch experiments, it was possible to monitor “memories” of previous environmental encounters. LTEI experiments combined with forward genetic screens revealed not only a conventional paradigm of diet-induced plasticity but also novel mechanisms of transgenerational inheritance. LTEI was able to demonstrate that an environmental response is transmitted across generations and eventually improves descendant fitness that has an adaptive potential.

P. pacificus, with its tractable genetics and clearly defined plastic traits, offers an ideal system to dissect these forms of nongenetic inheritance, linking environmentally-induced plasticity to heritable and multigenerational outcomes. Here, I provide an integrated overview of the approaches I have applied, the key insights gained from my research to date. The work is organized around several major findings, each presented in separate chapters, addressing critical gaps in plasticity research: (1). Diet-induced plastic responses resulting in transgenerational effects mediated by a non-canonical mechanism involving destabilization of microRNAs, (2). identification of a bacterial trigger for transgenerational inheritance, linking vitamin B12 through methionine-dependent processes and vitellogenin provisioning from mothers to offspring, and (3) canalization of environmentally-induced traits. Together, these results illustrate how environmental cues, development, and epigenetic mechanisms interact to shape phenotypic evolution over multiple generations.

1.10. TEI in the light of evolution

The evolutionary significance of plasticity has been widely explored, however there are considerably fewer studies that examine the potential role of transgenerational inheritance in shaping adaptive trajectories^{77,127}. By bridging the gap between plastic responses and stable genetic change, TEI expands the classical gene-centered view of evolution, underscoring the dynamic and multifaceted nature of inheritance. Theoretical work on this topic has largely revolved on two central questions: first, whether TEI can enable populations to track rapidly changing environments when genetic adaptation alone may be too slow; and second, how natural selection acts not only on phenotypic outcomes but also on the mechanisms of TEI under fluctuating environmental conditions^{36,128}.

In broader terms, TEI can be adaptive with the possibility of responding to environmental change, posing major implications on heredity and evolution. It appears to be that evolution has its way to effectively reverse any deleterious effects imposed on the parental generation's lifetime experience. In plants, nematodes and fruit flies, TEI has been proposed to facilitate rapid adaptation to fluctuating environments²². More broadly, the transmission of epigenetic information across generations can result in canalization of the offspring development. This allows fast-reproducing organisms to adapt to rapidly changing environmental conditions. However, in cases where the "predicted" environment fails to align to actual conditions, such inherited epigenetic effects may result in maladaptation.

2. RESEARCH OBJECTIVES

Borrowing the concept of experimental evolution to investigate the consequences of dietary change^{129,130}, I combined long-term dietary induction with food-reversal experiments across more than 100 generations of isogenic lines. My work started with an open-ended, completely exploratory study of experimental evolution. The overarching aim of my thesis was to elucidate the mechanisms by which environmental factors induced phenotypic plasticity and transgenerational epigenetic inheritance (TEI) in *Pristionchus pacificus*. Specifically, I aimed to:

- I conducted long-term experimental evolution with 110 clonal *P. pacificus* RSC011 lines on *Novosphingobium* for 101 generations, tracking mouth-form plasticity across generations.
- I performed dietary reversal experiments by transferring RSC011 worms back to *E. coli* OP50 after 15-95 generations on *Novosphingobium* and monitored mouth-form phenotypes to assess reversibility.
- I combined long-term induction with EMS mutagenesis, screened ~3000 F2 lines for transgenerational inheritance-defective (Tid) phenotypes with reduced Eu mouth-form, and isolated 75 candidate lines for further study.
- I generated mutations in candidate genes identified from the EMS screen and produced large deletions in the miR-2235a locus of *P. pacificus* RSC011 via CRISPR-Cas9.
- I identified dietary triggers, including bacterial vitamin B12, that induced transgenerational inheritance of the predatory morph and investigated the metabolic and maternal contributions, such as maternal vitellogenin provisioning.
- I uncovered novel mechanisms of EBAX-1-mediated target-directed microRNA degradation (TDMD) in *P. pacificus* by exploiting the binary mouth-form phenotype as a functional assay, combined with small RNA profiling, to identify in vivo TDMD triggers, reveal lineage-specific regulatory sites, and map miRNA-mRNA networks controlling cognate miRNA cluster dynamics.

- I evaluated the evolutionary significance of TEI of the predatory morph by showing that these inherited traits enhanced descendant fitness using lifetime reproductive success.
- I investigated how TEI responded to variable environmental conditions by performing Alternative Induction Experiments, comparing observed patterns to predictive models, and testing whether environmental trigger concentration influenced TEI induction and its onset.

To summarise, my aim was to leverage natural organismal readouts of plasticity through long-term environmental induction experiments. This approach provided a foundation for investigating molecular mechanisms and evolutionary principles of inheritance, culminating in the following papers and manuscripts.

3. RESULTS

3.1 Application of ALFA-Tagging in the Nematode Model Organisms *Caenorhabditis elegans* and *Pristionchus pacificus*

Catia Igreja, Tobias Loschko, Alejandra Schäfer, Radhika Sharma, **Shiela Pearl Quiobe**, Elbin Alosky, Hanh Witte, Ralf J Sommer

Cells 11(23), (2022). DOI: 10.3390/cells11233875

3.1.1 Synopsis

Protein detection, manipulation, and purification are central to modern life sciences research and rely heavily on epitope tagging strategies across diverse model organisms. While a wide range of tags has been developed, each comes with specific limitations depending on the experimental context and model system. The recently introduced ALFA-tag is a small, rationally designed epitope that enables sensitive and versatile protein detection through high-affinity camelid-derived nanobodies, supporting applications from advanced imaging to biochemical analyses. In this study, we adapt the ALFA-tag for use in the nematode model organisms *Caenorhabditis elegans* and *Pristionchus pacificus*. We demonstrate that CRISPR-mediated insertion of the 13-amino acid ALFA-tag sequence is efficient and well tolerated in both species. Using multiple examples, we show high-resolution visualization of protein expression, and we further validate the approach by successfully performing pulldown experiments with the GW182 ortholog *Ppa-AIN-1* in *P. pacificus*. Together, our results introduce the ALFA-tag as a robust and versatile epitope tag for nematode research, expanding the toolkit for in vivo and biochemical protein studies.

3.1.2 Own contribution

I designed the gRNA and a corresponding repair template to insert two copies of the ALFA-tag at the C-terminal end of *Ppa-AIN-1*, and tracked these animals for mouth-form phenotype for at least 4 to 5 generations by screening 200 animals (20 animals in 10 replicates) per generation. I estimate my contribution at 10%.

3.2 EBAX-1/ZSWIM8 destabilizes miRNAs, resulting in transgenerational inheritance of a predatory trait.

Shiela Pearl Quiobe, Ata Kalirad, Waltraud Röseler, Hanh Witte, Yinan Wang, Christian Rödelsperger, Ralf J Sommer

Science Advances 11(11): eadu0875, (2025). DOI: 10.1126/sciadv.adu0875

3.2.1 Synopsis

Phenotypic plasticity can be transmitted across generations, yet the molecular mechanisms underlying this transgenerational epigenetic inheritance (TEI) remain poorly understood. We utilized long-term dietary induction experiments involving different diets using *P. pacificus* nematodes that exhibit mouth-form plasticity. Propagation of 110 isogenic lines over 101 generations, together with food-reversal experiments, revealed that diet induces the predatory morph and that this state is subsequently memorized across generations. Establishment of this memory requires at least five consecutive generations of induction, a feature that is phenomenologically distinct from previously reported TEI cases. By combining dietary induction and mutagenesis, we identified EBAX-1 as a key regulator of TEI in the nematode *Pristionchus pacificus*. Although *Ppa-ebax-1* mutants responded to dietary cues, they failed to retain transgenerational memory. We further show that *Ppa*-EBAX-1 destabilizes the miR-2235/miR-35 microRNA family. Consistently, deletion of a cluster containing 44 identical copies of miR-2235 led to precocious onset and prolonged TEI of the predatory morph. These findings implicate ubiquitin-mediated regulation, likely through microRNA turnover, as a critical mechanism linking environmental signals to heritable phenotypic change.

3.2.2 Own contribution

I carried out all experiments including long-term environmental induction with *P. pacificus*, maintaining 110 clonal lines on a *Novosphingobium* diet for 101 generations. I was involved in writing drafts of the manuscript, from the initial version to its final submitted form, integrating data, analyses, and interpretations throughout the process. I estimate my overall contribution at 85%.

3.3 Vitamin B12 induces memory of predation through vitellogenin provisioning

Shiela Pearl Quiobe, Ata Kalirad, Raphaela Zurheide, Hanh Witte, Christian Rödelsperger, Ralf J. Sommer

Nature Communications 17:3408, (2026). DOI: 10.1038/s41467-026-71494-w

3.3.1 Synopsis

Dietary influences can strongly shape animal traits, and in some cases these effects persist across generations, as seen in humans and other animals. While such environmental inheritance is well documented, the molecular signals responsible remain poorly understood. In *Pristionchus pacificus*, a nematode with diet-dependent mouth-form plasticity, a multigenerational diet of *Novosphingobium* bacteria induces a predatory morph that is inherited by subsequent generations. We show that bacterial vitamin B12 is both necessary and sufficient for transgenerational memory of the predatory morph. *Novosphingobium* mutants defective in vitamin B12 production fail to induce inheritance, while vitamin B12 supplementation rescues the phenotype. Notably, different levels of vitamin B12 are required for the initial induction versus the maintenance of the inherited predatory morph. Genetic analysis reveals that vitamin B12 acts through methionine synthase and methionine metabolism, independently of vitamin B9. The inherited response is associated with increased multigenerational vitellogenin expression, indicating enhanced maternal nutrient provisioning. Consistently, mutants lacking the vitellogenin receptor *Ppa-rme-2* fail to transmit the memory. Together, these findings show that vitamin B12 regulates maternal provisioning to control transgenerational changes in physiology and behavior.

3.3.2 Own contribution

I performed all experiments, generated and analyzed the data. I was involved in writing the manuscript from initial drafts to the final version. I estimate my overall contribution at 85%.

3.4 Lessons learned from manual curation of thousands of gene models in the nematode *Pristionchus pacificus*

Christian Rödelberger, Neha Agyal, **Shiela Pearl Quiobe**, Hao Wu, Waltraud Röseler, Dafne Ibarra, Ralf J. Sommer

Plos one, 21(3):e0344513, (2026). DOI: 10.1371/journal.pone.0344513

3.4.1 Synopsis

Despite major advances in sequencing technologies that allows chromosome-scale genome assemblies, genome annotation often remains complicated by the fact that numerous genes are unexpressed. When this occurs, transcriptome data alone is insufficient and must be supplemented with computational gene predictions and protein homology evidence. We conducted a case study involving community curation of the *Pristionchus pacificus* RSC011 strain. By combining Iso-Seq, RNA-Seq, and genome-wide screening, we identified and corrected over 7,500 (~24%) gene models. The curation revealed frequent annotation errors, including assembly mistakes, artificial transcript fusions from overlapping or polycistronic genes, falsely predicted open reading frames, and error propagation from homology-based predictions. The improved annotation enhances the RSC011 gene set and highlights broader challenges that may affect annotations in other species. These findings offer guidance for more accurate genome annotation across diverse taxa.

3.4.2 Own contribution

I manually curated approximately one fourth of the candidate genes, grown animals in specific diets for RNA-Seq and prepared cultures for Iso-seq processing. I estimate my contribution to be 15%.

3.5 Bacterial diet and genetic background influence mutation rate in *Pristionchus pacificus*

Shiela Pearl Quiobe*, Yinan Wang*, Penghieng Theam*, Waltraud Röseler, Hanh Witte, Christian Rödelsperger, Ralf J. Sommer

(published as Wang *et al.*)

G3 Genes|Genomes|Genetics: jkag038, (2026). DOI: 10.1093/g3journal/jkag038

3.5.1 Synopsis

Mutation drives evolution, and its accumulation is influenced by both genetic and environmental factors. While temperature effects on mutation rates especially in multicellular organisms are well studied, the impact of diet is less understood. We used mutation accumulation (MA) lines of unique natural isolates of *Pristionchus pacificus* and compared MA rates across different bacterial isolates naturally derived from *P. pacificus*-associated environments. Whole-genome sequencing revealed that the average single-nucleotide variant accumulation rates ranged from 0.46×10^{-9} and 2.27×10^{-9} nucleotide site⁻¹generation⁻¹, and insertion/deletion rates varied from 1.92×10^{-10} and 1.19×10^{-9} nucleotide site⁻¹generation⁻¹. We also found the accumulation rate difference to be a fivefold difference among genetic backgrounds and a twofold difference across diets. Despite these rate changes, mutation spectra remained largely consistent, highlighting that environmental and genetic factors can modulate MA rates without altering the overall mutational landscape.

3.5.2 Own contribution

I performed all mutation accumulation experiments with the *P. pacificus* RSC011 strain in distinct diets, and prepared animals for DNA extraction and WGS. I conducted the brood size assays between diets. I wrote corresponding parts of the manuscript. I estimate my contribution to be 20%.

3.6 The logic of transgenerational inheritance of cannibalism

Shiela Pearl Quiobe, Ata Kalirad, Ralf J. Sommer

Under Review in iScience

3.6.1 Synopsis

Epigenetic inheritance of environmentally induced traits is widespread across taxa, yet the dynamics of non-genetic inheritance under fluctuating environments remain poorly understood. We exploited the transgenerational epigenetic inheritance (TEI) of an environmentally induced cannibalistic trait in the nematode *Pristionchus pacificus* to address this gap. To explore how environmental variability affects the TEI of the predatory morph, we designed alternative induction experiments in which *P. pacificus* was exposed to *Novosphingobium* L76 or *E. coli* OP50 across multiple generations. We first developed theoretical models to predict TEI patterns under variable conditions and then implemented a two-pronged experimental strategy: (a) alternative induction experiments to systematically track TEI dynamics under fluctuating environments, and (b) superinduction experiments to test how the intensity of the environmental cue influences TEI. Together, our studies reveal how TEI responds to environmental variability and provide a mechanistic framework linking experimental observations to theoretical predictions, advancing our understanding of transgenerational mouth-form plasticity in *P. pacificus*.

3.6.2 Own contribution

I conceptualized, performed all experiments, analyzed the data and contributed to manuscript preparation. I estimate my contribution at 60%.

3.7 Darwinian games in a cannibalistic polyphenism and its transgenerational inheritance

Ata Kalirad, **Shiela Pearl Quiobe**, Ralf J. Sommer

Under Review in Nature Communications

3.7.1 Synopsis

Non-genetic inheritance of environmentally induced traits across generations is increasingly recognized in nature. However, its evolutionary significance remains debated. In particular, the adaptive value of transgenerational epigenetic inheritance (TEI), defined as effects that persist for three or more generations, is difficult to assess because its fitness consequences and associated trade-offs are rarely measurable. Although theory predicts that TEI is favored under relatively stable environmental conditions, its broader eco-evolutionary implications remain unclear. The nematode *Pristionchus pacificus* provides a powerful system to address these questions due to its environmentally regulated mouth-form polyphenism, in which individuals develop either a non-predatory or a predatory morph depending on environmental cues such as diet. Previous work demonstrated that bacterial diet can induce the predatory morph and that this effect can be transmitted across generations through TEI, mediated by specific molecular mechanisms including microRNA regulation and dietary vitamin B12. The ability to experimentally measure both the molecular underpinnings of this plastic trait and its associated fitness outcomes makes this system ideal for evaluating how TEI impacts adaptation. The present study integrates experimental and modeling approaches to examine how diet-induced TEI influences fitness and how environmental variability shapes the evolutionary dynamics of TEI strategies. Together, this work provides new insight into the ecological and evolutionary relevance of transgenerational inheritance.

3.7.2 Own contribution

I performed all experiments, analyzed parts of the data and contributed to final manuscript preparation. I estimate my contribution at 30%.

3.8 A phenotypically anchored map of endogenous triggers controlling miRNA destabilization in *Pristionchus pacificus*

Shiela Pearl Quiobe, Hanh Witte, Ralf J. Sommer

Ready for Submission

3.8.1 Synopsis

Target-directed miRNA degradation (TDMD) is a key mechanism regulating miRNA stability, yet most characterized triggers have been identified based on molecular signatures without clear links to whole-organism phenotypes. In this study, the binary mouth-form plasticity of the nematode *Pristionchus pacificus* is leveraged as a phenotypic readout to examine TDMD *in vivo*. The ubiquitin ligase *Ppa*-EBAX-1 is shown to control transgenerational inheritance of the predatory mouth morph by modulating the stability of the expanded *miR*-35/2235a cluster. Combining computational predictions, genetic perturbations, and small RNA profiling, endogenous transcripts with 3' UTR binding sites acting as TDMD triggers are identified. Disruption of these sites stabilizes the corresponding miRNAs and alters the dynamics of transgenerational inheritance of the predatory morph. Small RNA sequencing further reveals that loss of individual triggers leads to coordinated upregulation of multiple cluster members. While many predicted target genes are conserved across related species, the TDMD-relevant binding sites are often lineage-specific, indicating rapid evolutionary change in this regulatory layer. Overall, these results establish a direct connection between TDMD, miRNA cluster dynamics, and organismal phenotypes, and illustrate how phenotypically grounded approaches can uncover functional TDMD triggers that may be overlooked by traditional sequence- or biochemistry-based screens.

3.8.2 Own contribution

I conceptualized the experimental design, performed all experiments, generated and analyzed the data. I was involved in writing the manuscript from initial drafts to the final version. I estimate my overall contribution at 90%.

3.9 Genetic analysis of the Weismann Barrier in *Pristionchus pacificus*

Shiela Pearl Quiobe, Hanh Witte, Christian Rödelberger, Ralf J. Sommer

Ready for Submission

3.9.1 Synopsis

Transgenerational epigenetic inheritance (TEI) challenges the traditional Weismann barrier by demonstrating that environmentally induced states in somatic tissues can influence progeny phenotypes. In oviparous species, maternal provisioning via vitellogenins- a family of yolk proteins synthesized in the intestine and transported to oocytes- provides a direct route for soma-to-germline communication. Using *Pristionchus pacificus*, we show that diet-derived vitamin B12 induces the predatory mouth form and its transgenerational inheritance through elevated vitellogenin expression and RME-2-dependent transport. Deletion of multiple vitellogenin genes or disruption of RME-2 progressively reduces the initial morph induction and abolishes TEI, while vitamin B12 supplementation cannot rescue these defects. Maternal vitellogenin deposition likely delivers a combination of nutrients, lipids, vitamins, and small RNAs to embryos, initiating transgenerational signaling. These findings establish vitellogenins as key mediators of maternal-to-progeny communication and provide a mechanistic framework for how environmental cues can cross the soma-germline barrier to shape offspring physiology and behavior.

3.9.2 Own contribution

I conceptualized the experimental design, performed all experiments, generated and analyzed the data. I was involved in writing the manuscript from initial drafts to the final version. I estimate my overall contribution at 90%.

4. DISCUSSION

Environmental influences on trait inheritance have long been proposed but remain contentious due to challenges in distinguishing true transgenerational effects from transient or parental influences. While mammalian studies are limited by technical and ethical constraints, nematode models, particularly *C. elegans*, have shown that even brief environmental exposures can induce heritable changes^{9,131}, though the consequences of longer or multigenerational exposures remain largely unexplored. Fundamental insight could be obtained by integrating experiments that visualize plasticity in response to environmental stimuli with experimental evolution.

Here, I addressed these gaps by studying a discrete, environmentally controlled developmental decision of mouth-form plasticity in the nematode *Pristionchus pacificus* RSC011. Borrowing the concept of experimental evolution to investigate the consequences of dietary change¹²⁹, I combined long-term dietary induction with food-reversal experiments across more than 100 generations of isogenic lines. My work started with an open-ended, completely exploratory study of experimental evolution. Here, I define experimental evolution as the study of evolutionary changes happening in experimental populations as a consequence of these environmental changes. Specifically, unlike canonical experimental evolution experiments^{129,130}, standing genetic variation is trivial in this approach. This definition excludes artificial selection since no explicit choice among phenotypes is possible when the environment itself fully biases the selected trait.

My work highlights that propagation of 110 isogenic lines for 101 generations combined with food-reversal experiments, revealed i) dietary induction of the predatory morph, ii) subsequent TEI of this phenotype and iii) low-frequency canalization. TEI requires minimal exposure of five generations and is induced by diet-derived vitamin B12^{96,132}. Given that these observations are phenomenologically different from what occurs in *C. elegans*, I performed large-scale genetic screening and identified the role of EBAX-1/ZSWIM8. Specifically, *Ppa*-EBAX-1 destabilizes microRNAs of the *miR*-2235a family, which underwent a ~ 10 kb expansion in *P. pacificus* relative to its *C. elegans* homolog. I further showed the repressive role of the *miR*-2235a by generating

an entire deletion of the locus, resulting in precocious and extended TEI of the predatory morph.

Focusing on the aspects of the dietary induction and transgenerational inheritance, a major advance was identifying vitamin B12 as a key inducing agent ¹³². However, the current gap lies on how vitamin B12 would relate to the overall function of EBAX-1/ZSWIM8 activity. The necessity of multigenerational dietary exposure (of at least five generations) to induce TEI highlights the complexity of environmental cue integration. *Novosphingobium* exposure and vitamin B12- supplemented *E. coli* diets similarly induces the expression of *ebax-1*, the *miR-2235a* cluster and even other members of the *miR-35* family. For the time being, our current TEI model assumes that certain trigger/s from *Novosphingobium* induces EBAX-1 activity that will result in the upregulation of target gene/s in *P. pacificus* leading to the TEI of the Eu morph. Consistent with this model, the large or complete deletion of the *miR-2235a* locus results in precocious and extended TEI, while mutations in *Ppa-ebax-1* result in the absence of memory.

Dietary triggers are either endogenous (*Ppa*-sourced) or exogenous (dietary-sourced), and the identity of these triggers, and how vitamin B12 modulates EBAX-1-mediated miRNA destabilization remains the major gaps. One possibility is the progressive accumulation of triggers over generations may be crucial for target-directed miRNA degradation (TDMD), which is the increase in “trigger” expression across generations following exposure to *Novosphingobium*. This aligns with previous findings showing that accumulation of mRNA triggers to a certain threshold directs TDMD or TDMD-like processes leading to miRNA degradation ^{133–135}, mirroring the concentration-dependent effects of vitamin B12 to induce TEI of the Eu morph.

Motivated by this threshold-dependent mechanism, I performed a systematic *in-silico* search for potential TDMD triggers and miRNA targets. This analysis revealed a broad spectrum of RNAs with predicted miRNA-binding sites that could function either as triggers for miRNA degradation or as conventional targets for repression. Despite this extensive landscape, few of the predicted targets correspond to known components of the mouth-form gene regulatory network, suggesting that *miR-2235a* may act indirectly or more likely, through currently uncharacterized pathways. These findings highlight the complexity of miRNA-mediated regulation and underscore the need for

experimental validation. Importantly, exploration of this trigger space in *P. pacificus* demonstrated that mutations in candidate triggers produce pronounced phenotypic effects, providing new avenues for TDMD.

Building on this framework, my LTEI experiments in *P. pacificus* have opened new research directions spanning both immediate and long-term steps needed for a comprehensive understanding of transgenerational epigenetic inheritance. Immediate priorities include defining the trigger space by identifying dietary and maternal factors underlying TEI. A second focus is the unexplored interplay between vitamin B12 and vitellogenin (*vit*), particularly whether the observed transgenerational increase in *vit* expression corresponds to elevated protein levels in progeny. However, inability to directly quantify vitamin B12 and determine its precise levels in nematode tissues remains.

To address the concentration-dependent effects of B12 and its sufficiency to induce TEI, I implemented two experimental approaches. First, an alternative induction experiment that allowed a systematic exploration of TEI under variable environmental conditions. Second, a superinduction experiment which assessed how TEI responds to the intensity of the environmental cue. These experiments showed that the induction of the Eu morph and the patterns of TEI are not identical. While the induction of predatory mouth form is induced systemically and immediately, the onset of TEI depends both on the number of generations exposed to the environmental trigger and the concentration of that trigger.

TEI is widespread, yet its adaptive significance remains debated¹³⁶. Rather than persisting indefinitely, TEI often exhibits a tunable duration, suggesting evolutionary optimization of its persistence¹³⁷. Skepticism surrounding TEI largely arises from the frequent assumption of adaptiveness without sufficient experimental validation. Here, I investigated the strain-specific effects after the original dietary induction of the predatory mouth form and found both the Eu morph and progeny increase are transgenerationally inherited. The last two sets of experiments were tightly linked to modelling studies performed by Dr. Ata Kalirad in our lab.

More broadly, understanding the processes underlying variation in complex traits has long been a central challenge in evolutionary biology. The progress I presented here

lays foundation for investigating natural variation in TEI in *P. pacificus*. Apart from the central focus on the RSC011 strain, I found that the EBAX-1 sensitive *miR-2235a* cluster exhibits natural variation among *P. pacificus* wild isolates and is rapidly evolving within the genus *Pristionchus*. Indeed, some strains with expanded miRNA copies (with >10 kb of miRNA expansion) lacked TEI, highlighting its repressive role. Preliminary analyses suggest that the TEI trait is indeed complex, presumably controlled by multiple genetic loci. Although, the caveat of expanding and testing more strains and species still exists. Whether the miRNA cluster itself has evolved in the lines I experimentally evolved at generation 100 compared to the ancestral P0 remains ongoing.

The conceptual and experimental contributions of my work over the past five years has been substantial, highlighting several novel aspects of the experimental design. First, such a design leverages a natural organismal readout of plasticity that exists in *P. pacificus*. Second, the implementation of long-term environmental induction experiments has provided insights into developmental plasticity, the associated transgenerational inheritance and low-frequency canalization of traits. Finally, these features will set foundations in deciphering broader evolutionary questions, including potential roles of TEI and genetic assimilation in shaping trait evolution, offering a mechanistic link between environmental responsiveness and heritable phenotypic changes.

5. REFERENCES

1. Heard, E. & Martienssen, R. A. Transgenerational Epigenetic Inheritance : Myths and Mechanisms. *Cell* **157**, 95–109 (2014).
2. Corsi, P. & Mandelbaum, J. The age of Lamarck: evolutionary theories in France, 1790-1830. *J. Hist. Biol.* **23**, (1990).
3. Liu, Y. A new perspective on Darwin's Pangenesis. *Biological Reviews* vol. 83 141–149 Preprint at <https://doi.org/10.1111/j.1469-185X.2008.00036.x> (2008).
4. Watson, J. D. & Crick, F. H. C. Molecular structure of nucleic acids: A structure for deoxyribose nucleic acid. *Nature* **171**, 737–738 (1953).
5. Weismann, A. *The Germ-Plasm: A Theory of Heredity*. (Scribner's, 1893).
6. Mousseau, T. A. & Fox, C. W. The adaptive significance of maternal effects. **13**, 403–407 (1998).
7. Marshall, D. J. & Uller, T. When is a maternal effect adaptive? *Oikos* vol. 116 1957–1963 Preprint at <https://doi.org/10.1111/j.2007.0030-1299.16203.x> (2007).
8. Uller, T. Developmental plasticity and the evolution of parental effects. *Trends in Ecology and Evolution* vol. 23 432–438 Preprint at <https://doi.org/10.1016/j.tree.2008.04.005> (2008).
9. Baugh, L. R. & Day, T. Nongenetic inheritance and multigenerational plasticity in the nematode *C. elegans*. *Elife* **9**, 1–13 (2020).
10. Waddington, C. H. The epigenotype. *Endeavour* **1**, 18–20 (1942).
11. Haig, D. The (dual) origin of epigenetics. in *Cold Spring Harbor symposia on quantitative biology* vol. 69 67–70 (Cold Spring Harbor Laboratory Press, 2004).
12. Rando, O. J. & Verstrepen, K. J. Timescales of Genetic and Epigenetic Inheritance. *Cell* vol. 128 655–668 Preprint at <https://doi.org/10.1016/j.cell.2007.01.023> (2007).
13. Jablonka, E. Epigenetic inheritance and plasticity: The responsive germline. *Prog. Biophys. Mol. Biol.* **111**, 99–107 (2013).
14. Jaenisch, R. & Bird, A. Epigenetic regulation of gene expression: How the genome integrates intrinsic and environmental signals. *Nature Genetics* vol. 33 245–254 Preprint at <https://doi.org/10.1038/ng1089> (2003).
15. Harvey, Z. H., Chen, Y. & Jarosz, D. F. Protein-Based Inheritance: Epigenetics beyond the Chromosome. *Molecular Cell* vol. 69 195–202 Preprint at <https://doi.org/10.1016/j.molcel.2017.10.030> (2018).
16. Martire, S. & Banaszynski, L. A. The roles of histone variants in fine-tuning chromatin organization and function. *Nature Reviews Molecular Cell Biology* vol. 21 522–541 Preprint at <https://doi.org/10.1038/s41580-020-0262-8> (2020).

17. Fitz-James, M. H. & Cavalli, G. Molecular mechanisms of transgenerational epigenetic inheritance. *Nature Reviews Genetics* vol. 23 325–341 Preprint at <https://doi.org/10.1038/s41576-021-00438-5> (2022).
18. Ewe, C. K. & Rechavi, O. The third barrier to transgenerational inheritance in animals: somatic epigenetic resetting. *EMBO Rep.* **24**, (2023).
19. Harvey, W. *Exercitationes de Generatione Animalium*. (Typis Johannis Balthasar, Frankfurt, 1651).
20. Wolff, J. F. *Theoria Generationis*. (Sumptibus Academiae Imperialis, St. Petersburg, 1759).
21. Reik, E., Dean, W. & Walter, J. Epigenetic reprogramming in mammalian development. *Science* (1979). **293**, 1089–1093 (2001).
22. Horsthemke, B. A critical view on transgenerational epigenetic inheritance in humans. *Nature Communications* vol. 9 Preprint at <https://doi.org/10.1038/s41467-018-05445-5> (2018).
23. Gainey, D. P., Shubin, A. V & Hunter, C. P. Reported transgenerational responses to *Pseudomonas aeruginosa* in *Caenorhabditis elegans* are not robust. *Elife* **13**, (2025).
24. Kaletsky, R. *et al.* *Molecular Requirements for C. Elegans Transgenerational Epigenetic Inheritance of Pathogen Avoidance*.
25. Fire, A. *et al.* Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806–811 (1998).
26. Vastenhouw, N. L. *et al.* Long-term gene silencing by RNAi. *Nature* **442**, 882–822 (2006).
27. Alcazar, R. M., Lin, R. & Fire, A. Z. Transmission dynamics of heritable silencing induced by double-stranded RNA in *Caenorhabditis elegans*. *Genetics* **180**, 1275–1288 (2008).
28. Schott, D., Yanai, I. & Hunter, C. P. Natural RNA interference directs a heritable response to the environment. *Sci. Rep.* **4**, (2014).
29. Rechavi, O. *et al.* Starvation-induced transgenerational inheritance of small RNAs in *C. elegans*. *Cell* **158**, 277–287 (2014).
30. Jobson, M. A. *et al.* Transgenerational effects of early life starvation on growth, reproduction, and stress resistance in *Caenorhabditis elegans*. *Genetics* **201**, 201–212 (2015).
31. Ashe, A. *et al.* PiRNAs can trigger a multigenerational epigenetic memory in the germline of *C. elegans*. *Cell* **150**, 88–99 (2012).
32. Greer, E. L. *et al.* Transgenerational epigenetic inheritance of longevity in *Caenorhabditis elegans*. *Nature* **479**, 365–371 (2011).
33. Katz, D. J., Edwards, T. M., Reinke, V. & Kelly, W. G. A *C. elegans* LSD1 Demethylase Contributes to Germline Immortality by Reprogramming Epigenetic Memory. *Cell* **137**, 308–320 (2009).

34. Buckley, B. A. *et al.* A nuclear Argonaute promotes multigenerational epigenetic inheritance and germline immortality. *Nature* **489**, 447–451 (2012).
35. Luteijn, M. J. *et al.* Extremely stable Piwi-induced gene silencing in *Caenorhabditis elegans*. *EMBO Journal* **31**, 3422–3430 (2012).
36. Bonduriansky, R. & Day, T. Nongenetic inheritance and its evolutionary implications. *Annu. Rev. Ecol. Evol. Syst.* **40**, 103–125 (2009).
37. Champagne, F. A. & Curley, J. P. Epigenetic mechanisms mediating the long-term effects of maternal care on development. *Neuroscience and Biobehavioral Reviews* vol. 33 593–600 Preprint at <https://doi.org/10.1016/j.neubiorev.2007.10.009> (2009).
38. Newburg, D. S. & Walker, W. A. Protection of the neonate by the innate immune system of developing gut and of human milk. *Pediatric Research* vol. 61 2–8 Preprint at <https://doi.org/10.1203/01.pdr.0000250274.68571.18> (2007).
39. Fogarty, L. & Kandler, A. The fundamentals of cultural adaptation: implications for human adaptation. *Sci. Rep.* **10**, (2020).
40. Skinner, M. K., Guerrero-Bosagna, C. & Haque, M. M. Environmentally induced epigenetic transgenerational inheritance of sperm epimutations promote genetic mutations. *Epigenetics* **10**, 762–771 (2015).
41. Bogdanović, O. & Veenstra, G. J. C. DNA methylation and methyl-CpG binding proteins: Developmental requirements and function. *Chromosoma* vol. 118 549–565 Preprint at <https://doi.org/10.1007/s00412-009-0221-9> (2009).
42. Zhang, H., Lang, Z. & Zhu, J. K. Dynamics and function of DNA methylation in plants. *Nature Reviews Molecular Cell Biology* vol. 19 489–506 Preprint at <https://doi.org/10.1038/s41580-018-0016-z> (2018).
43. Cubas, Pi., Vincent, C. & Coen, E. An epigenetic mutation responsible for natural variation in floral symmetry. *Nature* **401**, 157–161 (1999).
44. Morgan, H. D., Sutherland, H. G. E., Martin, D. I. K. & Whitelaw, E. Epigenetic inheritance at the agouti locus in the mouse. *Nat. Genet.* **23(3)**, 314–318 (1999).
45. Rošić, S. *et al.* Evolutionary analysis indicates that DNA alkylation damage is a byproduct of cytosine DNA methyltransferase activity. *Nat. Genet.* **50**, 452–459 (2018).
46. Gao, F. *et al.* Differential DNA methylation in discrete developmental stages of the parasitic nematode *Trichinella spiralis*. *Genome Biol.* **13**, R100 (2012).
47. Schiffer, P. H. *et al.* *The Genome of Romanomermis Culicivora: Revealing Fundamental Changes in the Core Developmental Genetic Toolkit in Nematoda.* *BMC Genomics* vol. 14 <http://www.biomedcentral.com/1471-2164/14/923> (2013).
48. Zhang, G. *et al.* N6-methyladenine DNA modification in *Drosophila*. *Cell* **161**, 893–906 (2015).
49. Wu, T. P. *et al.* DNA methylation on N6-adenine in mammalian embryonic stem cells. *Nature* **532**, 329–333 (2016).

50. Greer, E. L. *et al.* DNA methylation on N6-adenine in *C. elegans*. *Cell* **161**, 868–878 (2015).
51. Hao, Z. *et al.* N6-Deoxyadenosine Methylation in Mammalian Mitochondrial DNA. *Mol. Cell* **78**, 382-395.e8 (2020).
52. Kong, Y., Mead, E. A. & Fang, G. Navigating the pitfalls of mapping DNA and RNA modifications. *Nature Reviews Genetics* vol. 24 363–381 Preprint at <https://doi.org/10.1038/s41576-022-00559-5> (2023).
53. O'Brown, Z. K. *et al.* Sources of artifact in measurements of 6mA and 4mC abundance in eukaryotic genomic DNA. *BMC Genomics* **20**, (2019).
54. Douvlataniotis, K., Bensberg, M., Lentini, A., Gylemo, B. & Nestor, C. E. *No Evidence for DNA N 6-Methyladenine in Mammals*. <https://www.science.org> (2020).
55. Musheev, M. U., Baumgärtner, A., Krebs, L. & Niehrs, C. The origin of genomic N 6-methyl-deoxyadenosine in mammalian cells. *Nat. Chem. Biol.* **16**, 630–634 (2020).
56. Allis, C. D. & Jenuwein, T. The molecular hallmarks of epigenetic control. *Nature Reviews Genetics* vol. 17 487–500 Preprint at <https://doi.org/10.1038/nrg.2016.59> (2016).
57. Allshire, R. C. & Madhani, H. D. Ten principles of heterochromatin formation and function. *Nature Reviews Molecular Cell Biology* vol. 19 229–244 Preprint at <https://doi.org/10.1038/nrm.2017.119> (2018).
58. Simon, M. *et al.* Reduced Insulin/IGF-1 Signaling Restores Germ Cell Immortality to *Caenorhabditis elegans* Piwi Mutants. *Cell Rep.* **7**, 762–773 (2014).
59. Miska, E. A. & Ferguson-Smith, A. C. Transgenerational inheritance: Models and mechanisms of non–DNA sequence–based inheritance. *Science* (1979). **354**, 54–63 (2016).
60. Lev, I. & Rechavi, O. Germ Granules Allow Transmission of Small RNA-Based Parental Responses in the “Germ Plasm”. *iScience* **23**, (2020).
61. Voronina, E., Seydoux, G., Sassone-Corsi, P. & Nagamori, I. RNA granules in germ cells. *Cold Spring Harbor Perspectives in Biology* vol. 3 Preprint at <https://doi.org/10.1101/cshperspect.a002774> (2011).
62. Dodson, A. E. & Kennedy, S. Phase Separation in Germ Cells and Development. *Developmental Cell* vol. 55 4–17 Preprint at <https://doi.org/10.1016/j.devcel.2020.09.004> (2020).
63. Banani, S. F., Lee, H. O., Hyman, A. A. & Rosen, M. K. Biomolecular condensates: Organizers of cellular biochemistry. *Nature Reviews Molecular Cell Biology* vol. 18 285–298 Preprint at <https://doi.org/10.1038/nrm.2017.7> (2017).
64. Ciabrelli, F. *et al.* Stable Polycomb-dependent transgenerational inheritance of chromatin states in *Drosophila*. *Nat. Genet.* **49**, 876–886 (2017).
65. Bonev, B. & Cavalli, G. Organization and function of the 3D genome. *Nature Reviews Genetics* vol. 17 661–678 Preprint at <https://doi.org/10.1038/nrg.2016.112> (2016).

66. van Steensel, B. & Belmont, A. S. Lamina-Associated Domains: Links with Chromosome Architecture, Heterochromatin, and Gene Repression. *Cell* vol. 169 780–791 Preprint at <https://doi.org/10.1016/j.cell.2017.04.022> (2017).
67. Holla, S. *et al.* Positioning Heterochromatin at the Nuclear Periphery Suppresses Histone Turnover to Promote Epigenetic Inheritance. *Cell* **180**, 150-164.e15 (2020).
68. Yin, Y. *et al.* Impact of cytosine methylation on DNA binding specificities of human transcription factors. *Science* (1979). **356**, (2017).
69. Kremisky, I. & Corces, V. G. Protection from DNA re-methylation by transcription factors in primordial germ cells and pre-implantation embryos can explain trans-generational epigenetic inheritance. *Genome Biol.* **21**, (2020).
70. Jung, Y. H. *et al.* Recruitment of CTCF to an Fto enhancer is responsible for transgenerational inheritance of BPA-induced obesity. *Proc. Natl. Acad. Sci. U. S. A.* **119**, (2022).
71. Pilu, R. Paramutation phenomena in plants. *Seminars in Cell and Developmental Biology* vol. 44 2–10 Preprint at <https://doi.org/10.1016/j.semcdb.2015.08.015> (2015).
72. Sapetschnig, A., Sarkies, P., Lehrbach, N. J. & Miska, E. A. Tertiary siRNAs Mediate Paramutation in *C. elegans*. *PLoS Genet.* **11**, (2015).
73. De Vanssay, A. *et al.* Paramutation in *Drosophila* linked to emergence of a piRNA-producing locus. *Nature* **490**, 112–115 (2012).
74. Yuan, S., Oliver, D., Schuster, A., Zheng, H. & Yan, W. Breeding scheme and maternal small RNAs affect the efficiency of transgenerational inheritance of a paramutation in mice. *Sci. Rep.* **5**, (2015).
75. Schlichting, C. D. & Pigliucci, M. *Phenotypic Evolution*. (Sunderland, MA, USA, Sinauer Associates, 1998).
76. Pigliucci, M. *Phenotypic Plasticity: Beyond Nature and Nurture*. (2001).
77. West-Eberhard, M. J. *Developmental Plasticity and Evolution*. (Oxford University Press, 2003).
78. deWitt, T. J. & Scheiner, S. M. *Phenotypic Plasticity: Functional and Conceptual Approaches*. (Oxford University Press, Oxford, UK, 2004).
79. Whitman, D. W. & Ananthakrishnan, T. N. *Phenotypic Plasticity of Insects: Mechanisms and Consequences*. (Science Publishers, Jersey, USA, 2009).
80. Moczek, A. P. *et al.* The role of developmental plasticity in evolutionary innovation. *Proceedings of the Royal Society B: Biological Sciences* 2705–2713 (2011) doi:10.1098/rspb.2011.0971.
81. Woltereck, R. Weitere experimentelle Untersuchungen über Artveränderung, speziell über das Wesen quantitativer Artunterschiede bei Daphniden. *Verhandlungen der Deutschen Zoologischen Gesellschaft* 110–172 (1909).
82. Abouheif, E. *et al.* Eco-evo-devo: the time has come. in *Ecological genomics: Ecology and the evolution of genes and genomes* 107–125 (2013).

83. Lande, R. Evolution of phenotypic plasticity and environmental tolerance of a labile quantitative character in a fluctuating environment. *J. Evol. Biol.* **27**, 866–875 (2014).
84. Laland, K. *et al.* Does evolutionary theory need a rethink? *Nature* **514**, 161–164 (2014).
85. Laland, K. N. *et al.* The extended evolutionary synthesis: Its structure, assumptions and predictions. *Proceedings of the Royal Society B: Biological Sciences* vol. 282 Preprint at <https://doi.org/10.1098/rspb.2015.1019> (2015).
86. Moczek, A. P. *et al.* The significance and scope of evolutionary developmental biology: A vision for the 21st century. *Evol. Dev.* **17**, 198–219 (2015).
87. Nijhout, H. F. To plasticity and back again. *Elife* **4**, (2015).
88. Sieriebriennikov, B. & Sommer, R. J. Developmental Plasticity and Robustness of a Nematode Mouth-Form Polyphenism. *Front. Genet.* **9**, 1–8 (2018).
89. Uller, T., Moczek, A. P., Watson, R. A., Brakefield, P. M. & Laland, K. N. Developmental bias and evolution: A regulatory network perspective. *Genetics* **209**, 949–966 (2018).
90. Lafuente, E. & Beldade, P. Genomics of Developmental Plasticity in Animals. *Front. Genet.* **Volume 10-2019**, (2019).
91. Levis, N. A. & Pfennig, D. W. Plasticity-led evolution: evaluating the key prediction of frequency-dependent adaptation. *Proceedings of the Royal Society B: Biological Sciences* **286**, 20182754 (2019).
92. Williams, G. C. Adaptation and natural selection. *Princeton, NJ* (1966).
93. Charlesworth, B., Lande, R. & Slatkin, M. A neo-Darwinian commentary on macroevolution. *Evolution (N Y)*. **36**, 474–498 (1982).
94. Wund, M. A. Assessing the Impacts of Phenotypic Plasticity on Evolution. *Integr. Comp. Biol.* **52**, 5–15 (2012).
95. Sommer, R. J. Phenotypic plasticity: From theory and genetics to current and future challenges. *Genetics* **215**, 1–13 (2020).
96. Quiobe, S. P. *et al.* EBAX-1/ZSWIM8 destabilizes miRNAs resulting in transgenerational memory of a predatory trait. *Sci. Adv.* **11**, 1–17 (2025).
97. Sommer, R. J., Carta, L., Kim, S.-Y. & Sternberg, P. W. Morphological, genetic and molecular description of *Pristionchus pacificus* sp. n. (Nematoda: Neodiplogastridae). *Fundam. apply. Nematol.* **19**, 511–521 (1996).
98. Kanzaki, N. *et al.* *Nine New Pristionchus (Nematoda: Diplogastridae) Species from China*. *Zootaxa* vol. 4943 (2021).
99. Herrmann, M., Mayer, W. E. & Sommer, R. J. Nematodes of the genus *Pristionchus* are closely associated with scarab beetles and the Colorado potato beetle in Western Europe. *Zoology* **109**, 96–108 (2006).

100. Herrmann, M. *et al.* The Nematode *Pristionchus pacificus* (Nematoda: Diplogastridae) Is Associated with the Oriental Beetle *Exomala orientalis* (Coleoptera: Scarabaeidae) in Japan. *Zoolog. Sci.* **24**, 883–889 (2007).
101. Schlager, B., Wang, X., Braach, G. & Sommer, R. J. Molecular cloning of a dominant roller mutant and establishment of DNA-mediated transformation in the nematode *Pristionchus pacificus*. *genesis* **47**, 300–304 (2009).
102. Witte, H. *et al.* Gene inactivation using the CRISPR/Cas9 system in the nematode *Pristionchus pacificus*. *Dev. Genes Evol.* **225**, 55–62 (2014).
103. Han, Z. *et al.* Improving transgenesis efficiency and CRISPR-associated tools through codon optimization and native intron addition in *pristionchus* nematodes. *Genetics* **216**, 947–956 (2020).
104. Nakayama, K. ichi, Ishita, Y., Chihara, T. & Okumura, M. Screening for CRISPR/Cas9-induced mutations using a co-injection marker in the nematode *Pristionchus pacificus*. *Dev. Genes Evol.* **230**, 257–264 (2020).
105. Rödelberger, C. *et al.* Single-Molecule Sequencing Reveals the Chromosome-Scale Genomic Architecture of the Nematode Model Organism *Pristionchus pacificus*. *Cell Rep.* **21**, 834–844 (2017).
106. Dieterich, C. *et al.* The *Pristionchus pacificus* genome provides a unique perspective on nematode lifestyle and parasitism. *Nat. Genet.* **40**, 1193–1198 (2008).
107. Rödelberger, C. *et al.* Phylotranscriptomics of *Pristionchus* Nematodes Reveals Parallel Gene Loss in Six Hermaphroditic Lineages. *Current Biology* **28**, 3123–3127.e5 (2018).
108. Werner, M. S. *et al.* Young genes have distinct gene structure, epigenetic profiles, and transcriptional regulation. *Genome Res.* **28**, 1675–1687 (2018).
109. Brown, A. L. *et al.* Characterization of the *Pristionchus pacificus* “ epigenetic toolkit ” reveals the evolutionary loss of the histone methyltransferase complex PRC2. *Genetics* **227**, 1–19 (2024).
110. Ragsdale, E. J., Müller, M. R., Rödelberger, C. & Sommer, R. J. A developmental switch coupled to the evolution of plasticity acts through a sulfatase. *Cell* **155**, 922–933 (2013).
111. Harry, C. J., Messar, S. M. & Ragsdale, E. J. Comparative reconstruction of the predatory feeding structures of the polyphenic nematode *Pristionchus pacificus*. *Evol. Dev.* **24**, 16–36 (2022).
112. Susoy, V. *et al.* Large-scale diversification without genetic isolation in nematode symbionts of figs. *Sci. Adv.* **2**, 1–11 (2016).
113. Renahan, T. & Sommer, R. J. Nematode Interactions on Beetle Hosts Indicate a Role of Mouth-Form Plasticity in Resource Competition. *Front. Ecol. Evol.* **9**, 1–9 (2021).
114. Brakefield, P. M. *et al.* Development, plasticity and evolution of butterfly eyespot patterns. *Nature* **384**, 236–242 (1996).

115. Brakefield, P. M. & Reitsma, N. Phenotypic plasticity, seasonal climate and the population biology of *Bicyclus* butterflies (Satyridae) in Malawi. *Ecol. Entomol.* **16**, 291–303 (1991).
116. Givnish, T. J. COMPARATIVE STUDIES OF LEAF FORM: ASSESSING THE RELATIVE ROLES OF SELECTIVE PRESSURES AND PHYLOGENETIC CONSTRAINTS. *New Phytologist* **106**, 131–160 (1987).
117. Werner, M. S. *et al.* Environmental influence on *Pristionchus pacificus* mouth form through different culture methods. *Sci. Rep.* **7**, 1–12 (2017).
118. Lenuzzi, M. *et al.* Influence of environmental temperature on mouth-form plasticity in *Pristionchus pacificus* acts through daf-11-dependent cGMP signaling. *J. Exp. Zool. B Mol. Dev. Evol.* **340**, 214–224 (2023).
119. Bose, N. *et al.* Complex small-molecule architectures regulate phenotypic plasticity in a nematode. *Angewandte Chemie - International Edition* **51**, 12438–12443 (2012).
120. Ptashne, M. Lambda's Switch: Lessons from a Module Swap. *Current Biology* **16**, R459–R462 (2006).
121. Yoshida, K. *et al.* Chromosome fusions repatterned recombination rate and facilitated reproductive isolation during *Pristionchus* nematode speciation. *Nat. Ecol. Evol.* **7**, 424–439 (2023).
122. Yoshida, K. *et al.* Rapid chromosome evolution and acquisition of thermosensitive stochastic sex determination in nematode androdioecious hermaphrodites. *Nat. Commun.* **15**, 9649 (2024).
123. Sommer, R. J. *Pristionchus* – Beetle associations : Towards a new natural history. *J. Invertebr. Pathol.* **209**, 108243 (2025).
124. Dardiry, M., Piskobulu, V., Kalirad, A. & Sommer, R. J. Experimental and theoretical support for costs of plasticity and phenotype in a nematode cannibalistic trait. *Evol. Lett.* **7**, 48–57 (2023).
125. Akduman, N., Rödelberger, C. & Sommer, R. J. Culture-based analysis of *Pristionchus*-associated microbiota from beetles and figs for studying nematode-bacterial interactions. *PLoS One* **13**, 1–13 (2018).
126. Akduman, N. *et al.* Bacterial vitamin B12 production enhances nematode predatory behavior. *ISME Journal* **14**, 1494–1507 (2020).
127. Lande, R. Adaptation to an extraordinary environment by evolution of phenotypic plasticity and genetic assimilation. *J. Evol. Biol.* **22**, 1435–1446 (2009).
128. Bonduriansky, R., Crean, A. J. & Day, T. The implications of nongenetic inheritance for evolution in changing environments. *Evol. Appl.* **5**, 192–201 (2012).
129. Lenski, R. E. Experimental evolution and the dynamics of adaptation and genome evolution in microbial populations. *ISME Journal* **11**, 2181–2194 (2017).

130. Lenski, R. E., Rose, M. R., Simpson, S. C. & Tadler, S. C. Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2,000 generations. *Am. Nat.* **138**, 1315–1341 (1991).
131. Filipowicz, A. & Allard, P. *Caenorhabditis Elegans* as a Model for Environmental Epigenetics. *Current Environmental Health Reports* vol. 12 Preprint at <https://doi.org/10.1007/s40572-025-00472-z> (2025).
132. Quiobe, S. P. *et al.* Diet-derived vitamin B12 induces transgenerational inheritance of nematode predation through elevated vitellogenin provisioning. *bioRxiv* 2025.05.21.655301 (2025).
133. Shi, C. Y. *et al.* The ZSWIM8 ubiquitin ligase mediates target-directed microRNA degradation. *Science* (1979). **370**, eabc9359 (2020).
134. de la Mata, M. *et al.* Potent degradation of neuronal miRNA s induced by highly complementary targets . *EMBO Rep.* **16**, 500–511 (2015).
135. Ghini, F. *et al.* Endogenous transcripts control miRNA levels and activity in mammalian cells by target-directed miRNA degradation. *Nat. Commun.* **9**, (2018).
136. Santilli, F. & Boskovic, A. Mechanisms of transgenerational epigenetic inheritance: lessons from animal model organisms. *Curr. Opin. Genet. Dev.* **79**, 1–8 (2023).
137. Houri-Ze'evi, L., Korem, Y., Sheftel, H., Degani, L. & Alon, U. A Tunable Mechanism Determines the Duration of the Transgenerational Small RNA Inheritance in *C. elegans*. *Cell* **165**, 88–99 (2016).

6. APPENDIX

**Application of ALFA-Tagging in the Nematode Model
Organisms *Caenorhabditis elegans* and *Pristionchus pacificus***

Catia Igreja, Tobias Loschko, Alejandra Schäfer, Radhika Sharma, **Shiela Pearl
Quiobe**, Elbin Alosky, Hanh Witte, Ralf J Sommer

Published in:

Cells 11(23), (2022). DOI: 10.3390/cells11233875

Article

Application of ALFA-Tagging in the Nematode Model Organisms *Caenorhabditis elegans* and *Pristionchus pacificus*

Catia Igreja , Tobias Loschko, Alejandra Schäfer, Radhika Sharma , Shiela Pearl Quiobe , Elbin Aloshy, Hanh Witte and Ralf J. Sommer * 

Department for Integrative Evolutionary Biology, Max Planck Institute for Biology Tübingen, Max Planck Ring 9, 72076 Tübingen, Germany

* Correspondence: ralf.sommer@tuebingen.mpg.de

Abstract: The detection, manipulation and purification of proteins is key in modern life sciences studies. To achieve this goal, a plethora of epitope tags have been employed in model organisms from bacteria to humans. Recently, the introduction of the rationally designed ALFA-tag resulted in a highly versatile tool with a very broad spectrum of potential applications. ALFA-tagged proteins can be detected by nanobodies, the single-domain antibodies of camelids, allowing for super-resolution microscopy and immunoprecipitation in biochemical applications. Here, we introduce ALFA-tagging into the two nematode model organisms *Caenorhabditis elegans* and *Pristionchus pacificus*. We show that the introduction of the DNA sequence, corresponding to the 13 amino acid sequence of the ALFA-tag, can easily be accommodated by CRISPR engineering. We provide examples of high-resolution protein expression in both nematodes. Finally, we use the GW182 ortholog *Ppa-ain-1* to show successful pulldowns in *P. pacificus*. Thus, the ALFA-tag represents a novel epitope tag for nematode research with a broad spectrum of applications.

Keywords: ALFA-tag; nematodes; *Caenorhabditis elegans*; *Pristionchus pacificus*; immunohistochemistry; immunoprecipitation



Citation: Igreja, C.; Loschko, T.; Schäfer, A.; Sharma, R.; Quiobe, S.P.; Aloshy, E.; Witte, H.; Sommer, R.J. Application of ALFA-Tagging in the Nematode Model Organisms *Caenorhabditis elegans* and *Pristionchus pacificus*. *Cells* **2022**, *11*, 3875. <https://doi.org/10.3390/cells11233875>

Academic Editor: Paola Fabrizio

Received: 15 October 2022

Accepted: 28 November 2022

Published: 1 December 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

The introduction of various epitope tags has helped to transform research in biotechnology, as well as many other areas of the modern life sciences [1,2]. Short peptide epitope tags, such as Hemagglutinin (HA), FLAG[®] and myc-tags, can be used to detect a protein of interest (POI) for which no antibody is available with minimal impact on protein function, and they also assist in (i) the purification of recombinant proteins in biotechnological applications, (ii) protein detection in cell and developmental biology and, finally, (iii) the immunoprecipitation of proteins—or macromolecular complexes—in biochemical studies with living cells or organisms. The recent implementation of ALFA-tag has resulted in a highly versatile tool for nanobody-based applications [3]. The ALFA-tag is a rationally designed 13 amino acid sequence that is hydrophilic and uncharged at physiological pH and devoid of residues that might react with fixatives. It forms stable α -helices that re-fold after chemical treatment and are compatible with protein function in living cells [3]. Importantly, an ALFA-tag can be added at any position in a POI. Given its sequence: Ser-Arg-Leu-Glu-Glu-Glu-Leu-Arg-Arg-Arg-Leu-Thr-Glu (SRLEEELRRRLTE), this tag is physically smaller than many others tags, and can be recognized by camelid single-domain nanobodies specifically targeting the ALFA peptide [3], further enhancing its performance in various applications.

Since its introduction in 2019, ALFA-tagging has been applied in various systems [3–5], but to the best of our knowledge not yet in nematodes. Nematodes represent one of the major animal phyla with an estimated species number of more than a million [6], with free-living, as well as animal and plant parasitic species [7]. *Caenorhabditis elegans* is an important

nematode model system in many areas of the life sciences [8]. Several key characteristics of *C. elegans* fostered its use as a model organism. For example, *C. elegans* completes its development in 3–5 days (20 °C), can be cultured indefinitely on *Escherichia coli* bacteria on agar plates and propagates as self-fertilizing hermaphrodites, resulting in isogenic cultures [9]. Work over the last few decades has provided a detailed insight into the genetic and molecular mechanisms, among others, of *C. elegans* development, neurobiology and aging [8].

The enhanced understanding of the various biological processes in *C. elegans* has initiated comparative approaches in closely and more distantly related nematode species [10]. One species that has emerged as a powerful model system is *Pristionchus pacificus* [11,12]. Indeed, *P. pacificus* shares many functional tools with *C. elegans* and is another self-fertilizing species, with the two species thought to have diverged around 200 million years ago [13]. The development of CRISPR-associated technologies has also allowed precise manipulations of the nematode genomes [14,15], rapidly enhancing the application of various epitope tags. While the introduction of large fragments of DNA into the genome of *C. elegans* is easily achieved, size restriction of CRISPR-mediated genome editing is still a limitation in *P. pacificus* research [16]. Therefore, versatile, but physically short tags will be a major asset for research into this nematode.

Here, we apply ALFA-tagging in the two nematode models *C. elegans* and *P. pacificus*. Introduction of the DNA sequence corresponding to ALFA-tag can easily be accommodated by CRISPR engineering and we provide several examples of high-resolution protein expression in both species. Finally, we use the GW182 ortholog *Ppa-ain-1* to show successful pulldowns in *P. pacificus*, indicating that ALFA-tagging represents a new nematode tagging method with a broad spectrum of applications.

2. Materials & Methods

2.1. Maintenance of Worm Cultures

Stock cultures of *C. elegans* N2 and *P. pacificus* PS312 or RSC011 were reared at room temperature (20 °C) on nematode growth medium (NGM) in 6 cm Petri dishes, as originally described [9,11]. *Escherichia coli* OP50 were used as a food source. Bacteria were grown overnight at 37 °C in LB medium, and 200 µL of the overnight culture was pipetted onto NGM agar plates and left for several days at room temperature to grow bacterial lawns. *P. pacificus* PS312 worms were also grown in liquid cultures, as previously described [17]. Briefly, eggs-J1 animals from three fully grown plates obtained after bleach synchronization were grown in 10 mL of S medium [18] in autoclaved Erlenmeyer flasks with a volume of 50 mL, and containing a pellet of 100 mL overnight culture of *E. coli* OP50 grown in LB to an optical density (OD₆₀₀) of 0.5. The liquid cultures were incubated at 22 °C with constant shaking (180 rpm) for 68 hrs and 72 hrs to obtain J4 and young adult worms, respectively.

2.2. Nomenclature of Tagged Genes and Proteins

We followed standard *C. elegans* and *P. pacificus* nomenclature rules. Tagged genes are referred to as i.e. *Cel-dlg-1(tu1782[dlg-1::2xALFA])*; tagged proteins are indicated as *Ppa-EUD-1(tu1729[Ppa-EUD-1::2xALFA])*.

2.3. CRISPR/Cas9 Mutagenesis

We followed a previously published protocol for *P. pacificus* with subsequently introduced modifications [15,19]. All target-specific CRISPR RNAs (crRNAs) were designed to target 20 bp upstream of the protospacer adjacent motifs (PAMs). Ten µL of the 100 µM stock of crRNA (CRISPR/Cas9 RNA; IDT) was combined with 10 µL of the 100 µM stock of tracrRNA (catalog# 1072534; IDT, USA), denatured at 95 °C for 5 min, and allowed to cool to room temperature and anneal. The hybridization product was combined with Cas9 protein (catalog# 1081058; IDT, USA) and incubated at room temperature for 5 min. The mix was diluted with Tris-EDTA buffer to a final concentration of 18.1 µM for the RNA hybrid and 2.5 µM for Cas9. For the induction of specific site-directed mutations

via CRISPR/Cas9, an ssDNA oligo template was included in the mix at a concentration of 4 μ M. The repair template (ssDNA donor) contained the desired modifications flanked by 35 nucleotide long homology arms at either side of the edited sequences. To avoid recutting of the edited allele, the PAM motif or the gRNA binding sequence included silent mutations. The ssDNA donor contained two copies of the sequence encoding the ALFA peptide codon optimized for *P. pacificus* and *C. elegans*. To minimize sequence identity, the two copies of the ALFA sequence in the ssDNA donor were designed with different codons for the same amino acid. The plasmid carrying the *Ppa-efl-3* promoter and the modified TurboRFP sequences [19] was used as co-injection marker. The sgRNAs, associated primers and repair templates for the specific targeted knock-ins utilized in this study can be found in Tables S1 and S2. The complete list of worm mutants generated in this study is present in Table 1.

Table 1. Organisms/strains used in this study.

Strain	Source	Modified Gene/ID	Comment
<i>Caenorhabditis elegans</i>			
N2 (wild type)	<i>C. elegans</i> Genetics Center	–	–
RS4102	Sommer Lab	<i>unc-10(tu1781[unc-10::2xALFA])</i>	C-terminally tagged <i>Ce</i> UNC-10
RS4103		<i>dlg-1(tu1782[dlg-1::2xALFA])</i>	C-terminally tagged <i>Ce</i> DLG-1
<i>Pristionchus pacificus</i>			
312 (wild type)	Sommer Lab	–	–
RSC011 (wild type)		–	–
RS4031		<i>eud-1(tu1729[eud-1::2xALFA])</i>	C-terminally tagged <i>Ppa</i> EUD-1
RS4068		<i>ain-1(tu1753[ain-1::2xALFA])</i>	C-terminally tagged <i>Ppa</i> AIN-1
RS4085		<i>sult-1(tu1760[sult-1::2xALFA])</i>	C-terminally tagged <i>Ppa</i> SULT-1

Injections were performed on a Zeiss Axiovert microscope (Zeiss, Germany) coupled to an Eppendorf TransferMan micromanipulator and an Eppendorf FemtoJet injector (Eppendorf AG., Hamburg, Germany). The microinjection mixture was injected in the rachis of the gonad of an approximately one day old N2, PS312 and RSC011 (in the case of *Ppa ain-1(tu1753[ain-1::2xALFA])* adult hermaphrodites ($n = 40$ – 50 *P. pacificus* worms; $n = 30$ *C. elegans* worms). Eggs laid by injected animals within a 12–16 h period post injection were recovered. After two days, the P0 plates containing the F1 (first generation) animals with fluorescent signals of co-injection marker were isolated, and 10–12 F1 progenies from each of the isolated P0 plates were singled out on individual plates. After the F1 animals had laid eggs, they were placed in 10 μ L of single worm lysis buffer (10 mM Tris-HCl at pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 0.45% NP-40, 0.45% Tween 20, 120 μ g/ml Proteinase K), incubated in a thermocycler at 65 °C for 1 h, followed by heat deactivation of the proteinase at 95 °C for 10 min. The resulting lysate was used as a template in subsequent PCR steps. The genotype of the F1 animals were subsequently analyzed via Sanger sequencing and any mutations were identified before re-isolation in homozygosis. In *P. pacificus*, the CRISPR/Cas9 mediated knock-in efficiency varied between 1–3%.

2.4. Mouth-Form Phenotyping in *P. pacificus*

P. pacificus mouth-form phenotyping was performed as previously described [20]. Briefly, 30 adult hermaphrodite worms were mounted onto slides with 5% Noble Agar pads with 0.3% NaN₃ added as an anesthetic and examined using DIC microscopy. Individual worms were distinguished by the presence (Eurystomatous, Eu) or absence (Stenostomatous, St) of a large right sub ventral tooth and a curved dorsal tooth. The number of

biological replicates (n) was ≥ 3 for all conditions, with each replicate comprising at least 30 animals. Error bars represent the standard deviation.

2.5. ALFA-Tag Immunostaining

Healthy (not starved) and fully grown worms were collected from 6 cm Petri dishes ($n = 10$) with M9 medium or from liquid cultures and filtered through a 5 μm Nylon net filter (Milipore Merck, Darmstadt, Germany), as previously described [17]. All *P. pacificus* developmental stages were retained by the 5 μm filter, while bacteria passed through. Worms were fixed with 4% para-formaldehyde, 1xPBS (500 μL) in 1.5 mL Eppendorf tubes overnight on a tube rotator. After fixation, the samples were washed three times with 0.5% Triton X-100, 1x PBS (washing solution). To chemically reduce the disulfide bonds between collagen fibers in the cuticle and to enhance the diffusion of the chemical reagents into the specimen, animals were incubated overnight in 500 μL of buffer containing 5% β -mercaptoethanol, 1% Triton X-100 and 0.1 M Tris-HCl pH 7.4, with gentle shaking (300 rpm). Following two washes with 1% Triton X-100, 0.1 M Tris-HCl pH 7.4 buffer and one wash with collagenase buffer (1 mM CaCl_2 , 1% Triton X-100, 0.1 M Tris pH 7.4), worms were digested in 200 μL of collagenase buffer with 200 units of collagenase type IV (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C for 2–3 hrs or until the thinning of the cuticle caused the worms to break at the vulva region. Afterwards, the samples were washed three times with the washing solution. Digested animals were then incubated for 1 h at RT in 100 μL of blocking solution (1% BSA, 0.5% Triton X-100, 1x PBS). After two washes in washing solution, animals were stained with the primary antibody (FluoTag-X2 anti-ALFA-AbberiorStar580, NanoTag Biotechnologies, Göttingen, Germany, 1:100) in 50 μL of blocking solution, overnight at 4 °C in a tube rotator. Worms were washed thrice with the washing solution, resuspended in VectaShield mounting medium (Vector Laboratories, USA) containing 1 $\mu\text{g/mL}$ 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes, Thermo Fisher Scientific) and gently mounted on freshly prepared 5% Noble agar pads. Images were acquired on a Leica TCS SP8 microscope and assembled on Fiji (Image J, NIH, Bethesda, MD, USA).

2.6. ALFA-Tag Immunoprecipitation

P. pacificus PS312 and ALFA-tagged AIN-1 (*Ppa-ain-1(tu1753[ain-1::2xALFA])*) worms were collected from 10 cm Petri dishes ($n = 100$) with M9 medium and filtered through a 5 μm Nylon net filter (Milipore Merck, Darmstadt, Germany), dropped into liquid nitrogen to produce frozen “worm popcorns/pellets”, and stored at -80 °C. The worm pellets were ground into a fine powder using a mortar and pestle pre-cooled with liquid nitrogen and the powder was then transferred to a Falcon tube with 10 mL of cold egg buffer (118 mM NaCl, 48 mM KCl, 2 mM CaCl_2 , 2 mM MgCl_2 , 25 mM HEPES, pH 7.3) [21]. This worm suspension was centrifuged at 2000 rpm, 4 °C for 2 min, and the resulting worm pellet was resuspended in 8 mL of lysis buffer [50 mM Tris-HCl pH7.5, 150 mM KCl, 5 mM MgCl_2 , 2 mM EDTA, 0.1% Triton X-100, 2 mM DTT, 10% glycerol, complete protease inhibitor (Roche, Switzerland), PMSF] after discarding the supernatant. The worm solution was homogenized with a Wheaton Dounce Dura-Grind tissue grinder (DWK Life Sciences, Mainz, Germany) (10 strokes) to break the cuticle. The lysate was cleared from full worms by centrifugation (100 g, 4 °C, 6 min); the supernatant was transferred to a fresh 15 mL Falcon tube and the remaining pellet was resuspended in 8 mL of lysis buffer and further ground on the Wheaton Dounce with 25 strokes. Following a short centrifugation to remove non-homogenized worms (100 g, 4 °C, 6 min), the supernatants (worm lysates) were combined into one tube and cleared with a high-speed centrifugation (19,000 g, 4 °C, 20 min). The clarified extract was transferred to a fresh tube and input samples were collected for western blotting. To reduce non-specific protein binding to the beads, we precleared the lysate with Protein G sepharose resin (75 μL of 50% slurry) for an hour at 4 °C in a tube rotator. After centrifugation at 1000 g for 2 min, the supernatant was transferred to a fresh tube containing 75 μL of previously equilibrated ALFA selector^{PE} resin (NanoTag

Technologies, Göttingen, Germany) and incubated for 1–2 hrs at 4 °C in a tube rotator. The beads were sedimented by centrifugation (1 min, 1000 g, 4 °C) and washed thrice with the lysis buffer. To elute the ALFA-tagged proteins, the beads were resuspended in 200 µL of lysis buffer, transferred to a Mini Spin column (NanoTag Biotechnologies, Göttingen, Germany) and centrifuged for 1 min at 1000 g. The flow-through was discarded and the beads were washed twice with 400 µL of lysis buffer without DTT, glycerol and protease inhibitor and once with TBS 1x. The Mini Spin column was capped with a bottom plug and transferred to a fresh Eppendorf tube. Elution of the ALFA protein was performed with five column bed volumes of elution buffer (400 µM of the ALFA peptide in 200 µL of lysis buffer) for 20 min at room temperature with subtle shaking. The Mini Spin column bottom plug was removed to collect the eluate by centrifugation (1 min, 1000 g, 4 °C). To achieve the highest yields, the elution step was repeated and the eluates were combined. An aliquot of the eluate was collected for analysis by Western blot. To maximize the elution efficiency, an additional elution step with a denaturing sample buffer (NuPAGE LDS sample buffer, Life Technologies, Carlsbad, CA, USA) can also be performed on the ALFA resin.

2.7. Western Blot

Western blot was performed using standard methods. Briefly, worms were collected from 6 cm Petri dishes ($n = 10$) with M9 medium and filtered through a 5 µm Nylon net filter (Milipore Merck, Darmstadt, Germany), as previously described [17]. The worm pellet was flash-frozen in liquid nitrogen and stored at −80 °C. To obtain worm extracts, frozen worm pellets were ground to a powder on a mortar and pestle pre-cooled with liquid nitrogen before the addition of 2X Laemmli Sample Buffer (100 mM Tris-HCl pH = 6.8, 4% SDS, 20% glycerol, 0.2 M DTT; 3x the volume of the worm pellet). Samples were stored at −20 °C prior to the analysis. Before SDS-PAGE, the samples were boiled for 5 min at 95 °C and vortexed to shear genomic DNA. Proteins were transferred from the SDS-PAGE gel onto a nitrocellulose membrane (Santa Cruz Biotechnology, Dallas, TX, USA) by tank transfer. Primary antibodies were incubated overnight at 4 °C, secondary antibodies for an hour at room temperature in blocking solution (5% milk, 0.3% Tween 20, PBS 1x). All Western blots were developed with Clarity Western ECL substrate solutions (Bio-Rad, USA). The antibodies used in this study are as follows: sdAB anti-ALFA-HRP (N1505-HRP, NanoTag Biotechnologies, Göttingen, Germany, 1:1000), mouse monoclonal anti-Tubulin (Sigma Aldrich, St. Louis, MI, USA, T6199, 1:500), rabbit anti-GRP78 (Thermo Fisher Scientific, USA, PA5-22967, 1:1000), sheep polyclonal anti-mouse IgG-HRP (GE Healthcare, Chicago, IL, USA, RPN4201, 1:5000) and anti-rabbit IgG-HRP (GE Healthcare, USA, NA934V, 1:5000).

2.8. Mass Spectrometry Measurements

For analysis of the *Ppa*-AIN-1::2xALFA interactome, following the in-gel protein digestion with trypsin of the two replicas of PS312 and AIN-1::2xALFA elutes, the peptides were separated and analyzed on a Proxeon Easy-nLC 1200 UHPLC (Thermo Fisher Scientific, USA) coupled to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific, USA). Eluted peptides were ionized on an electrospray ionization source. The MS data acquisition was conducted in the positive ion mode and data-dependent acquisition mode. The 12 most intense multiple-charged ions were selected for higher-energy collision dissociation (HCD) fragmentation. Raw data files were processed with the MaxQuant software suite v.1.5.2.28 [22,23]. Using the Andromeda search engine integrated in the software, the spectra were searched against the Uniprot *P. pacificus* complete proteome database and a database comprising the ALFA tag sequence and commonly observed contaminants. The processing of the raw data was performed with a setting of 1% for the FDR (False Discovery Rate).

3. Results and Discussion

3.1. CRISPR-Based ALFA Tagging in *C. Elegans* and *P. Pacificus*

We selected a total of five genes in *C. elegans* and *P. pacificus* to generate ALFA-tagged fusion proteins in both species (Table 1). For all genes, we added two copies of the ALFA-tag sequence, resulting in an addition of 78 nucleotides to the coding region of the gene of interest that translated into an extra 26 amino acids in the protein. Specifically, in *C. elegans* we chose *dlg-1* (*discs large-1*), a membrane-associated guanylate kinase-like protein that is localized to the adherens junctions of all epithelia [24,25], and *unc-10* which is expressed at synapse-dense regions, such as the nerve ring and the dorsal and ventral nerve cords [26]. *Cel-UNC-10* is the nematode homolog of the Rab3-interacting molecule (RIM)-binding protein and is involved in the presynaptic localization of the voltage-gated calcium channel (VGCC) UNC-2 [27]. The expression pattern of both of these proteins has been characterized in multiple publications via the use of monoclonal antibodies or GFP-transgenic animals [24–26,28]. We tagged both genes at the C-terminus, immediately before the stop codon (Table 1). In *P. pacificus*, we targeted two mouth-form regulatory genes, *Ppa-eud-1* and *Ppa-seud-1/sult-1*, encoding for proteins involved in sulfation processes [29–32], and *Ppa-ain-1*, the nematode homolog of the Argonaute interacting protein GW182, which is known to be broadly expressed [33,34]. Spatial expression profiling of the *Ppa-EUD-1* sulfatase and the sulfotransferase *Ppa-SEUD-1/SULT-1* has been determined using fluorescent transcriptional reporters or fluorescent in situ hybridization (FISH) [29–31,35–37]. The three *P. pacificus* proteins were also C-terminally tagged (Table 1).

CRISPR-engineering worked efficiently for all five targeted genes with all the resulting lines being viable. All the data shown in this manuscript was obtained with CRISPR-engineered worm strains that resembled the parental wild-type strains, i.e., the worms containing ALFA-tagged proteins were without any visible abnormal phenotype. As changes in the function of the sulfation-related enzymes affect mouth-form ratios in *P. pacificus* [29,31], we determined the Eu/St mouth-form ratios of the *Ppa-eud-1*(*tu1729*[*eud-1::2xALFA*]) and *Ppa-sult-1*(*tu1760*[*sult-1::2xALFA*]) strains, when grown on solid agar plates and liquid cultures, respectively. Similar to the laboratory California strain (PS312) of *P. pacificus*, *Ppa-eud-1*(*tu1729*[*eud-1::2xALFA*]) adult worms were mainly Eu ($97.7 \pm 1.7\%$ Eu; Figure 1A) when grown on solid agar. In contrast, PS312 and the *Ppa-sult-1*(*tu1760*[*sult-1::2xALFA*]) worms developed largely into St morphs in liquid culture (only $6.7 \pm 5.8\%$ Eu; Figure 1B), as previously observed [17]. Thus, CRISPR-based ALFA tagging in *C. elegans* and *P. pacificus* did not interfere with protein function.

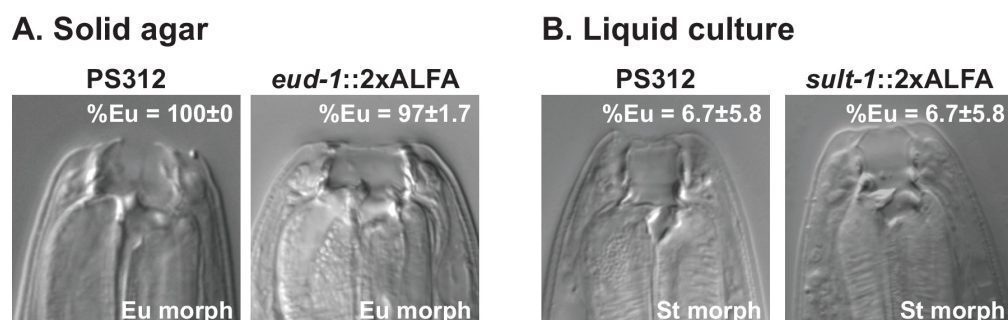
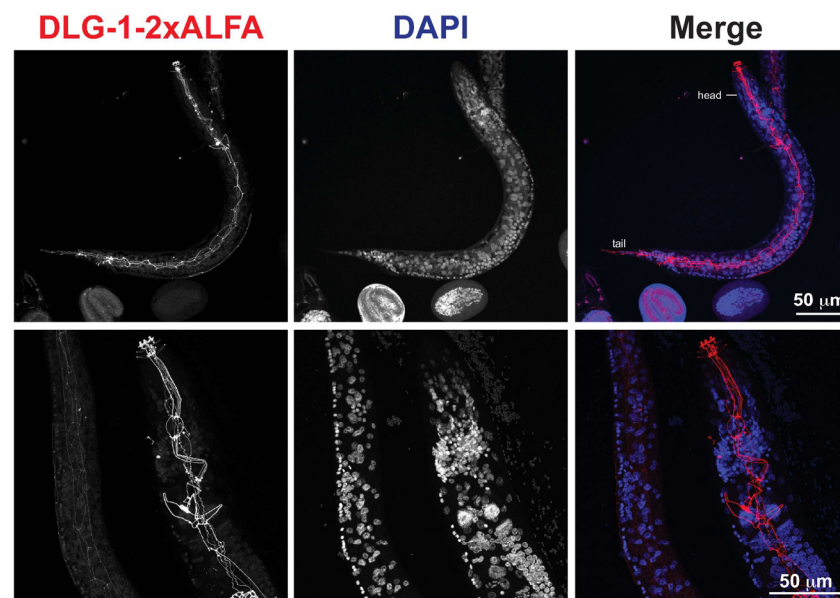


Figure 1. EUD-1 and SULT-1 ALFA tagging in *P. pacificus* (A,B) Images depicting the eurystomatous (Eu) (A) or stenostomatous (St) (B) morphs of PS312, *eud-1::2xALFA* and *sult-1::2xALFA* animals grown on agar plates (A) and liquid cultures (B). Eu worms have a wide-mouth with two teeth. The bacterial feeding St worms show a narrow mouth with a single tooth. The numbers in the images represent the mean value $\pm$ the standard deviation of mouth-form ratio in the different environmental conditions and worm strains presented as percent Eu (% Eu).

3.2. High-Resolution Immunofluorescence of ALFA-Tagged Proteins in *C. Elegans*

We first analyzed the expression of *Cel*-DLG-1 using the *dlg-1::2xALFA* line. In adult animals, we observed strong staining of *Cel*-DLG-1::2xALFA in epithelial cells of the epidermis, pharynx and intestine, forming a continuous belt around the cells from the head to the tail (Figure 2A, upper panels). In the head region of adult hermaphrodites, *Cel*-DLG-1::2xALFA is seen in the pharynx with a high resolution of the protein localization (Figure 2A, bottom panels). The observed expression is similar to previous reports [25] and is present throughout worm development (data not shown), which indicates the high structural-resolution obtained by visualization with nanobodies. In contrast, *Cel*-UNC-10::2xALFA staining was restricted to the synapse-rich areas of the nervous system, including the nerve ring and the dorsal and ventral nerve cords (Figure 2B), as previously reported [26]. These data demonstrate the specificity of the anti-ALFA nanobody. Taken together, ALFA-tagging is a simple and reliable method for high-resolution in situ protein detection, and it can be combined with the analysis of transcript expression in order to provide a comprehensive comparison of gene expression at all levels.

A. *C. elegans* *dlg-1::2xALFA*



B. *C. elegans* *unc-10::2xALFA*

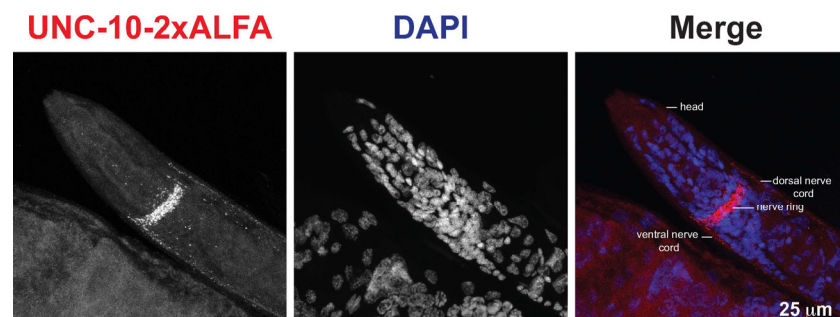


Figure 2. Spatial expression patterns of *Ce* *dlg-1::2xALFA* and *Ce* *unc-10::2xALFA* in adult worms. (A,B) Representative confocal fluorescent micrographs of the body and head of fixed *C. elegans* *dlg-1(tu1782[dlg-1::2xALFA])* (A) and *unc-10(tu1781[unc-10::2xALFA])* (B) animals. The worms were stained with anti-ALFA fluorescent nanobody. The merged images show the anti-ALFA signal in red and the nuclei in blue (DAPI staining). Images depict L4/adult worms.

3.3. Visualization of ALFA-Tagged Proteins in *P. pacificus* Reveals Sub-Localization of Mouth-Form Regulators

Next, we wanted to generate ALFA-tagged proteins in *P. pacificus* to study if this type of epitope tag works across nematode species borders. Note that the implementation of several methodologies that work routinely in *C. elegans* had proven difficult in other nematodes. In *P. pacificus*, for example, the establishment of DNA-mediated transformation required major modifications of the original protocol and took more than a decade [38]. In contrast, CRISPR-mediated gene knock-out was easily transferred from *C. elegans* [39], although this was later improved by several laboratories [15,19].

We used the two sulfation-associated proteins *Ppa*-EUD-1 and *Ppa*-SEUD-1/SULT-1, representing a sulfatase and sulfotransferase, respectively. In agreement with the spatial information revealed by fluorescent transcriptional reporters [29,31,35] and FISH³⁶, *Ppa*-EUD-1::2xALFA protein was detected in different somatic and pharyngeal neurons in the head of the animals (Figure 3A). The higher resolution of the anti-ALFA nanobody also revealed the expression of *Ppa*-EUD-1::2xALFA in the long axonal processes leading towards the mouth opening or the excretory gland cell (Figure 3A, asterisk). At the anterior of the worm, SEUD-1/SULT-1::2xALFA was expressed in cells that make up the face (anterior body wall), the stoma (mouth), the anterior pharynx (Figure 3B) in the feeding apparatus of *P. pacificus*. The *Ppa*-SEUD-1/SULT-1::2xALFA protein was preferentially detected in experimental conditions that promote the St morph (Figure 1B). *Ppa*-SEUD-1/SULT-1 was present in the cytoplasm of cells of the anterior epidermal (hyp) syncytia, the arcade cells at the interface of the mouth and the pharynx, and the pharyngeal muscle cells that secrete the extracellular teeth of the animal (Figure 3B), as previously observed [30,31,40].

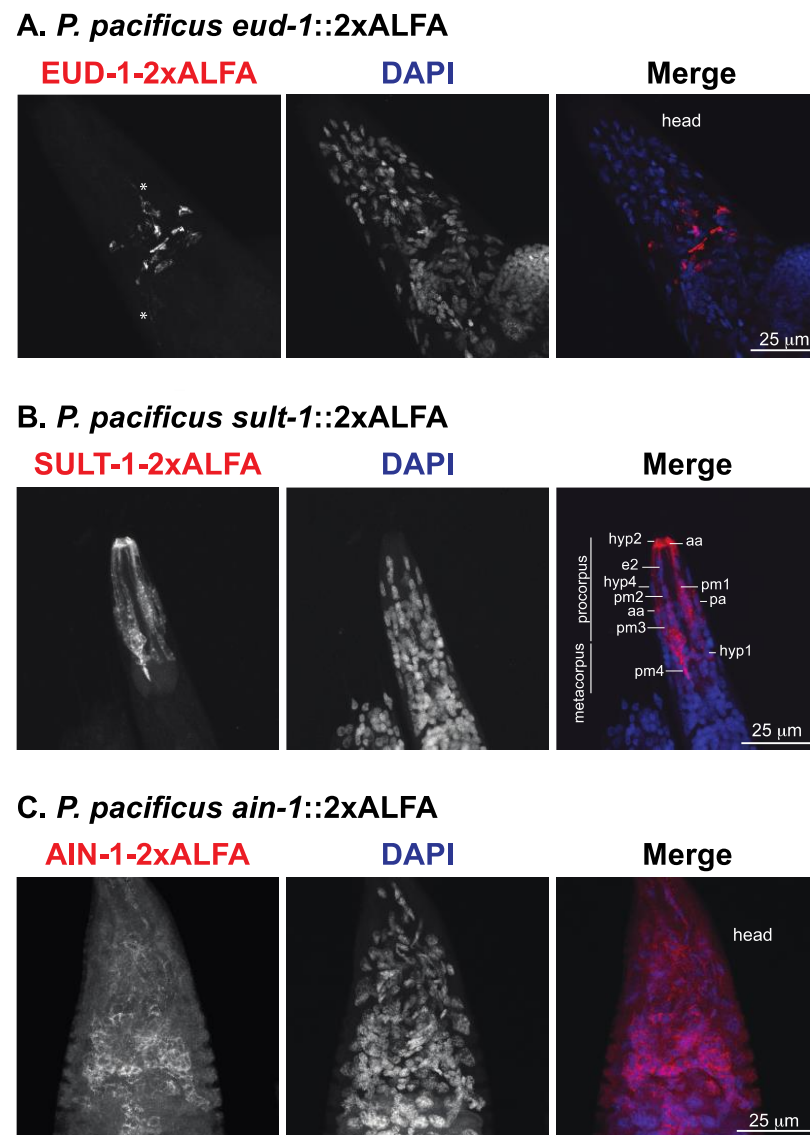


Figure 3. ALFA-tagged proteins show distinct expression patterns in *P. pacificus* (A–C) Representative

confocal fluorescent micrographs of the head of fixed *P. pacificus* eud-1(tu1729[eud-1::2xALFA]) (A), sult-1(tu1760[sult-1::2xALFA]) (B) and ain-1(tu1753[ain-1::2xALFA]) (C) animals. The worms were stained with anti-ALFA fluorescent nanobody. The merged images show the anti-ALFA signal in red and the nuclei in blue (DAPI staining). To maximize the expression of the sulfotransferase, sult-1(tu1760[sult-1::2xALFA]) animals were grown under liquid culture conditions. Images depict J4/adult worms. In A, asterisks indicate neuronal projections. In B, cell types were identified based on the reconstruction of the head hypodermis in *P. pacificus* [40]. aa: anterior arcade cell, e: epidermal cell, hyp: hypodermal syncytia, pm: pharyngeal muscle cell.

Finally, we targeted the nematode ortholog of the human GW182 gene *Ppa-ain-1*, a broadly expressed protein that localizes as discrete cytoplasmic ribonucleoprotein particles known as processing (P)-bodies [33]. Indeed, we observed that *Ppa-AIN-1::2xALFA* was a cytoplasmic protein expressed in the majority of the cells in adult worms (Figure 3C). Based on the fact that the analyzed ALFA-tagged proteins show distinct spatial expression patterns, which is in agreement with prior studies, we conclude that ALFA-tagging allows high-resolution and specific in situ visualization of proteins in *P. pacificus* and *C. elegans*.

3.4. Detection of ALFA-Tagged Proteins by Western Blot

We then tested the nematode ALFA tagged proteins using Western blot. Animals from *Cel-dlg-1(tu1782[dlg-1::2xALFA])*, *Ppa-sult-1(tu1760[sult-1::2xALFA])* and *Ppa-ain-1(tu1753[ain-1::2xALFA])* worms were collected, lysed and used in SDS-PAGE. Wild type N2 and PS312 worms were included as control lysates lacking ALFA-tagged proteins. All worm lysates were probed with the nanobody specific for the ALFA peptide and additionally with an anti-TUBULIN or anti-GRP78 (an endoplasmic reticulum chaperone) as protein loading controls. First, the ALFA-tagged proteins were detected only in the respective ALFA-tagged strains (Figure 4A–C), whereas the loading control was present in all the worm lysates. Second, the molecular weight of the ALFA-tagged proteins was in agreement with the predicted size for each protein (*Cel-DLG-1* = 107 kDa; *Ppa-SULT-1* = 53.3 kDa; *Ppa-AIN-1* = 70 kDa), highlighting the specificity of the anti-ALFA nanobody. Third, we only observed a cross-reaction of the anti-ALFA nanobody with an endogenous protein of about 55 kDa in *P. pacificus* PS312 lysates following long exposures to the chemiluminescent signal (data not shown). However, the non-specific signal was always much weaker than the one observed for the ALFA-tagged proteins. Thus, the ALFA system is a reliable and straightforward approach to evaluate and quantify the levels of endogenous proteins in nematode model organisms.

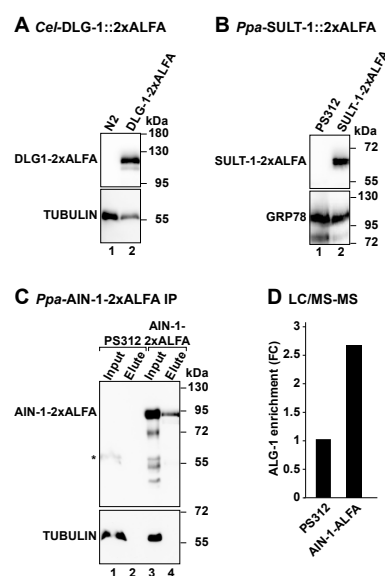


Figure 4. Immunoblotting and pulldown of ALFA-tagged proteins (A,B) Western blot showing the

expression levels of the ALFA-tagged proteins in the respective worm strains. *C. elegans* N2 and *P. pacificus* PS312 worms were used as mock controls for the absence of ALFA-tagged proteins. The blots were developed with anti-ALFA nanobody directly coupled with horse radish peroxidase (HRP) or with a mouse anti-TUBULIN primary antibody followed by anti-mouse IgG-HRP antibody. Molecular weight markers (kDa) are shown next to the blot. (C) Affinity purification of *Ppa-AIN-1::2xALFA* using ALFA resins. *Ppa-ain-1(tu1753[ain-1::2xALFA])* worm lysates were incubated with ALFA selector^{PE} resin. After washing, the resin was incubated with 400 μ M of ALFA peptide to elute the *Ppa-AIN-1::2xALFA* bound to the resin. Input (30% of worm lysate) and elute (2.5% of eluted *Ppa-AIN-1::2xALFA* protein) fractions were analyzed by SDS-PAGE and Western blot. The membrane was first incubated with anti-TUBULIN primary antibody and the corresponding secondary antibody coupled with HRP. After acquisition of the TUBULIN signal, ALFA-tagged protein was detected using the anti-ALFA nanobody. The control reaction (no ALFA-tag on AIN-1) was set with *P. pacificus* PS312 worm lysate. The asterisk indicates the signal remaining from the TUBULIN protein. (D) Bar plot showing the difference (fold change) in the number of *Ppa-ALG-1* peptides present in duplicated samples of *Ppa-AIN-1::2xALFA* and wild type (PS312) worm strains quantified using LC/MS-MS.

3.5. Capture of ALFA-Tagged Proteins from Worm Lysates

Affinity purification coupled with mass spectrometry is a commonly used approach to purify protein complexes from cells. We applied the ALFA system to capture, for the first time, an ALFA-tagged protein and its associated proteins in a multicellular organism. We incubated *P. pacificus* PS312 and *Ppa-ain-1(tu1753[ain-1::2xALFA])* worm lysates with the anti-ALFA nanobody immobilized on an agarose-based resin. After washing, the nanobody-bound proteins were competed with an excess of free ALFA peptide. The resulting eluted proteins were analyzed by SDS-PAGE and immunoblotting (Figure 4C). The *Ppa-AIN-1-2xALFA* protein successfully bound to the ALFA resin and was specifically eluted following the incubation with the free ALFA peptide (Figure 4C, upper panel, lane 4). In contrast, TUBULIN did not bind to the resin and therefore was only detected in the worm lysates (Figure 4C, bottom panel, lanes 1 vs. 2 and 3 vs. 4).

To study the efficiency of the ALFA system in the capture of ALFA-tagged proteins from worm lysates, we used immunoblotting and tracked the *Ppa-AIN-1-2xALFA* protein along the affinity purification protocol (Figure S1A,B). Once again, the *Ppa-AIN-1-2xALFA* present in the worm pellet bound to the ALFA resin and was specifically eluted with an excess of free ALFA peptide (Figure S1B, lanes 1 and 5). Nevertheless, we observed that two rounds of incubation with an excess of ALFA peptide was not sufficient to elute all the ALFA-tagged protein bound to the ALFA resin. In fact, enhanced elution of the *Ppa-AIN-1-2xALFA* protein was achieved after an additional incubation of the ALFA resin with a denaturing SDS sample buffer (Figure S1A,B). Thus, improved elution steps can provide higher yields of the protein captured using the ALFA system. Future experiments will indicate whether the efficiency of elution using the ALFA system depends on the ALFA-tagged protein or solely on the ALFA resin.

To examine the specificity and sensitivity of the ALFA system in the isolation of protein complexes in *P. pacificus*, we analyzed the elute fractions from PS312 and *Ppa-AIN-1::2xALFA* by mass spectrometry. We specifically searched for peptides corresponding to the *P. pacificus* homolog of the Argonaute protein, *Ppa-ALG-1*, a known interactor of AIN-1/GW182 proteins [33]. In comparison to the PS312 sample, *Ppa-ALG-1* protein was 2.6x fold enriched in the pulldown sample of AIN-1::2xALFA (Figure 4D). As expected, *Ppa-AIN-1* peptides were only detected in the *Ppa-AIN-1::2xALFA* samples. These results indicated that the ALFA system can also be applied in nematodes to specifically purify a POI and its binding partners.

In conclusion, this study reports the versatile application of the ALFA-tag epitope in the two nematode model organisms *C. elegans* and *P. pacificus*. With their rapid and simple growth and hermaphroditic mode of reproduction, epitope-tagged worms can easily be generated and used for genetic and biochemical characterization. Combined with the advantages of using fluorophore-labelled nanobodies in microscopy [41], the small size and the unique sequence of the tag, our work shows that the ALFA system is suitable for studying the spatial distribution and expression of endogenous proteins in a multicellular organism with a high resolution. In addition, we were able to reproducibly and specifically detect, quantify and capture POIs. We envision that the ALFA tag will be particularly powerful in multiple biochemical and cellular applications. These include genome-scale mapping of protein–protein interactions, protein–DNA/chromatin, and protein–mRNA/small RNA/ncRNA interactions coupled with mass spectrometry and DNA/RNA sequencing approaches, and the sorting of cells carrying surface tagged proteins. The combination of different small tags and respective nanobodies may also open an opportunity to simultaneously visualize multiple proteins in situ. Thus, the ALFA-tag adds another powerful tool to the already existing toolkit of *C. elegans*.

4. Limitations of the Current Study

There are two limitations in the current study that can only be addressed through additional applications. First, it remains unknown if C-terminal tags will be applicable in the majority of proteins. In particular, if functional domains in the C-terminus of a POI

exist, an ALFA-tag in its proximity might interfere with protein function. At the same time, it is one of the advantages of the ALFA-tag that, in principle, can be added in any position of the protein. Second, for biochemical applications, the pulldown of additional proteins and/or RNAs from a macromolecular complex labeled through ALFA-tagging of one of its protein components, might depend on the expression level of the POI. While the protein used in this study is highly abundant and expressed broadly in most cells of the worm, many POIs are known to have restricted expression patterns. Therefore, future studies will indicate if there are any limits for these types of applications. However, the increasing sensitivity of HPLC/MS-MS analysis for protein identification and the sequencing depth for RNA molecules are likely to help in overcoming these shortcomings.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cells11233875/s1>, Figure S1: Affinity purification of ALFA-tagged *Ppa*-AIN-1; Table S1: CRISPR/Cas9 sgRNA target sequences and ssDNA donor used in this study; Table S2: Primer sequences used in this study.

Author Contributions: Conceptualization, C.I. and R.J.S.; methodology and investigation, C.I., T.L., H.W., A.S., S.P.Q., R.S. and E.A.; writing—original draft preparation, C.I. and R.J.S.; writing—review and editing, C.I. and R.J.S.; supervision, C.I. and R.J.S.; project administration and funding acquisition, R.J.S. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by the Max Planck Society. R.S., S.P.Q. and E.A. were supported by the IMPRS “From Molecules to Organisms”.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: We are grateful to Heike Haussmann for her assistance in the freezing of all nematode strains produced in this study. We would like to thank Kohta Yoshida for discussion and suggestions on the project. We thank Aurora Panzera for her help with the confocal microscope and Johannes Madlung for assistance in the proteomic measurements by mass spectrometry.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Brizzard, B. Epitope tagging. *Biotechniques* **2008**, *44*, 693–695. [\[CrossRef\]](#) [\[PubMed\]](#)
2. Vandemoortele, G.; Eyckerman, S.; Gevaert, K. Pick a Tag and Explore the Functions of Your Pet Protein. *Trends Biotechnol.* **2019**, *37*, 1078–1090. [\[CrossRef\]](#) [\[PubMed\]](#)
3. Gotzke, H.; Kilisch, M.; Martinez-Carranza, M.; Sograte-Idrissi, S.; Rajavel, A.; Schlichthaerle, T.; Engels, N.; Jungmann, R.; Stenmark, P.; Opazo, F.; et al. The ALFA-tag is a highly versatile tool for nanobody-based bioscience applications. *Nat. Commun.* **2019**, *10*, 4403. [\[CrossRef\]](#) [\[PubMed\]](#)
4. Rudolph, M.; Carsten, A.; Kulnik, S.; Aepfelbacher, M.; Wolters, M. Live imaging of Yersinia translocon formation and immune recognition in host cells. *PLoS Pathog.* **2022**, *18*, e1010251. [\[CrossRef\]](#) [\[PubMed\]](#)
5. Akhuli, D.; Dhar, A.; Viji, A.S.; Bhojappa, B.; Palani, S. ALIBY: ALFA Nanobody-Based Toolkit for Imaging and Biochemistry in Yeast. *mSphere* **2022**, e0033322. [\[CrossRef\]](#)
6. van den Hoogen, J.; Geisen, S.; Routh, D.; Ferris, H.; Traunspurger, W.; Wardle, D.A.; de Goede, R.G.M.; Adams, B.J.; Ahmad, W.; Andriuzzi, W.S.; et al. Soil nematode abundance and functional group composition at a global scale. *Nature* **2019**, *572*, 194–198. [\[CrossRef\]](#)
7. Glazer, I.; Shapiro-Ilan, D.; Sternberg, P.W. *Nematodes as Model Organisms*; CABI: Wallingford, UK, 2022; p. 376.
8. Corsi, A.K.; Wightman, B.; Chalfie, M. A Transparent Window into Biology: A Primer on *Caenorhabditis Elegans*. *Genetics* **2015**, *200*, 387–407. [\[CrossRef\]](#)
9. Brenner, S. The genetics of *Caenorhabditis elegans*. *Genetics* **1974**, *77*, 71–94. [\[CrossRef\]](#)
10. Haag, E.S.; Fitch, D.H.A.; Delattre, M. From “the Worm” to “the Worms” and Back Again: The Evolutionary Developmental Biology of Nematodes. *Genetics* **2018**, *210*, 397–433. [\[CrossRef\]](#)
11. Sommer, R.J.; Sternberg, P.W. Apoptosis and change of competence limit the size of the vulva equivalence group in *Pristionchus pacificus*: A genetic analysis. *Curr. Biol. CB* **1996**, *6*, 52–59. [\[CrossRef\]](#)
12. Schroeder, N.E. Introduction to *Pristionchus pacificus* anatomy. *J. Nematol.* **2021**, *53*, e2021-91. [\[CrossRef\]](#) [\[PubMed\]](#)

13. Howard, R.J.; Giacomelli, M.; Lozano-Fernandez, J.; Edgecombe, G.D.; Fleming, J.F.; Kirstensen, R.M.; Ma, X.; Olesen, J.; Sorensen, M.V.; Thomsen, P.F.; et al. The Ediacaran origin of Ecdysozoa: Integrating fossil and phylogenomic data. *J. Geol. Soc.* **2022**, *179*. [[CrossRef](#)]
14. Dickinson, D.J.; Goldstein, B. CRISPR-Based Methods for *Caenorhabditis elegans* Genome Engineering. *Genetics* **2016**, *202*, 885–901. [[CrossRef](#)] [[PubMed](#)]
15. Nakayama, K.I.; Ishita, Y.; Chihara, T.; Okumura, M. Screening for CRISPR/Cas9-induced mutations using a co-injection marker in the nematode *Pristionchus pacificus*. *Dev. Genes Evol.* **2020**, *230*, 257–264. [[CrossRef](#)]
16. Han, Z.; Sieriebriennikov, B.; Susoy, V.; Lo, W.S.; Igreja, C.; Dong, C.; Berasategui, A.; Witte, H.; Sommer, R.J. Horizontally Acquired Cellulases Assist the Expansion of Dietary Range in *Pristionchus* Nematodes. *Mol. Biol. Evol.* **2022**, *39*, msab370. [[CrossRef](#)] [[PubMed](#)]
17. Werner, M.S.; Sieriebriennikov, B.; Loschko, T.; Namdeo, S.; Lenuzzi, M.; Dardiry, M.; Renahan, T.; Sharma, D.R.; Sommer, R.J. Environmental influence on *Pristionchus pacificus* mouth form through different culture methods. *Sci. Rep.* **2017**, *7*, 7207. [[CrossRef](#)]
18. Stiernagle, T. Maintenance of *C. elegans*. In *Wormbook*; Fay, D., Ed.; Oxford University Press: Oxford, UK, 2006.
19. Han, Z.; Lo, W.S.; Lightfoot, J.W.; Witte, H.; Sun, S.; Sommer, R.J. Improving Transgenesis Efficiency and CRISPR-Associated Tools Through Codon Optimization and Native Intron Addition in *Pristionchus* Nematodes. *Genetics* **2020**, *216*, 947–956. [[CrossRef](#)]
20. Sun, S.; Theska, T.; Witte, H.; Ragsdale, E.J.; Sommer, R.J. The oscillating Mucin-type protein DPY-6 has a conserved role in nematode mouth and cuticle formation. *Genetics* **2022**, *220*, iyab233. [[CrossRef](#)]
21. Sangaletti, R.; Bianchi, L. A method for culturing embryonic *C. elegans* cells. *J. Vis. Exp.* **2013**, *79*, e50649. [[CrossRef](#)]
22. Cox, J.; Mann, M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* **2008**, *26*, 1367–1372. [[CrossRef](#)]
23. Cox, J.; Neuhauser, N.; Michalski, A.; Scheltema, R.A.; Olsen, J.V.; Mann, M. Andromeda: A peptide search engine integrated into the MaxQuant environment. *J. Proteome Res.* **2011**, *10*, 1794–1805. [[CrossRef](#)] [[PubMed](#)]
24. Bossinger, O.; Klebes, A.; Segbert, C.; Theres, C.; Knust, E. Zonula adherens formation in *Caenorhabditis elegans* requires *dlg-1*, the homologue of the *Drosophila* gene discs large. *Dev. Biol.* **2001**, *230*, 29–42. [[CrossRef](#)] [[PubMed](#)]
25. McMahon, L.; Legouis, R.; Vonesch, J.L.; Labouesse, M. Assembly of *C. elegans* apical junctions involves positioning and compaction by LET-413 and protein aggregation by the MAGUK protein DLG-1. *J. Cell Sci.* **2001**, *114* Pt 12, 2265–2277. [[CrossRef](#)] [[PubMed](#)]
26. Koushika, S.P.; Richmond, J.E.; Hadwiger, G.; Weimer, R.M.; Jorgensen, E.M.; Nonet, M.L. A post-docking role for active zone protein Rim. *Nat. Neurosci.* **2001**, *4*, 997–1005. [[CrossRef](#)] [[PubMed](#)]
27. Kushibiki, Y.; Suzuki, T.; Jin, Y.; Taru, H. RIMB-1/RIM-Binding Protein and UNC-10/RIM Redundantly Regulate Presynaptic Localization of the Voltage-Gated Calcium Channel in *Caenorhabditis elegans*. *J. Neurosci.* **2019**, *39*, 8617–8631. [[CrossRef](#)] [[PubMed](#)]
28. Yu, C.J.; Barry, N.C.; Wassie, A.T.; Sinha, A.; Bhattacharya, A.; Asano, S.; Zhang, C.; Chen, F.; Hobert, O.; Goodman, M.B.; et al. Expansion microscopy of *C. elegans*. *Elife* **2020**, *9*, e46249. [[CrossRef](#)] [[PubMed](#)]
29. Ragsdale, E.J.; Muller, M.R.; Rodelsperger, C.; Sommer, R.J. A developmental switch coupled to the evolution of plasticity acts through a sulfatase. *Cell* **2013**, *155*, 922–933. [[CrossRef](#)]
30. Bui, L.T.; Ivers, N.A.; Ragsdale, E.J. A sulfotransferase dosage-dependently regulates mouthpart polyphenism in the nematode *Pristionchus pacificus*. *Nat. Commun.* **2018**, *9*, 4119. [[CrossRef](#)]
31. Namdeo, S.; Moreno, E.; Rodelsperger, C.; Baskaran, P.; Witte, H.; Sommer, R.J. Two independent sulfation processes regulate mouth-form plasticity in the nematode *Pristionchus pacificus*. *Development* **2018**, *145*, dev166272. [[CrossRef](#)]
32. Igreja, C.; Sommer, R.J. The Role of Sulfation in Nematode Development and Phenotypic Plasticity. *Front. Mol. Biosci.* **2022**, *9*, 838148. [[CrossRef](#)]
33. Ding, L.; Spencer, A.; Morita, K.; Han, M. The developmental timing regulator AIN-1 interacts with miRISCs and may target the argonaute protein ALG-1 to cytoplasmic P bodies in *C. elegans*. *Mol. Cell* **2005**, *19*, 437–447. [[CrossRef](#)] [[PubMed](#)]
34. Jonas, S.; Izaurralde, E. The role of disordered protein regions in the assembly of decapping complexes and RNP granules. *Genes Dev.* **2013**, *27*, 2628–2641. [[CrossRef](#)] [[PubMed](#)]
35. Sieriebriennikov, B.; Prabh, N.; Dardiry, M.; Witte, H.; Roseler, W.; Kieninger, M.R.; Rodelsperger, C.; Sommer, R.J. A Developmental Switch Generating Phenotypic Plasticity Is Part of a Conserved Multi-gene Locus. *Cell Rep.* **2018**, *23*, 2835–2843 e4. [[CrossRef](#)] [[PubMed](#)]
36. Kieninger, M.R.; Ivers, N.A.; Rodelsperger, C.; Markov, G.V.; Sommer, R.J.; Ragsdale, E.J. The Nuclear Hormone Receptor NHR-40 Acts Downstream of the Sulfatase EUD-1 as Part of a Developmental Plasticity Switch in *Pristionchus*. *Curr. Biol. CB* **2016**, *26*, 2174–2179. [[CrossRef](#)] [[PubMed](#)]
37. Seroby, V.; Xiao, H.; Namdeo, S.; Rodelsperger, C.; Sieriebriennikov, B.; Witte, H.; Roseler, W.; Sommer, R.J. Chromatin remodelling and antisense-mediated up-regulation of the developmental switch gene *eud-1* control predatory feeding plasticity. *Nat. Commun.* **2016**, *7*, 12337. [[CrossRef](#)]
38. Schlager, B.; Wang, X.; Braach, G.; Sommer, R.J. Molecular cloning of a dominant roller mutant and establishment of DNA-mediated transformation in the nematode *Pristionchus pacificus*. *Genesis* **2009**, *47*, 300–304. [[CrossRef](#)]

-
39. Witte, H.; Moreno, E.; Rodelsperger, C.; Kim, J.; Kim, J.S.; Streit, A.; Sommer, R.J. Gene inactivation using the CRISPR/Cas9 system in the nematode *Pristionchus pacificus*. *Dev. Genes Evol.* **2015**, *225*, 55–62. [[CrossRef](#)]
 40. Harry, C.J.; Messar, S.M.; Ragsdale, E.J. Comparative reconstruction of the predatory feeding structures of the polyphenic nematode *Pristionchus pacificus*. *Evol. Dev.* **2022**, *24*, 16–36. [[CrossRef](#)]
 41. de Beer, M.A.; Giepmans, B.N.G. Nanobody-Based Probes for Subcellular Protein Identification and Visualization. *Front. Cell Neurosci.* **2020**, *14*, 573278. [[CrossRef](#)]

Supplementary Information

Application of ALFA-tagging in the nematode model organisms *Caenorhabditis elegans* and *Pristionchus pacificus*

Catia Igreja, Tobias Loschko, Alejandra Schäfer, Radhika Sharma, Shiela Pearl Quiobe, Elbin Aloshy, Hanh Witte & Ralf J. Sommer*

Department for Integrative Evolutionary Biology, Max Planck Institute for Biology Tübingen, Max Planck Ring 9, 72076 Tübingen, Germany

*Author for correspondence at: ralf.sommer@tuebingen.mpg.de

Running head: Nematode ALFA-tagging

ORCID numbers:

Catia Igreja: <https://orcid.org/0000-0003-3563-1788>

Ralf J. Sommer: <https://orcid.org/0000-0003-1503-7749>

Igreja *et al.* Supplementary Figure 1.

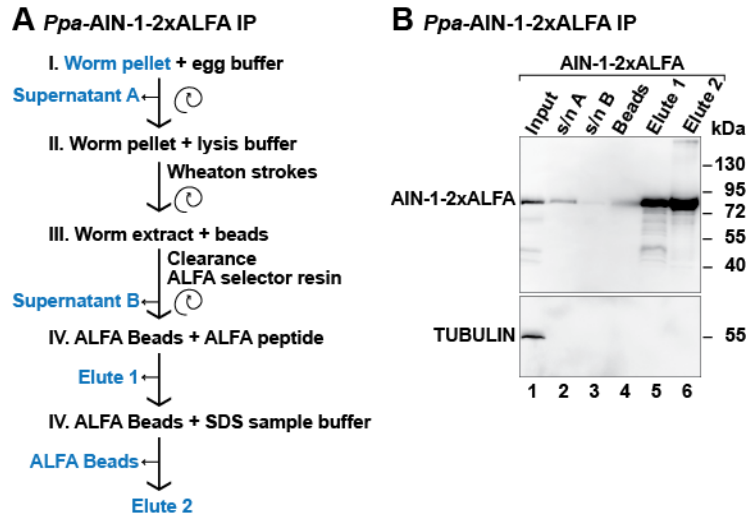


Figure S1. Affinity purification of ALFA-tagged *Ppa*-AIN-1

(A) Simplified schematic representation of the experimental protocol used to capture ALFA-tagged proteins from worm lysates (for details see Materials & Methods section). Curved arrows represent centrifugation steps. Aliquots of samples highlighted in blue were collected throughout the purification steps and analyzed by immunoblotting in B.

(B) Detection of AIN-1-2xALFA by Western blotting. Samples were collected throughout the affinity purification of *Ppa*-AIN-1::2xALFA using ALFA resins. Input: Worm pellet (Input; 10% of worm pellet dissolved in SDS sample buffer), supernatant A (s/n A; 0.1% of worm pellet dissolved in 10 ml of egg buffer), supernatant B (s/n B; 0.1% of worm pellet lysed in 8 ml of lysis buffer), elute 1 (7.5% of ALFA-tagged protein eluted from the ALFA resin following incubation with 400 μ M of ALFA peptide); elute 2 (10% of ALFA-tagged protein eluted from the ALFA resin with denaturing SDS sample buffer after collection of elute 1) and beads (0.5% of the ALFA resin at the end of the two elution steps, i.e., ALFA-tagged protein still bound to the beads at the end of the affinity purification protocol) samples were analyzed by SDS-PAGE and Western blot. The membrane was first incubated with anti-TUBULIN primary antibody and corresponding secondary antibody coupled with HRP. After acquisition of the TUBULIN signal, ALFA-tagged protein was detected using the anti-ALFA nanobody.

Table S1. CRISPR/Cas9 sgRNA target sequences and ssDNA donor used in this study

Locus	sgRNA	ssDNA donor
<i>Ce-dlg-1</i> (C25F6/ G5ECY0)	5'-TGAATCGCAGACGCCAATT-3'	5'- AGTGTACTCCATCATCAGCCG GAATCAAAACACCGATCT GGGTGC CACGTCATT CACGACTCGAAGAAGAACTCCGACGACGACTTACAGA ATCCCGTCTCGAGGAGGAGCTCCGTCGTCGTCACCGAGTAG ATGA AATTGGATATTTTTAGACAAATAATCGTTT-3'
<i>Ce-unc-10</i> (T10A3/ Q22366)	5'-AAAGATTCCGATGTATCAGT-3'	5'- GCTGGCACTGGTCCAGTTCGA AAAGATTCAGACGTTTCCGT CGGAGG TGCTCAGCAGT CACGACTCGAAGAAGAACTCCGACGACGACTTACA GAATCCCGTCTCGAGGAGGAGCTCCGTCGTCGTCACCGAGTAA CA AATTCATATGTTTTGTTTGTCTTA-3'
<i>Ppa-eud-1</i> (PPA43535/ A0A4X3PQV1)	5'-TCGAGAAGGAGGAGCTCAA-3'	5'- CGCGCTTCGAAGAGCCATGCTCGAGAAGGAGGAGCTCAA TTGGAGT GATCAAGAATACGATCAGCTTGAAT CTAGACTCGAGGAGGAGCTCA GAAGAAGACTCACTGAGTCCCGTCTCGAGGAGGAGCTCCGTCGTCG TCTACCGAGTAA GAATAAATATACCTAACGATTTATCTCGTTCCATC AGTTC-3'
<i>Ppa-ain-1</i> (PPA22409/ H3FJF8)	5'-CAACTATCAGTACTCCTCC-3'	GTCTCAGAGTCGGCCACGA CAACTATCAGTATTGTTCTT GGGTCT AGACTCGAGGAGGAGCTCAGAAGAAGACTCACTGAGTCCCGTCTCG AGGAGGAGCTCCGTCGTCGTCACCGAGTAA GCATCGATGATCTTT GATCTATCGACTCTCCCTTCCCCTTC
<i>Ppa-sult-1</i> (PPA12547/ H3ERY0)	5'-GAAGATCGACGACGCTACAG-3'	5'- TCAATTTCCCTTGACTCCCTGAAGATCGACGACGCTACAG AAGAGCA GGACT CTAGACTCGAGGAGGAGCTCAGAAGAAGACTCACTGAGTCC CGTCTCGAGGAGGAGCTCCGTCGTCGTCACCGAGTAAT CTATTTT ATCACTTATGTATTCCATTAGAT-3'

Gene names are also identified by the corresponding Wormbase (*Ce*) or El Paco_v3 (*Ppa*) and Uniprot IDs. In the ssDNA donor, the two copies of the Alfa tag are highlighted in bold characters, the STOP codon is depicted in red and the modified PAM motif or gRNA is shown in green.

Table S2. Primer sequences used in this study

Locus	Forward primer	Reverse primer	Sequencing primer
<i>Ce-dlg-1</i>	5'-TTTGTTACGCGTATGTCTTT-3'	5'-GAACTTGGAAAGGTCATGTAA-3'	Forward primer
<i>Ce-unc-10</i>	5'-TGGAGGGCAAACAATGCATTGC-3'	5'-CAAGTACCCATAATGCATAACG-3'	Forward primer
<i>Ppa-eud-1</i>	5'-CACTTCAACGCATCTGTTAT-3'	5'-AAGCGAGATAAATCGTTAGG-3'	Forward primer
<i>Ppa-ain-1</i>	5'-GATGACGACAGCTAAGATCG-3'	5'-AAATCGGAACGATAATAGGG-3'	Forward primer
<i>Ppa-sult-1</i>	5'-CCCGCTGTGGAGGGTCCGCTGG-3'	5'-ACATTGCTCGTGGAGCAAAAGC-3'	Forward primer

**EBAX-1/ZSWIM8 destabilizes miRNAs, resulting in
transgenerational inheritance of a predatory trait.**

Shiela Pearl Quiobe, Ata Kalirad, Waltraud Röseler, Hanh Witte, Yinan
Wang, Christian Rödelsperger, Ralf J Sommer

Published in:

Science Advances 11(11): eadu0875, (2025). DOI: 10.1126/sciadv.adu0875

GENETICS

EBAX-1/ZSWIM8 destabilizes miRNAs, resulting in transgenerational inheritance of a predatory trait

Shiela Pearl Quiobe, Ata Kalirad, Waltraud Röseler, Hanh Witte, Yinan Wang, Christian Rödelsperger, Ralf J. Sommer*

Environmental influences on traits and associated transgenerational epigenetic inheritance have widespread implications but remain controversial and underlying mechanisms poorly understood. We introduce long-term environmental induction experiments on alternative diets in *Pristionchus pacificus*, a nematode exhibiting mouth-form plasticity including predation, by propagating 110 isogenic lines for 101 generations with associated food-reversal experiments. We found dietary induction and subsequent transgenerational inheritance of the predatory morph and identified a role of ubiquitin ligase EBAX-1/ZSWIM8 in this process. *Ppa-ebax-1* mutants are transgenerational inheritance defective, and *Ppa-EBAX-1* destabilizes the clustered microRNA family *miR-2235a/miR-35*. Deletions of a cluster of 44 identical *miR-2235a* copies resulted in precocious and extended transgenerational inheritance of the predatory morph. These findings indicate that EBAX-1/ZSWIM8 destabilizes miRNAs, resulting in transgenerational inheritance, suggesting a role for target-directed miRNA degradation.

INTRODUCTION

Environmental influences on trait inheritance in animals including humans have long been suggested but remain controversial, largely because of the many confounding effects encountered when measuring such phenotypes over multiple generations (1, 2). However, laboratory studies in model organisms, most notably the nematode *Caenorhabditis elegans* with its short generation time, isogenic propagation, and simple husbandry has provided powerful demonstrations of transgenerational epigenetic inheritance (TEI) and started to provide mechanistic insight [for review, see (3–6)]. A growing body of evidence suggests that the environment can induce various phenotypic changes that are transmitted across generations. In this context, intergenerational and transgenerational inheritance are distinguished, with the former describing transmission that lasts for only two, whereas the latter describes inheritance that lasts for at least three generations beyond parental induction (3). Already transient, short-term environmental variation can induce TEI in *C. elegans* (7–14). For example, a single exposure to elevated temperature, starvation or pathogens induces changes of small interfering RNAs (siRNAs) that last for several generations (12, 15–19). In addition, Argonaute proteins (Ago) and histone modifications were shown to play a crucial role in siRNA signaling in *C. elegans* (7–9). However, a sophisticated machinery typically helps resetting such nongenetic effects after three to five generations (20).

Nonetheless, several experimental challenges impede the study of TEI and its long-term significance for the organism (6, 21–23). We try to overcome some of these challenges through two innovations in experimental design. First, we assay a natural, organismal readout in form of developmental plasticity in nematode feeding structures, which results in a binary morphological phenotype among genetically identical organisms (Fig. 1A). Second, we use a study system borrowed from experimental evolution (24) for the analysis of environmental influence on plastic trait formation and potential TEI. Specifically, we study the second nematode model

organism *Pristionchus pacificus* that shares with *C. elegans* its easy husbandry, short generation time, and hermaphroditic mode of reproduction, resulting in isogenic cultures (25, 26). However, unlike *C. elegans*, *P. pacificus* exhibits a mouth-form dimorphism that is an exemplar of developmental plasticity with morphological and behavioral implications (Fig. 1A) (27–29). By definition, the environment influences which of the two alternative phenotypes is expressed in a given individual as part of the “natural” biology of this organism, providing an ideal approach to investigate potential systems of TEI.

During the last decade, *P. pacificus* mouth-form plasticity has emerged as one of the study systems in plasticity research (30). During postembryonic development, genetically identical individuals adopt either the narrow-mouthed “stenostomatous” (St) morph with a single dorsal tooth or the wide-mouthed “eurystomatous” (Eu) form with a claw-like dorsal and an opposing sub-ventral tooth (Fig. 1A). Eu animals are omnivores that can feed on bacteria, fungi, and other nematodes as predators, whereas St worms are strict bacterial feeders (Fig. 1B) (31). Thus, the mouth-form decision of an individual animal has important behavioral and ecological consequences (32). This instance of plasticity, where environmental stimuli affect the likelihood of an individual developing one of the two alternative states, can be characterized as a bistable developmental switch (33, 34), distinct from other cases of more-or-less deterministic phenotypic plasticity [e.g., wing polyphenism in butterflies; (35)]. The gene regulatory network (GRN) regulating mouth-form plasticity in *P. pacificus* has been characterized in detail and involves a complex mixture of highly conserved as well as rapidly evolving genes (28, 36–44). In addition, multiple environmental cues influence mouth-form development. For example, culture methods (45), temperature (46), and small-molecule metabolites (47) were shown to influence the Eu versus St mouth-form ratio of isogenic *P. pacificus* strains. More recently, changes in diet were also found to profoundly affect the mouth form, establishing an easy study system for experimental manipulation (48). Here, we used long-term environmental induction through distinct diets by propagating 110 *P. pacificus* isogenic lines for 101 generations with associated food-reversal experiments.

Department for Integrative Evolutionary Biology, Max Planck Institute for Biology
Tübingen, Max-Planck Ring 9, Tübingen, 72076, Germany.

*Corresponding author. Email: ralf.sommer@tuebingen.mpg.de

Copyright © 2025 The Authors, some rights reserved; exclusive licensee American Association for the Advancement of Science. No claim to original U.S. Government Works. Distributed under a Creative Commons Attribution NonCommercial License 4.0 (CC BY-NC).

Downloaded from https://www.science.org at Max Planck Society on March 02, 2026

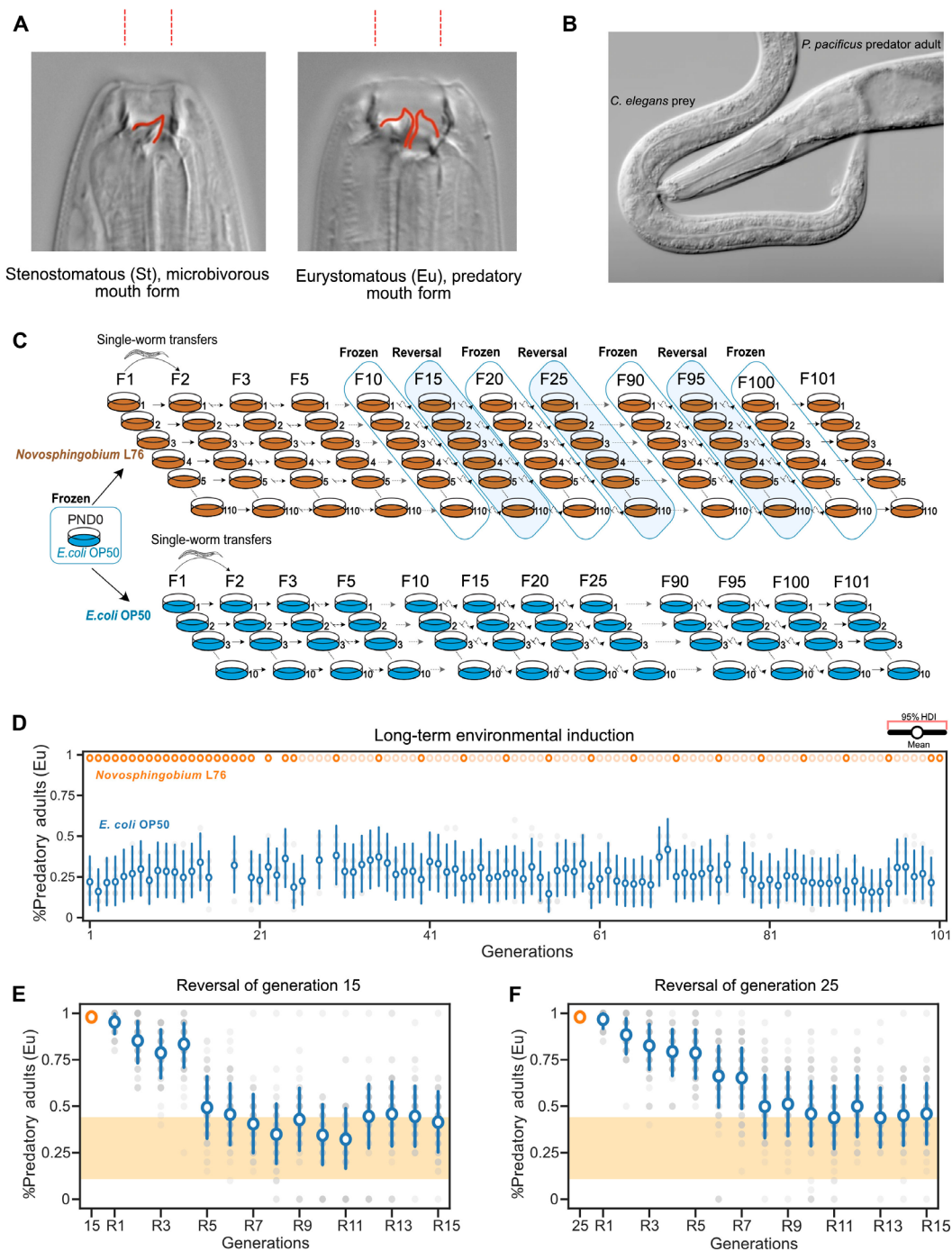


Fig. 1. Long-term environmental induction approach on alternative diets shows induction and TEI of the predatory morph in *Pristionchus pacificus*. (A) Mouth dimorphism of *P. pacificus*. The eurystomatous (Eu) morph has a wide mouth with two claw-like teeth and is an omnivore, whereas the stenostomatous (St) morph has a narrow mouth with a single flint-like dorsal tooth and feeds only on microbes. (B) Adult *P. pacificus* devouring a *C. elegans* prey. (C) Experimental design for long-term environmental induction using distinct diets. One hundred ten parallel lines derived from a single hermaphrodite were exposed to a *Novosphingobium* diet with 10 control lines remaining on the standard *E. coli* food source. All lines were propagated for 101 generations by single-worm descent. Periodic food reversal experiments back to the standard *E. coli* diet were performed all 10 generations starting in generation F15. In addition, lines were frozen all 10 generations starting in generation F10. (D) Mean probability of the predatory mouth form on *Novosphingobium* (orange) and *E. coli* (blue) during the long-term environmental induction experiment. The y axis indicates the observed Eu mouth form frequency, and x axis shows the number of generations. The 95% highest density interval (HDI) of the mean for every generation was used to visualize the probability of observing the Eu morph. (E) Mean probability of predatory mouth form on *E. coli* after 15 generations of exposure to *Novosphingobium*. (F) Mean probability of predatory mouth form on *E. coli* after 25 generations of exposure to *Novosphingobium*. The 95% HDI for the means was estimated using a Bayesian hierarchical model and indicates the probability of expressing the Eu morph inferred from the experimental data. The yellow area displays the upper and lower limits of the baseline Eu response of RSC011 [0.115 ≤ HDI($\bar{\theta}_{\text{Control}}$) ≤ 0.436], averaged across 101 generations on *E. coli*.

RESULTS

A long-term environmental induction experiment to visualize plasticity of feeding structures

We borrowed the concept of experimental evolution (24) to study the influence of diet on *P. pacificus* mouth-form plasticity. Specifically, we established a long-term environmental induction experiment for 101 generations with regular food reversals to investigate the consequences of dietary change (Fig. 1C). We previously showed that multiple bacterial isolates from nematode-associated environments can alter *P. pacificus* mouth-form ratios and predatory behaviors (48, 49). For example, growing *P. pacificus* for one generation on *Novosphingobium* L76 increased the efficiency of predatory behavior including successful killing of prey nematodes relative to *Escherichia coli*-grown worms and also changed mouth-form ratios toward Eu in certain *P. pacificus* strains (48, 49). Therefore, we performed the long-term environmental induction experiment by switching naïve *P. pacificus* cultures that were always grown on the standard laboratory food source *E. coli* OP50 to *Novosphingobium* (Fig. 1C). We used the strain *P. pacificus* RSC011 from La Réunion Island for the experiment because this strain is preferentially non-predatory (30% Eu:70% St) when grown on *E. coli* (48). *P. pacificus* RSC011 is isogenic after 10 generations of original inbreeding and was subsequently frozen as a reference strain. However, this strain has not undergone an inordinately long culture period in the laboratory that could have resulted in domestication. Together, the long-term environmental induction setup relies on (i) the propagation of isogenic cultures by self-fertilization, (ii) simple and rapid worm husbandry on monoxenic bacterial diets, (iii) the formation of large brood sizes for phenotyping, and (iv) the natural morphological readout of feeding structure plasticity.

To start the experiment, we singled out the complete brood of a single RSC011 adult hermaphrodite derived from an *E. coli* OP50 culture (Fig. 1C). We established 110 parallel lines on the *Novosphingobium* diet and 10 control lines that remained on standard *E. coli* food. All lines were propagated for a total of 101 generations. In each generation, a single healthy worm at the J4 juvenile stage was propagated to a new plate with the respective diet (Fig. 1C). We used single-worm descent to maximize founder effects and the chance that rare and stochastic, epigenetic events can manifest themselves in a population. Recent studies in *C. elegans* revealed that animals can stochastically assume transgenerational epigenetic states (13, 50). This approach is, by design, different from selection line experiments, where several animals are propagated together to draw on standing genetic variation already present in a population. In contrast, in our experiment, all animals are genetically identical, and propagation by single-worm descent allows capturing stochastic epigenetic variation, although genetic mutations will also accumulate during the experiment. Therefore, the naïve RSC011 culture, from which the single worm for starting the experiment was taken, was frozen as reference. Similarly, all 110 lines were frozen all 10 generations (F10, F20, etc.). Genome sequencing of some of these frozen lines after an exposure to *Novosphingobium* for 100 generations revealed a mutation rate of 1×10^{-9} to 3×10^{-9} (fig. S1K), which is similar to the previously reported mutation frequency in *P. pacificus* (51). Given the genome size of *P. pacificus*, this results in roughly one mutation in 10 generations of the experimental setup. With a generation time of 4 days, the complete long-term environmental induction experiment was carried out in ~1 year.

***Novosphingobium* induces the predatory mouth form and causes massive changes in gene expression**

The 110 sister lines of *P. pacificus* RSC011 developed a mouth-form ratio of 100% Eu animals already in the first generation cultured on *Novosphingobium* (Fig. 1D). In contrast, the probability of developing the Eu mouth form (θ) in *E. coli* control lines remained low in this generation and throughout the entire experiment [$0.115 \leq \text{HDI}(\theta) \leq 0.436$] (Fig. 1D). The immediate change in mouth-form ratio on *Novosphingobium* remained stable in all lines and in all subsequent generations with an all-Eu phenotype (Fig. 1D). In comparison to *E. coli*-grown worms, dietary induction of the predatory mouth form is immediate (starting in generation 1), systemic (occurred in all 110 lines), and complete (100% Eu). These findings are further supported by transcriptomic studies upon *Novosphingobium* exposure (fig. S2A and data S2, A to D). Specifically, RNA sequencing (RNA-seq) analysis identified 3043 differentially expressed genes (adjusted *P* value < 0.01) between both bacterial diets [bacterial diets (P0) on *E. coli* versus F3 on *Novosphingobium*]. These genes are mostly enriched in collagens, cadherins, and vitellogenin (fig. S2A and data S2A). Thus, exposure to *Novosphingobium* induces a massive transcriptomic response in RSC011 animals.

Dietary induction of the predatory morph shows TEI

Dietary effects can cause TEI in nematodes (12, 17, 52). Therefore, we started to revert all 110 lines back to standard *E. coli* OP50 food in generation F15 (Fig. 1, C and F). For that, we transferred one J4 juvenile per line to a nematode growth medium (NGM) agar plate supplemented with kanamycin and used a kanamycin-resistant *E. coli* strain for one generation. Kanamycin-supplemented plates and the kanamycin-resistant *E. coli* strain, which is a direct derivative of the OP50 strain, on their own had no effect on *P. pacificus* mouth form (fig. S1J). Subsequently, all lines were cultured for 14 additional generations on *E. coli* OP50 (Fig. 1E). We repeated these experiments all 10 generations with a total of nine reversals between F15 and F95 (Fig. 1, E and F, and fig. S1, C to I).

In the F15 reversal experiment, we observed high probability of developing the Eu mouth form in the first generation [$0.89 \leq \text{HDI}(\theta_{\text{F15R1}}) \leq 0.953$] (F15R1) and in the three subsequent generations [$0.733 \leq \text{HDI}(\theta_{\text{F15R2}}) \leq 0.957$, $0.652 \leq \text{HDI}(\theta_{\text{F15R3}}) \leq 0.913$, and $0.709 \leq \text{HDI}(\theta_{\text{F15R4}}) \leq 0.946$] (Fig. 1E). Only in generation F15R5, the mean mouth-form ratio dropped below 50% Eu [$\bar{\theta}_{\text{F15R5}} = 0.493$, $0.327 \leq \text{HDI}(\theta_{\text{F15R5}}) \leq 0.66$], which is not credibly different from the baseline level of naïve worms [$0.115 \leq \text{HDI}(\theta_{\text{Control}}) \leq 0.436$] (see Materials and Methods for details). These findings indicate a TEI of the Eu mouth form that lasts for four generations. When we repeated the reversal experiment in generation F25, we found even longer TEI. Specifically, in the F25R1 generation, all but one line had at least 85% Eu animals [$\bar{\theta}_{\text{F25R1}} = 0.968$, $0.917 \leq \text{HDI}(\theta_{\text{F25R1}}) \leq 0.999$] (Fig. 1F). The probability of developing the Eu mouth form remained high in the F25R5 generation [$0.649 \leq \text{HDI}(\theta_{\text{F25R5}}) \leq 0.913$] and only decreased considerably in generation F25R8 [$0.33 \leq \text{HDI}(\theta_{\text{F25R8}}) \leq 0.667$], whereas such a value was observed already in F15R5 [$0.327 \leq \text{HDI}(\theta_{\text{F15R5}}) \leq 0.66$] (Fig. 1, E and F). Thus, comparing the F15 and F25 reversal experiments suggests extended TEI of the predatory mouth form correlating with the length of dietary induction. Analysis of the subsequent reversal experiments indicated strong TEI and the

mean proportion of Eu animals at generation 15 after the reversal to fluctuate around 50 to 60% [e.g., $0.349 \leq \text{HDI}(\theta_{F15R35}) \leq 0.664$, $\leq 0.32 \leq \text{HDI}(\theta_{F15R65}) \leq 0.675$, and $0.457 \leq \text{HDI}(\theta_{F15R95}) \leq 0.773$; fig. S1, C to I]. Together, these observations indicate a strong transgenerational response of *P. pacificus* RSC011 to the *Novosphingobium* diet that results in multigenerational expression of the predatory mouth form.

TEI of the predatory mouth form requires a five-generation exposure

Next, we wanted to know the shortest duration of the exposure to a *Novosphingobium* diet that would induce the TEI of the predatory mouth form. Exposure of RSC011 to *Novosphingobium* for one, two, or three generations did not result in TEI of the Eu phenotype (Fig. 2, A to D). When we exposed naïve RSC011 worms to *Novosphingobium*

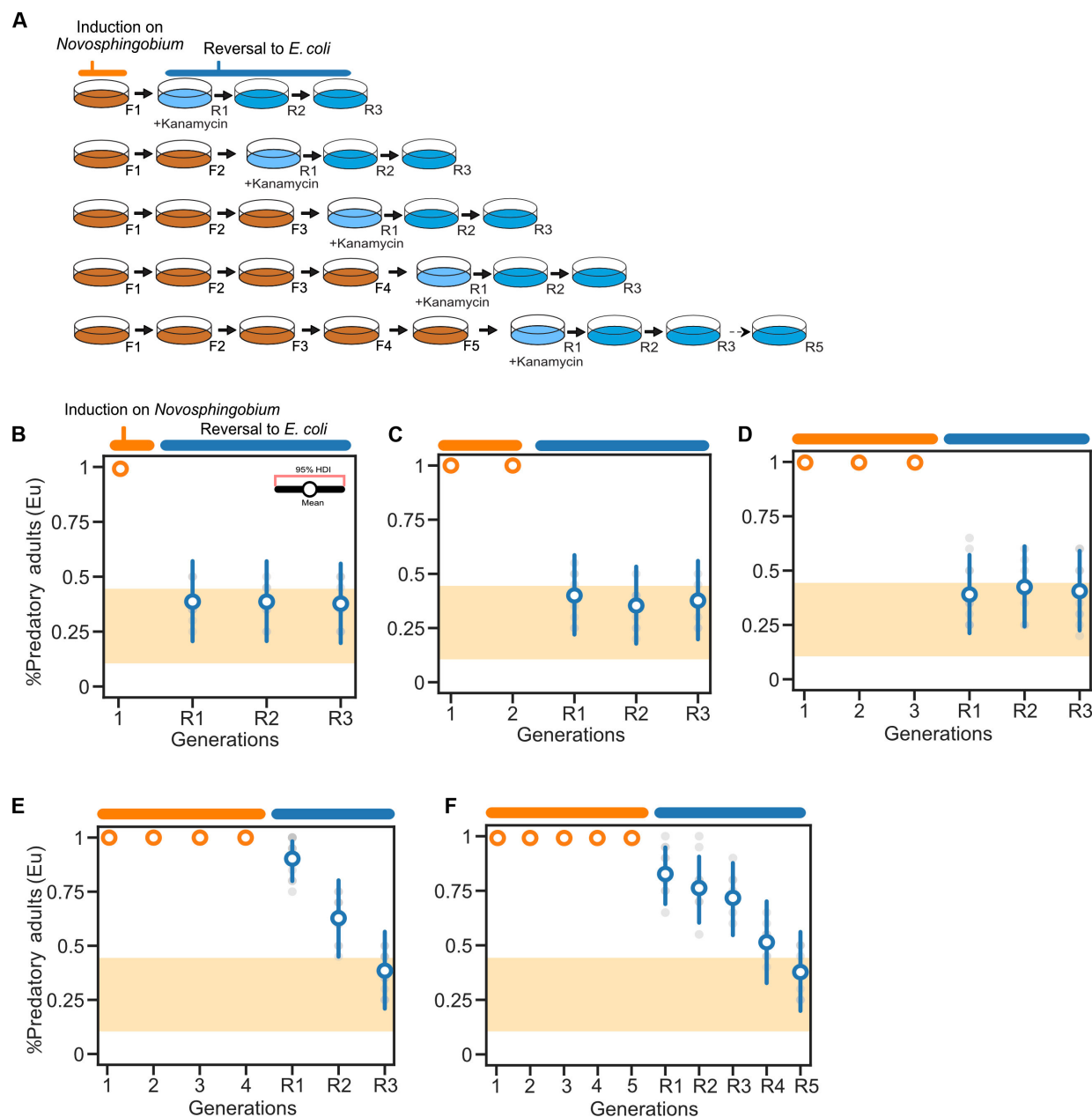


Fig. 2. Transgenerational inheritance of the predatory mouth form requires a minimum of five-generation exposure. (A) Graphical depiction of the tracking of the predatory morph responses after exposure to *Novosphingobium* (orange) for a certain number of generations and subsequent reversal to *E. coli* (blue). (B) Mean probability of predatory mouth-form after exposure to *Novosphingobium* for one generation, (C) two generations, (D) three generations, (E) four generations, and (F) five generations following reversal to *E. coli*. The 95% HDI estimates of the Eu mean that overlap with the upper limit of the baseline response (yellow) are considered not to be credibly different from the control on *E. coli*. Orange and blue ovals above [(B) to (F)] highlight generations on *Novosphingobium* and *E. coli*, respectively. Yellow area shows the RSC011 baseline response averaged across 101 generations on *E. coli*.

for four generations, we observed intergenerational inheritance that lasted for two generations (Fig. 2E). However, the mean probability of developing the Eu mouth form in the F4R3 generation dropped below 0.5 [$\bar{\theta}_{F4R3} = 0.385$, $0.21 \leq \text{HDI}(\theta_{F4R3}) \leq 0.565$] (Fig. 2E). In contrast, a five-generation exposure resulted in a mean probability of developing the Eu mouth form of $0.514 \leq \bar{\theta} \leq 0.83$ for four generations when reverted back to *E. coli* (Fig. 2F). Thus, TEI of the predatory mouth form builds up after multiple generations and requires an exposure of at least five generations on *Novosphingobium*. These findings are different from most of the studies in *C. elegans* that observe TEI already after transient environmental exposure (12, 15, 16); however, multigenerational exposure to pathogenic bacteria has been described to cause transgenerational inheritance of dauer formation (53).

Forward genetic screening identifies mutants defective in transgenerational inheritance of the predatory mouth form

To unravel the molecular machinery involved in the TEI of the predatory mouth form, we coupled the long-term environmental induction experiment with unbiased forward genetic screening for TEI-defective mutants. Specifically, we mutagenized 100 hermaphrodites that were grown on *Novosphingobium* for 14 generations with ethylmethane sulfonate (EMS) (Fig. 3A). After the F15 generation on *Novosphingobium*, we singled out 3000 F15R1 animals that harbor potential homozygous mutations at individual loci on kanamycin plates with kanamycin-resistant *E. coli*. We screened for mutants that would no longer show TEI of the predatory mouth form and, instead, have low probability of developing the Eu mouth form (θ) on *E. coli* similar to naïve worms [$0.115 \leq \text{HDI}(\theta_{\text{Control}}) \leq 0.436$] (Fig. 3A). After screening the F15R1 and rescreening the F15R2 to F15R4 generations, a total of 75 mutants were found with a transgenerational inheritance-defective (Tid) phenotype and a 20 to 40% Eu mouth-form ratio. We included mutant lines with additional morphological defects, i.e., egg-laying-defective (Egl) (see below) or uncoordinated-movement mutants, but excluded mutants that were completely Eu form defective (Eud), as these are likely mutants with mutations in mouth-form developmental switch genes, such as *eud-1* (28). We performed whole-genome sequencing of these mutants and searched for genes with multiple independent alleles. We set an arbitrary threshold of three alleles to consider a gene as a candidate based on previous experience (46, 54). We found mutations in *Ppa-ebax-1* and *Ppa-daf-21/Hsp90* in three independent Tid alleles (table S1). Both genes are 1:1 orthologous to the corresponding genes in *C. elegans* (fig. S3, A to B), where they have been implicated in several biological processes (55–59).

Substrate-receptor E3 ubiquitin ligase EBAX-1 is required for the TEI of the predatory morph

The identification of the Elongin BC-binding axon regulator *ebax-1* as a potential candidate gene involved in TEI of the predatory mouth form was intriguing. In *C. elegans*, *ebax-1* was first identified as part of the protein quality control mechanism that guards the SAX-3/Robo receptor in developing neurons (58). While this original study had suggested an interaction of EBAX-1 with the heat shock protein DAF-21/HSP90 in *C. elegans*, more recent studies indicated that *Cel-EBAX-1*, like its human and *Drosophila* homologs ZSWIM8 and Dora, is also involved in microRNA (miRNA) turnover (55–57). These studies suggested a different type of miRNA regulation,

referred to as “target-directed miRNA degradation” (TDMD). Current models assume that, after the pairing of an mRNA or long non-coding RNA trigger with an Ago-miRNA complex, a ubiquitin ligase will be recruited, causing the ubiquitination and proteasomal degradation of the Ago protein (Fig. 4A) (55, 56, 60). Subsequently, the miRNA is degraded by cellular nucleases, resulting in a reversed regulatory outcome from canonical miRNA-mediated gene silencing (61, 62). In mice, ZSWIM8 is known to destabilize many miRNAs that occur in miRNA clusters (60). In addition to EBAX-1/ZSWIM8, also DAF-21/Hsp90 has been reported to be involved in miRNA regulation, i.e., the possible loading of highly abundant miRNAs of the *miR-35* family into Ago proteins in *C. elegans* oocyte (63, 64). The isolation of *Ppa-ebax-1* mutants in our screen for a Tid phenotype could suggest a role of ubiquitin ligase and miRNA signaling in the transmission of epigenetic memory in *P. pacificus*.

To start testing these hypotheses, we first generated CRISPR alleles of *Ppa-ebax-1* in the RSC011 background, one of which has a frameshift mutation [*Ppa-ebax-1(tu1754)*] (Fig. 3B). While this allele results in a premature stop codon, two other alleles, *Ppa-ebax-1(tu1755)* and *Ppa-ebax-1(tu1756)*, are in-frame deletions of 60 base pairs (bp) and 12 bp, respectively. All three alleles are viable and grow normal on *E. coli*; however, the *Ppa-ebax-1(tu1754)* allele resulting in a frameshift has locomotion defects and a strong Egl phenotype similar to *Cel-ebax-1* mutants (fig. S3, C to E). When grown on an *E. coli* diet, all three *Ppa-ebax-1* mutants have an Eu mouth-form ratio similar to wild-type animals (Fig. 3C and fig. S3, F and G). Similarly, all three *Ppa-ebax-1* alleles are strongly but not completely induced on the *Novosphingobium* diet with the mean probability of developing the Eu mouth form $0.859 \leq \bar{\theta}_{tu1754} \leq 0.916$ (Fig. 3C and fig. S3, F and G). In contrast, all three mutant lines returned immediately to the baseline Eu mouth-form ratio of RSC011 [$0.113 \leq \text{HDI}(\theta_{R1(tu1754)}) \leq 0.423$] after food reversal (Fig. 3, C and D, and fig. S3, F and G). Thus, these experiments indicate a role for *Ppa-EBAX-1* in transgenerational inheritance of the predatory mouth form and provide the first evidence of a role of ubiquitin ligases in such processes.

The *daf-21/Hsp90* inhibitor radicicol eliminates TEI of the predatory mouth form

Next, we tested whether *Ppa-daf-21/hsp90* is also involved in TEI of the Eu mouth form. As previous work in the *P. pacificus* wild-type strain PS312 had indicated that CRISPR-induced frameshift mutations in *Ppa-daf-21/hsp90* resulting in loss-of-function alleles are lethal (59), we tried to rebuild the amino acid substitutions in *Ppa-daf-21* obtained after EMS mutagenesis using CRISPR editing. However, we were unsuccessful to rebuild such mutations, and all obtained frameshift mutations in *Ppa-daf-21* were lethal. Therefore, we used the HSP90 inhibitor “radicicol” that is known to specifically target only HSP90 family members in many organisms including *P. pacificus* (59). We grew wild-type RSC011 animals on *Novosphingobium* and added the radicicol inhibitor during the reversal to *E. coli* starting in the F5R2 and F10R2 generations, respectively (Fig. 3, E and F). In contrast to untreated animals (Fig. 3G), addition of radicicol in the F5R2 generation caused an immediate reduction in the probability of developing the Eu mouth form from $\theta_{R1} = 0.874$ [$0.752 \leq \text{HDI}(\theta_{R1}) \leq 0.975$] to $\theta_{R2} = 0.424$ [$0.239 \leq \text{HDI}(\theta_{R1}) \leq 0.609$] (Fig. 3E). We found a similar reduction in Eu frequency when applying radicicol after an exposure to *Novosphingobium* for 10 generations (Fig. 3F). These results indicate that, similar to *Ppa-EBAX-1*, *Ppa-DAF-21/HSP90* is required for TEI of the predatory mouth form.

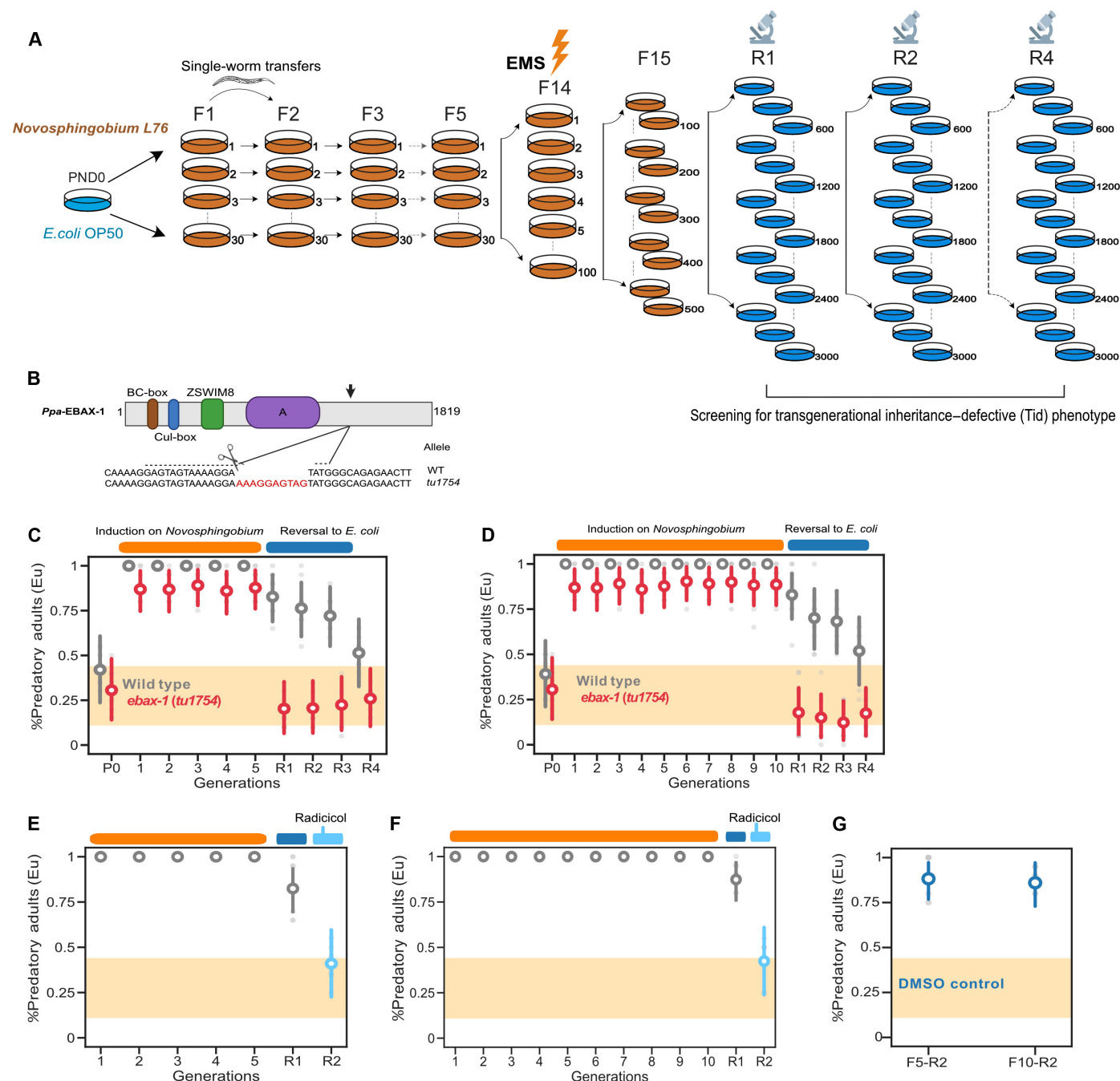


Fig. 3. Long-term environmental induction combined with forward genetic screening identifies regulators of transgenerational inheritance. (A) Graphical representation of the EMS mutagenesis experiment performed on the F14 generation on a *Novosphingobium* diet. Screening for transgenerational inheritance-defective (Tid) mutants was performed after reversal to *E. coli*, and mutant candidates were rescreened in subsequent generations. Some icons are from BioRender.com. (B) Domain composition of the substrate-receptor E3 ubiquitin ligase *Ppa-EBAX-1* that consists of 1819 amino acids. We generated CRISPR-Cas9-induced mutation in the predicted intrinsically disordered region of *Ppa-EBAX-1* as highlighted (arrow). Insertion of a 10-bp fragment results in a frameshift allele. WT, wild type. (C) Mean probability of the predatory mouth form in *Ppa-ebax-1* mutant animals upon *Novosphingobium* exposure for 5 generations and (D) 10 generations and reversal to *E. coli*. (E) Mean probability of the predatory morph upon radicicol treatment during reversal (R2) to *E. coli* after 5 generations and (F) 10 generations of *Novosphingobium* exposure. (G) Mean probability of the predatory morph on *E. coli* supplemented with dimethyl sulfoxide (DMSO) as controls for the radicicol supplementation experiment. Orange and blue ovals above [(C) to (F)] indicate generations on *Novosphingobium* and *E. coli*, respectively. Light blue ovals above [(E) and (F)] show generations exposed on *E. coli* supplemented with radicicol. The 95% HDI for the means is based on Bayesian model and shows the probability of developing the Eu morph inferred from the experimental data. Yellow area highlights the RSC011 baseline response averaged across 101 generations on *E. coli*.

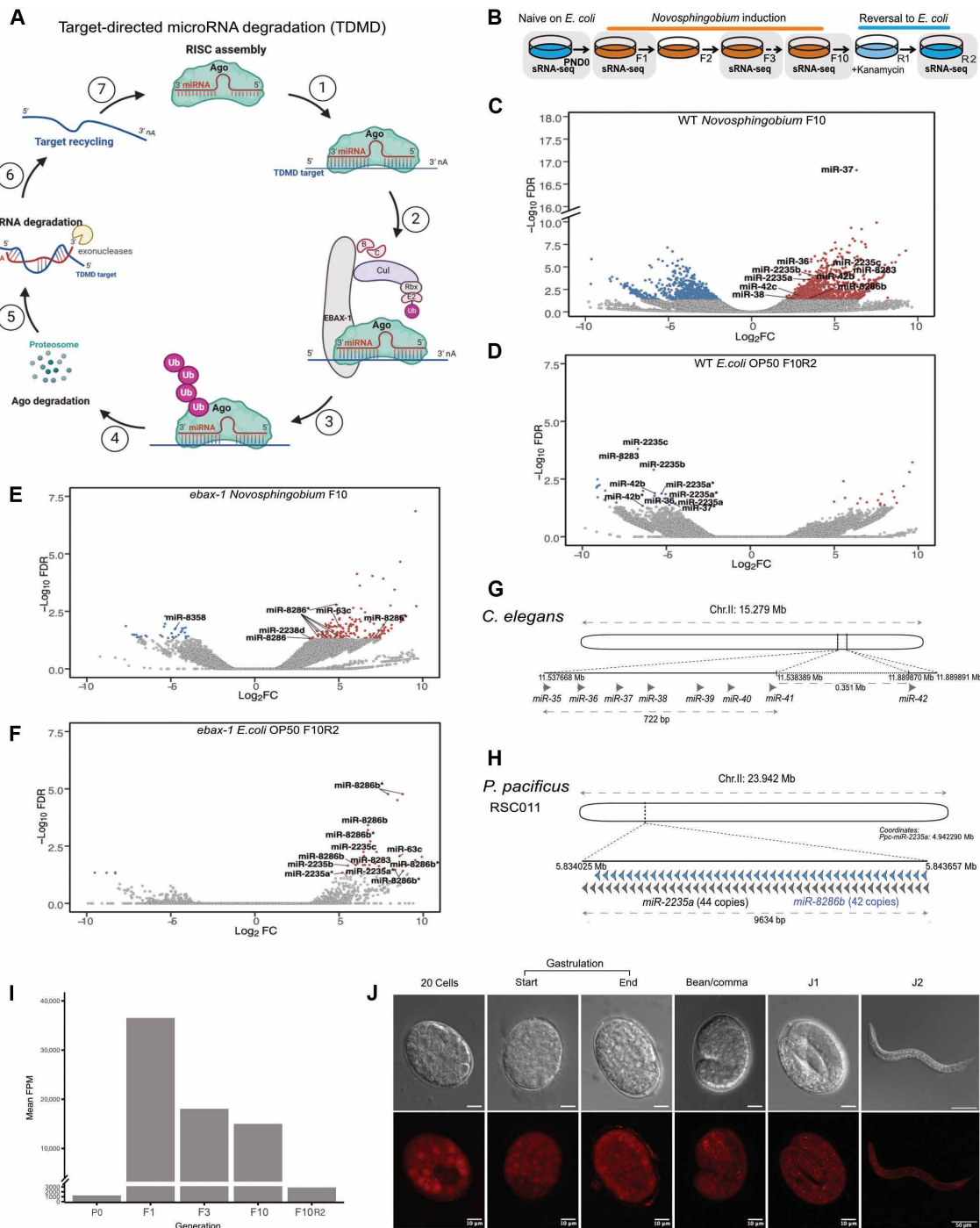


Fig. 4. *Ppa-ebax-1* destabilizes levels of *miR-2235/miR-35* family in *P. pacificus*. (A) Molecular model for target-directed miRNA degradation (TDMD) based on recent studies in mammals and flies. Extensive miRNA-target pairing is recognized by the ZSWIM8/EBAX-1 ubiquitin ligase and results in subsequent polyubiquitination of the Argonaute (Ago) protein, leading to Ago proteolysis and miRNA decay. Created with BioRender.com. RISC, RNA-induced silencing complex. (B) Experimental design for small RNA (sRNA) sequencing (sRNA-seq) before, during and after *Novosphingobium* induction. Worm generations used for sequencing are highlighted in gray. (C) sRNA-seq of lines exposed to *Novosphingobium* for 1, 3, or 10 generations compared with naïve unexposed animals ($n = 2$ biological replicates for F10 *Novosphingobium*, three biological replicates for P0). Log₂FC, log₂ fold change. (D) sRNA-seq of worms after reversal to *E. coli* for two generations compared with lines exposed to *Novosphingobium* for 10 generations ($n = 2$ biological replicates for each condition). (E) sRNA-seq of *Ppa-ebax-1* mutants compared to wild-type controls, both exposed to *Novosphingobium* for 10 generations ($n = 2$ biological replicates for each condition). Asterisk *, miRNA isoforms. (F) sRNA-seq of *Ppa-ebax-1* *E. coli* OP50 F10R2. Asterisk *, miRNA isoforms. (G) A schematic representation of the *miR-35* family members in *C. elegans* (*miR-35* to *miR-41*). Gray arrowheads, *miR-35* members. (H) A schematic representation of the heavily expanded *miR-2235a/miR-35* locus in *P. pacificus*. Gray arrowheads, *miR-2235a*; blue arrowheads, *miR-8286b*. (I) Mean FPM (fragments per million) counts of *miR-2235a* on naïve *E. coli* condition (P0), on *Novosphingobium* (F1, F3, and F10), and upon reversal to *E. coli* (F10R2). (J) The *Ppa-miR-2235a* promoter region drives ubiquitous expression during embryogenesis and early juvenile stages. *Ppa-miR-2235a*::TurboRFP transcriptional reporter construct is observed starting from 20-cell stage throughout the embryo.

A *Novosphingobium* diet results in up-regulation of the heavily expanded *miR-2235a/miR-35* family

Given the role of *Cel-ebax-1* and its *Drosophila* and human homologs in TDMD (Fig. 4A) (55–57), we wanted to know whether *Ppa-EBAX-1* also affects miRNA expression. Therefore, we first performed small RNA (sRNA) sequencing of wild-type animals at five different time points throughout the dietary induction experiment (Fig. 4B). Specifically, we sequenced naïve animals at the beginning of the experiment; the F1, F1, and F10 generations on *Novosphingobium*; and the F10R2 generation after reversal back to *E. coli*. Note that, because of nematode size, all sequenced mixed-stage samples had to grow for up to two more generations to collect sufficient material; thus, the “F3” samples were technically F4–F5 generations. To identify diet-sensitive sRNAs, we considered adjusted *P* value < 0.05 for capturing differential expression across conditions. Naïve worms exposed to *Novosphingobium* for 10 generations resulted in the differential expression of 2644 sRNAs, of which 1538 were up-regulated (Fig. 4C and data S1A). For the identification of miRNAs in this dataset, we used the previously established catalogue of *P. pacificus* miRNAs that are publicly available at miRBase (65–70) (for details, see Materials and Methods). Among the up-regulated sRNAs in all tested generations on *Novosphingobium* were 30 miRNA species, 9 of which belong to the *miR-35* family that is known to be regulated by EBAX-1 in *C. elegans* (Fig. 4C and data S1A) (55, 71). After the reversal of wild-type worms to the *E. coli* diet, there are only 45 differentially expressed sRNAs relative to the *Novosphingobium* F10 generation, most of which (26 of 45) is down-regulated (Fig. 4D and data S1B). This group of down-regulated sRNAs contains six of the nine *miR-35* family members that were previously significantly up-regulated. When we tracked the expression pattern of the most abundant *miR-35/miR-2235a* member over time, we observed an immediate *miR-2235a* induction in the F1 generation on *Novosphingobium* (Fig. 4I). This was followed by decreased expression in later generations and after reversal in the F10R2 generation (Fig. 4I). Most of the *miR-35* family members are similarly induced on *Novosphingobium*, and they are down-regulated in the F10R2 generation (fig. S4C). Thus, members of the *P. pacificus* *miR-35* family strongly respond to a change in diet.

In *C. elegans*, the *miR-35* family consists of eight miRNAs (*miR-35–miR-42*) whose genes are located on a single chromosome with seven of them being clustered within a 700-bp region in an intron of another gene (Fig. 4G) (71, 72). These miRNAs are loaded into the *C. elegans* oocyte where they represent the most abundantly expressed miRNA; however, during embryogenesis, these miRNAs are largely degraded (71). Deletion of the *miR-35–miR-41* family members results in strong phenotypic defects, whereas the additional deletion of *miR-42* causes embryonic lethality (72, 73). In *P. pacificus*, several but not all members of the *miR-35* family have been annotated in miRBase. When we mapped the entirety of the *miR-35* family onto the *P. pacificus* genome, we found three major differences compared to *C. elegans*. First, *P. pacificus* contains many more members of this miRNA family, several of which have been designated as *miR-2235a*, *miR-2235b*, and *miR-2235c* (fig. S5A). Second, single copies of *miR-35* family members are found on all but one chromosome (fig. S5A). Most notably, however, one locus in the *P. pacificus* RSC011 genome contains 44 identical copies of *miR-2235a* on chromosome II (Fig. 4H). These copies are evenly spaced with a nearly identical spacer of 202 bp. Intriguingly, we observed another miRNA, *miR-8286b*, from a different miRNA family in this spacer with

a total of 42 identical copies (Fig. 4H). As a result, this locus of ~10 kb in length contains more than 80 miRNAs in an otherwise gene desert (Fig. 4H). Note that, in contrast to *miR-2235a*, there are no other copies of *miR-8286b* elsewhere in the *P. pacificus* RSC011 genome. The levels of *miR-8286b* are also significantly up-regulated when worms are grown on *Novosphingobium* (Fig. 4C and data S1A). Genome-wide analysis revealed that the *miR-2235a* cluster on chromosome II is the largest cluster detected in the *P. pacificus* genome and is unique in harboring identical copies of the same miRNA and containing another interspersed miRNA in the spacer region (fig. S5B). Note that a recent developmental sRNA transcriptomic study in *P. pacificus* found that *miR-2235a* is one of the most abundantly expressed miRNAs and similar to *C. elegans*, and its expression ceases during early development (74). Together, dietary changes in *P. pacificus* result in differential expression of sRNAs including a heavily expanded family of miRNAs that in *C. elegans* is destabilized by the ubiquitin ligase EBAX-1 (55).

Absence of *Ppa-ebax-1* results in the up-regulation of the *miR-2235a* family

Next, we carried out sRNA-seq of *Ppa-ebax-1(tu1754)* mutant animals at the F10 and F10R2 time points during dietary induction and compared differential expression of sRNAs to that of wild-type animals. Growing *Ppa-ebax-1(tu1754)* mutants on *Novosphingobium* resulted in 206 sRNA being differentially expressed relative to wild-type animals (Fig. 4E and data S1C). Of these 206 sRNAs, 172 were significantly up-regulated in *Ppa-ebax-1(tu1754)* mutants. All *miR-35* family members, including *miR-2235a*, that were significantly up-regulated on *Novosphingobium* in the wild-type background, are similarly up-regulated in *Ppa-ebax-1(tu1754)* mutant animals (Fig. 4E and data S1C). In contrast, after reversal to *E. coli*, in the F10R2 generation, there are 35 sRNAs that are differentially expressed in *Ppa-ebax-1(tu1754)* mutants relative to wild type (Fig. 4F and data S1D). Of these 35 sRNAs, 31 are significantly up-regulated, including three members of the *miR-35* family with the exact mature sequence of *miR-2235c-3p*, *miR-2235b-3p*, and *miR-8283-3p* (Fig. 4F and data S1D).

Recent studies in mice and flies indicated that miRNA processing can result in trimmed and tailed miRNA versions (75). To capture tailing and trimming events at the 3' end of miRNAs during *Novosphingobium* exposure and reversal to *E. coli*, we used the first 18-nucleotide (nt) of the mature miRNA sequence in the *miR-2235a* locus and combined all miRNA isoforms into single counts. We found 10 miRNA variants associated with the *miR-2235a* locus are also significantly up-regulated in *Ppa-ebax-1(tu1754)* mutants relative to wild type (Fig. 4F and data S1D). This includes two variants that are longer (<23 nt) and shorter (21 nt) versions of *miR-2235a* and eight variants of *miR-8286b* that are longer 3' isoforms (20 to 26 nt long) (Fig. 4F and data S1D). These variants contain nucleotides that do not exist in the parental gene sequence and can, therefore, be considered as non-templated (76). Further analysis revealed that the tailed and trimmed versions of the mature *miR-2235a* and *miR-8286b* are up-regulated upon the loss of *Ppa-ebax-1* (fig. S4, A and B). Last, the 22-nt mature *miR-2235a* and 19-nt mature *miR-8286b* miRNAs are also up-regulated but not significantly (adjusted *P* value > 0.05). Together, these findings indicate that the loss of *Ppa-ebax-1* results in the up-regulation of several members of the highly expanded and repetitive *miR-2235a* locus. In addition, the observation of non-templated nucleotide additions and removals at the

3' end of *miR-2235a* and *miR-8286b* might suggest that they are subject to TDMD or TDMD-like processes. This would be consistent with a repressive role of the *miR-2235a* cluster in TEI of the predatory mouth form.

Target site prediction and expression of *miR-2235a* and *miR-8286b*

The identification of primary triggers of EBAX-1/ZSWIM8/Dora-mediated miRNA degradation has been shown to be challenging (77). Work in vertebrates so far identified only four (78–80), whereas studies in *Drosophila* found six endogenous RNA triggers (57). While TDMD-inducing targets usually exhibit extended base pairing with the miRNA 3' end (55–57), studies in *C. elegans* suggested that, for the decay of *miR-35* family members, the seed sequence of the miRNA is sufficient (71). Therefore, it is possible that mechanisms involved in the transgenerational inheritance of the predatory mouth form in *P. pacificus* might not require extensive pairing beyond the 3' end. We used existing bioinformatic pipelines to search for potential triggers (77) and found hundreds of predicted matches with full or partial complementarity to *miR-2235a* in the *P. pacificus* genome (fig. S5C and data S3). Expectedly, relaxation of sequence complementarity from 8 to 6 nt increased the number of seed matches in predicted 3' untranslated region (3'UTR) binding sites (fig. S5C). However, we did not identify matches with known genes in the mouth-form GRN. Note that a similar analysis with *miR-8286b* showed higher numbers of potential 3'UTR binding sites, while the number of potential matches with extended complementarity (TDMD) is lower for *miR-8286b* (fig. S5C and data S3).

To study the expression of the miRNAs in the *miR-2235a* cluster, we generated a reporter construct using a 1.4-kb fragment upstream of the first copy of *miR-2235a* in the cluster fused to a *Pristionchus*-codon optimized TurboRFP (81). We detected strong zygotic expression in early embryogenesis similar to *miR-35* expression in *C. elegans* (Fig. 4J) (82). In contrast to *C. elegans*, the red fluorescent protein (RFP) expression persisted throughout the J1 stage, which, in *P. pacificus*, remains in the egg, and the J2 stage after hatching (Fig. 4J). However, there is no detectable expression in later postembryonic stages, consistent with the miRNA abundance reported in developmental sRNA transcriptomics (74). The expression of this reporter responds to the *Novosphingobium* diet, resulting in a significantly higher expression in the F5 generation relative to naïve *E. coli*-grown cultures when comparing staged J1 juveniles (fig. S4D). Similar to the *miR-2235a* reporter construct, a *Ppa-ebax-1* transcriptional promoter construct showed a dynamic embryonic and juvenile expression starting from the comma stage and peaking in the J1 stage with expression in the pharynx and tail region (fig. S3H). In adult stages, *Ppa-ebax-1p::TurboRFP* is expressed in pharyngeal neurons, in the vulval region, and in the gonadal tissues (fig. S3, I and J).

Deletions of the *miR-2235a* locus are viable

Given the up-regulation of *miR-2235a* in *Ppa-ebax-1* mutants and the tailing and trimming of miRNAs of the *miR-2235a* cluster, we speculate that *miR-2235a* may play a repressive role in the TEI of the predatory mouth form. However, to the best of our knowledge, miRNAs have, so far, not been shown to be involved in transgenerational inheritance, which is different from siRNA and Piwi-interacting RNAs (piRNAs) (19). To study whether miRNA-mediated gene regulation including *miR-2235a* is involved in the TEI of the

predatory mouth form, we targeted the *miR-2235a* locus. Note that targeting the *miR-2235a* locus for deletion by CRISPR is challenging because large-scale deletions of more than 1 kb have not been regularly reported in *P. pacificus* (81). Nonetheless, the highly repetitive nature of the 10-kb locus might allow the use of single guide RNA (sgRNAs) that can bind repeatedly to target sequences in this region. We were able to generate a sgRNA that potentially binds to the *miR-2235a* sequence (Fig. 5A). To identify deletions of most or even the entire locus, we designed polymerase chain reaction (PCR) primers in the flanking regions with unique sequence specificity (Fig. 5A). Note that PCR experiments with these primers will not result in PCR products in wild-type animals because standard PCR conditions do not result in the amplification of 10-kb fragments (fig. S5D). In contrast, large deletions in the *miR-2235a* locus might result in smaller fragments that can be amplified. Using this strategy, we isolated two mutant lines out of 200 tested progenies of CRISPR-injected animals with large deletions in the *miR-2235a* locus. Sequencing analysis showed that both mutant lines exhibited nearly a complete deletion of the *miR-2235a* locus (Fig. 5B). Specifically, original sequencing of PCR-amplified products revealed that *miR-2235(tuDf9)* and *miR-2235(tuDf10)* carry deletions of 9408 and 9632 bp, respectively, with two and one copies of *miR-2235a* remaining (Fig. 5B). Whole-genome resequencing of both mutant lines confirmed the exact size of these deletions and the fact that only one and two copies of the miRNA are still present (Fig. 5C).

Both of these deficiencies did not result in the deletion of the entire cluster maybe because such lines would be embryonic lethal similar to the knockout of *C. elegans miR-35-miR-42*. To directly test this hypothesis, we performed another CRISPR experiments in the *miR-2235a(tuDf9)* mutant background to delete the remaining miRNA copies using the original sgRNA. We were able to isolate a mutant, *miR-2235(tuDf11)*, in which the entire *miR-2235a* cluster is deleted (Fig. 5B). *miR-2235(tuDf11)* mutant animals are viable and phenotypically wild type with no obvious growth defects when cultured on a *E. coli* or *Novosphingobium* diet. Deficiency alleles with partial and complete deletions of the cluster showed significant reduction of sRNA expression or negligible *miR-2235a* and *miR-8286b* expression relative to wild-type animals (fig. S5E). In the following, we used all three deficiencies at the *miR-2235a* locus to study a potential role of *miR-2235a* in transgenerational inheritance and to investigate the effect on global gene expression.

Deletions of the *miR-2235a* cluster cause precocious and extended transgenerational inheritance

If *miR-2235a* expression would repress TEI of the Eu mouth form after food reversal, then the deletion of the *miR-2235a* locus might result in an earlier onset of TEI and/or an extension of TEI after reversal. Therefore, we used all three *miR-2235a* alleles and reverted them back to *E. coli* after one and three generation exposures to *Novosphingobium*. In addition, we reverted mutants after five generations to investigate potential longer durations of TEI of the predatory mouth form. We found that all three *miR-2235a* mutants show precocious transgenerational inheritance. Specifically, in the F1R1 and F1R2 generations, mutants exhibit an elevated probability of developing the predatory mouth form [$0.844 \leq \text{HDI}(\theta_{\text{F1R1-tuDf11}}) \leq 0.994$, and $0.709 \leq \text{HDI}(\theta_{\text{F1R2-tuDf11}}) \leq 0.957$]. However, the F1R3 generation [$0.365 \leq \text{HDI}(\theta_{\text{F1R3-tuDf11}}) \leq 0.738$] was not credibly different from the base line [$0.115 \leq \text{HDI}(\theta_{\text{Control}}) \leq 0.436$] (Fig. 5D). Thus, a

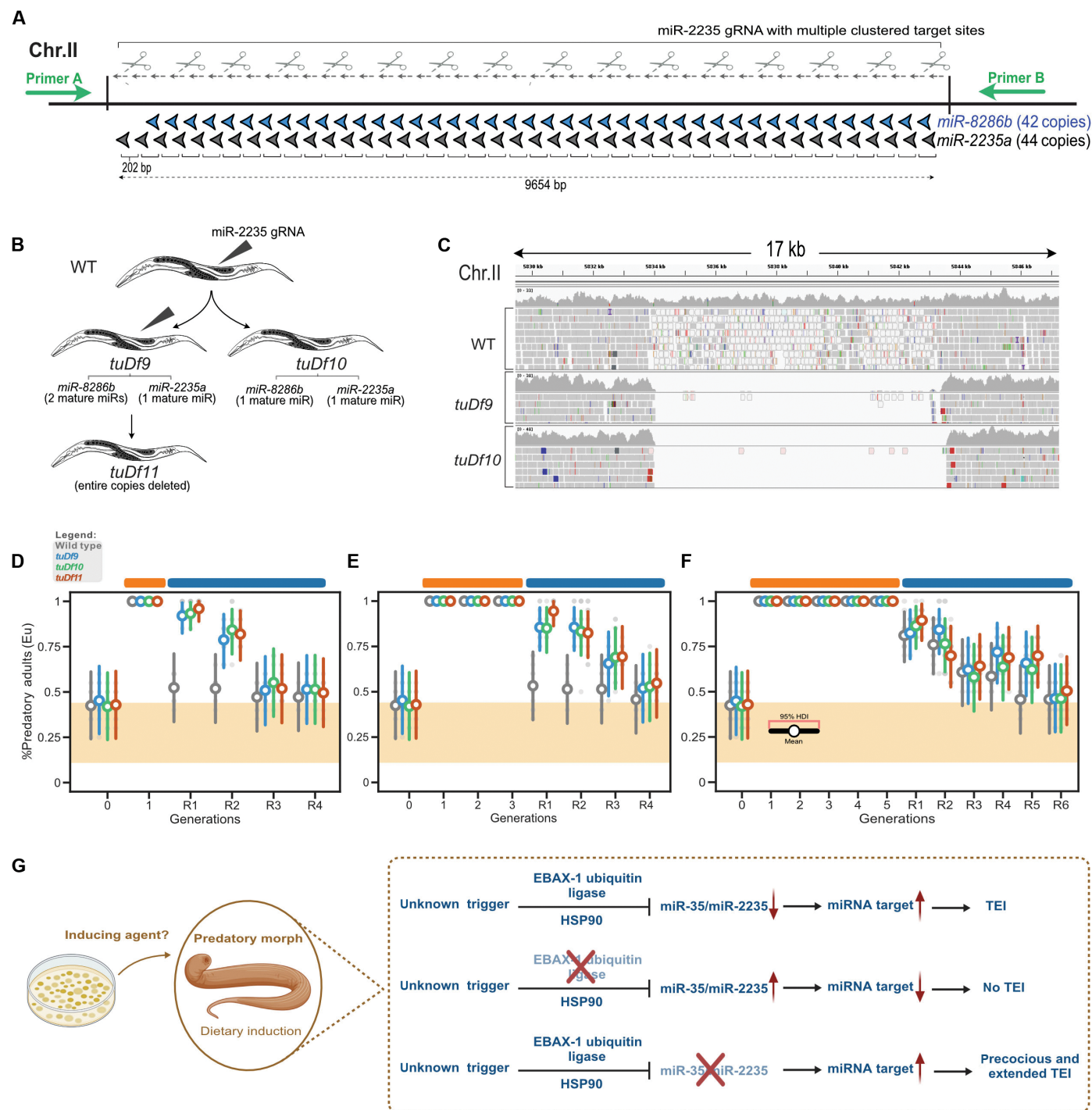


Fig. 5. Deletion of the *miR-2235a* locus results in precocious and extended transgenerational inheritance of the predatory mouth form. (A) Design of the CRISPR-Cas9 experiment to induce deletions of the *miR-2235a* locus. A sgRNA with multiple binding sites in *miR-2235a* was used. Primers flanking the target locus (green arrowheads) were designed to amplify shorter fragments as a consequence of large deletions. (B) A schematic for the CRISPR-Cas9-induced deletion for all three deficiency alleles. (C) Whole-genome resequencing confirms large deletions of the *miR-2235a* locus in the homozygous viable mutant lines *tuDf9* and *tuDf10*. (D) Mean probability of the predatory mouth form of all deficiency alleles after exposure to *Novosphingobium* for one generation, (E) three generations, and (F) five generations followed by reversal to *E. coli*. Wild type, gray; blue, *tuDf9*; green, *tuDf10*; red, *tuDf11*. Orange and blue ovals above (D) to (F) indicate generations on *Novosphingobium* and *E. coli*, respectively. The 95% HDI for the means is based on Bayesian model and shows the probability of developing the Eu morph inferred from the experimental data. The yellow area displays the upper and lower limits of the RSC011 baseline averaged across 101 generations on *E. coli*. (G) Proposed model depicting transgenerational inheritance of the predatory morph in wild-type animals and *ebax-1* and *miR-2235a* alleles as a result of *Novosphingobium* induction. A now unknown trigger after *Novosphingobium* exposure induces *Ppa-ebax-1* activity leading to *miR-2235a* destabilization. As a consequence of miRNA degradation, target gene(s) will be expressed resulting in TEI of the Eu morph.

one-generation exposure to *Novosphingobium* results in intergenerational inheritance of the Eu morph that lasts for two generations. In contrast, reversal after three generation exposure to *Novosphingobium* results in precocious transgenerational inheritance with $[0.863 \leq \text{HDI}(\theta_{\text{F3R1-tuDf11}}) \leq 0.998, 0.688 \leq \text{HDI}(\theta_{\text{F3R2}}) \leq 0.941, \text{ and } 0.518 \leq \text{HDI}(\theta_{\text{F3R3}}) \leq 0.86]$, whereas wild-type animals showed no TEI after such early reversals as shown previously (Fig. 5, D and E). Together, these results indicate that the deletion of the *miR-2235a* locus results in precocious transgenerational inheritance.

Given this phenotypic evidence, we assessed the expression dynamics of the *P. pacificus* transcriptome in the *miR-2235a* mutant condition. Specifically, we examined the differentially expressed genes between the *miR-2235a(tuDf9)* mutant and wild-type animals during precocious transgenerational inheritance in the F3R5 generation. We found 1714 genes that were differentially expressed, of which 1050 were significantly up-regulated in the *miR-2235a(tuDf9)* mutant (fig. S5F). Of the 1050 up-regulated genes, only 12 genes overlap with the predicted set of genes whose 3'UTRs have been predicted to have the *miR-2235a* TDMD binding site. However, some of the predicted *miR-2235a* targets showed a partial induction relative to expression in wild-type animals. We conclude that the deletion of the *miR-2235a* locus has a global effect on gene expression.

Last, we reverted *miR-2235a* mutants back to *E. coli* after 5 generations to test for a potential extension of the TEI of the predatory mouth form. We observed TEI of the Eu mouth form lasting for 5 generations $[0.524 \leq \text{HDI}(\theta_{\text{F5R5-tuDf11}}) \leq 0.863]$ (Fig. 5F), whereas wild-type animals returned to baseline level lower than 50% Eu in the F5R5 generation (Fig. 5F). Thus, deletions of the *miR-2235a* locus not only result in precocious but also extended TEI of the predatory mouth form. Together, our experiments support a role of *miR-2235a* as a repressor of transgenerational inheritance, and they are consistent with *Ppa-EBAX-1* being involved in TDMD.

DISCUSSION

This study establishes a synthesis of multiple innovative approaches to investigate transgenerational inheritance. Conceptually, the coupling of long-term environmental induction experiments with the analysis of a natural, phenotypically plastic trait and the subsequent involvement of forward genetic analysis revealed mechanistic insight into the machinery involved in memory transmission. This methodology will allow a detailed investigation of associated processes and signifies *P. pacificus* mouth-form plasticity as a model to investigate transgenerational inheritance. The use of forward mutagenesis provides an unbiased approach to identify genes involved in the transmission of non-genetic information across generations. The experiment described in this study used 3000 “F2” progeny of mutagenized animals and is, thus far, from saturation. However, saturation mutagenesis is feasible in *P. pacificus* because of the short generation time and easy husbandry similar to *C. elegans*. In addition, this study relies on a binary readout that builds on the developmental plasticity of feeding structures. This feeding structure plasticity is not limited to *P. pacificus* but is a specific characteristic of most of nematodes of the family Diplogastridae to which *P. pacificus* belongs (83). It is the diversity of feeding structures that has resulted in an extraordinary ecological diversification of these nematodes, highlighting the importance of plasticity as facilitator for evolutionary change (83–85). Therefore, this study provides

important findings for developmental plasticity, associated TEI, and the potential role of TEI for evolution.

Mutant analyses described in this work indicate that *Ppa-ebax-1* and the *miR-2235a* locus are involved in TEI of the Eu mouth form after induction through a *Novosphingobium* bacterial diet. One way to explain these findings is that the ubiquitin ligase is required for the induction of the predatory mouth form and its transgenerational inheritance by repressing miRNAs in *P. pacificus*, which would, otherwise, repress the TEI of the predatory morph. We speculate that this process involves TDMD or a TDMD-like mechanism by targeting Ago protein(s) that is(are) core component(s) of the RNA-induced silencing complexes (Fig. 5G). Specifically, a now unknown trigger, after exposure to *Novosphingobium*, induces *Ppa-EBAX-1* activity on an Ago-*miR-2235a* complex, which, in wild-type animals, will cause the degradation of Ago protein(s) and *miR-2235a*. This will result in the expression of target gene(s) that will lead to TEI of the Eu mouth form. In *Ppa-ebax-1* mutants, the Ago-*miR-2235a* complex is no longer destroyed, target gene(s) is(are) repressed, and TEI of the predatory mouth form does not occur (Fig. 5G). Similarly, the response to the *Novosphingobium* diet is incomplete in *Ppa-ebax-1* mutants, indicating a partial role of *Ppa-EBAX-1* for the dietary induction of the predatory mouth form. Consistent with this model, the large or complete deletion of the *miR-2235a* locus results in precocious and extended TEI of the predatory morph, whereas mutations in *Ppa-ebax-1* result in the absence of transgenerational inheritance.

It is important to note that the extent to which the observed phenotypes of *Ppa-ebax-1* mutants and deletions of the *miR-2235a* locus are due to processes involving TDMD with extended pairing of the miRNA and its trigger remains now unknown. Formally, we cannot rule out alternative models, in which *Ppa-EBAX-1* and *miR-2235a* are involved in different molecular processes associated with TEI of the Eu mouth form. *EBAX-1/ZSWIM8/Dora* are known to be involved in mechanisms of miRNA regulation other than TDMD (57, 71). Our differential gene expression analysis of *miR-2235a* mutants indicated global gene expression changes relative to wild-type animals, which would be consistent with a broader role of *miR-2235a*. This differential gene expression analysis, together with our computational target site prediction pipeline, is essential to prioritize potential *miR-2235a* target genes that will be systematically tested in future studies. Note that such experimental validation will require the generation of (i) deletion mutants of the 3'UTR binding sites, (ii) knockout mutants in the respective genes, and (iii) additional overexpression studies of the genes of interest.

While *P. pacificus* shares with *C. elegans* the viability of *ebax-1* mutants, it displays one genomic feature that partially limits some aspects of future analysis. *C. elegans* contains two copies of many major Ago genes, i.e., *alg-1/2* and *alg-3/4*. Although the reasons for these gene duplications are unknown, this “double gene set” has resulted in single mutants in these genes being viable in *C. elegans* [for review, see (19)]. In *P. pacificus*, in contrast, only one copy of *alg-1/2* and *alg-3/4* exists, similar to the miRNA-Ago complex cofactor *ain-1/2* known as GW182 in mammals (86). Any attempt to knockout *Ppa-alg-1/2* or *Ppa-ain-1/2* was unsuccessful as homozygous mutants are lethal. This genomic composition limits the genetic analysis of Ago proteins and their involvement in miRNA signaling and transgenerational inheritance in *P. pacificus*.

In conclusion, transgenerational phenomena come in different forms and flavors. Many of them last for three to five generations,

whereas others have been reported to last for more than 20 or even 300 generations (7, 16, 53, 87, 88). Some cases show stochastic inheritance, others exhibit distinct segregation patterns that have been described to follow major rules (13). The molecular machinery involved in TEI points toward an important role of sRNA molecules, Ago proteins, and also histone modifications. Already early studies suggested a key role of piRNAs (7) in *C. elegans*; however, a role of miRNAs has previously not been documented to regulate TEI. Our findings demonstrate a unique example of developmental plasticity in an ecologically relevant trait that is accompanied by TEI of previous environmental information. These findings and the subsequent genetic and molecular investigations demonstrate a role of miRNAs in TEI. Together, this work provides a platform and adds a distinct model system to better understand the importance of developmental plasticity and transgenerational inheritance both from a mechanistic perspective and in its evolutionary context.

MATERIALS AND METHODS

Nematode culture conditions

The ancestral *P. pacificus* RSC011 isolate used in this study was frozen within the first 5 to 10 generations after original isolation from Coteau Kerveguen on La Réunion to minimize domestication and, thereby, facilitate the investigation of diet-induced plasticity and TEI. Nematodes were grown under standard nematode growth conditions on NGM plates seeded with either *E. coli* OP50 or *Novosphingobium* L76 and maintained at 20°C. We refrained from exposing the RSC011 stock to all other types of stressors such as bleaching, starvation, and extreme temperature fluctuations as they are known to potentially influence mouth-form ratios.

Bacterial strain conditions

All bacterial strains were grown overnight in Lysogeny broth supplemented with kanamycin (50 µg/ml) where required. Bacteria were grown at 30° or 37°C depending on the species and 6-cm NGM plates were seeded with 300 µl of bacterial overnight cultures and were incubated for 2 days.

LTEI approach

Long-term experimental evolution (LTEI) was performed using the ancestral *P. pacificus* RSC011 strain that is preferentially St (20 to 40% Eu) on a standard *E. coli* OP50 diet. Using the complete brood of a single hermaphrodite, 110 clonal lines were established by picking single J4 larvae on a *Novosphingobium* L76 diet. J4 worms were initially left for at least an hour on a *Novosphingobium* diet to reduce traces of *E. coli* OP50 before transferring to final F1 *Novosphingobium* plates. All 110 lines were transferred every 4 days to new plates by picking a single J4 animal to start the new generation. Rigorous worm maintenance was carried out for an entire year allowing the completion of 101 generations on *Novosphingobium*. Populations on *Novosphingobium* were periodically frozen every 10 generations (up to F100) and stored in liquid nitrogen that allows accurate evaluation of the evolved states.

Dietary reversal experiments

Overnight cultures of *E. coli* OP50 and *Novosphingobium* L76 were spread to NGM plates and incubated at room temperature (RT) for 2 days. Reversal experiments to an *E. coli* OP50 diet were performed after exposing of RSC011 worms for 15, 25, 35, 45, 55, 65, 75, 85, and

95 generations on *Novosphingobium* L76. Worms were initially transferred from *Novosphingobium* L76 to NGM plates supplemented with kanamycin (50 µg/ml) for one generation and then back to *E. coli* OP50-seeded NGM plates for subsequent generations. Exposure to kanamycin for a single generation to eliminate traces of *Novosphingobium* did not affect mouth-form response. Mouth-form phenotyping was examined for the entirety of the reversal experiments (all 15 generations).

Mouth-form phenotyping

Mouth-form phenotyping was performed using Zeiss Discovery V.20 stereo microscope (×150 magnification) by observing the nematode buccal cavity based on mouth-form identities previously described (28). Final mouth-form frequencies are the mean of at least 10 to 20 replicates (i.e., 110 replicates for the LTEI experiment; 10 to 20 replicates for genetic mutants, and the radicicol supplementation experiments). In each replicate, 20 animals from a single plate were assayed. For the LTEI experiment, phenotyping was done continuously for 25 generations on *Novosphingobium* and periodically every 5 generations (after F25) until 101 generations for all 110 lines. To ensure continuous tracking, a subset of 30 of the 110 lines was assayed every generation (F26-F29, F31-F34, etc.).

Statistical analysis

To estimate the probability of developing the Eu mouth form in *P. pacificus* (θ), we constructed a hierarchical Bayesian model (fig. S1, A and B)

$$y_i \sim \text{Bernoulli}(\theta)$$

$$\theta \sim \text{Beta}(\omega(\kappa - 2) + 1, (1 - \omega)(\kappa - 2) + 1)$$

$$\omega \sim \text{Beta}(\alpha, \beta)$$

$$\kappa \sim \text{Gamma}(3, 1)$$

where θ is calculated for each replicate and hyperparameters ω and κ link the biological replicates for a given generation under a given experimental condition (89). The model was fitted to the laboratory measurements using PyMC (90) in Python 3.11 with Numpy 1.25.2 (91). The mean highest density interval (HDI) for θ for a group of observations was used to visualize the inferred probability of developing the Eu mouth form. We ensured that the estimated θ values were stable using common convergence diagnostics with effective sample size of $\geq 10,000$ (92).

Mutation rate calculation

Paired-end Illumina reads were mapped to the RSC011 PacBio genome with bwa (version 0.7.17-r1188; algorithm, mem) (93). Subsequently, the germline short variant discovery workflow (85) from the GATK (4.2.5.0) pipeline was used to mark and remove duplicates (MarkDuplicatesSpark), locally realign reads, call single-nucleotide polymorphisms (SNPs), and indels per-sample (HaplotypeCaller) and perform joint calling across multiple samples (GenomicsDBImport and GenotypeGVCFs) (94). SNPs were selected with GATK SelectVariants (--select-type-to-include SNP). Line-specific SNPs were considered to have arisen through mutation accumulation if the

alternative allele was homozygous, detected on chromosomes, and met high-quality criteria [depth (DP) ≥ 5 and genotype quality (GQ) ≥ 20]. Mutation rate per line was determined by dividing the number of accumulated mutations in each line by the number of generation (100) and the sample-specific total length of sufficiently covered genomic regions (genome region with DP ≥ 5 in gVCF from GATK HaplotypeCaller for each sample). To mitigate potential biases introduced by problematic genomic regions, the number of line-specific SNPs in sliding windows (length, 100 bp; step, 20 bp) across the chromosomes was calculated, and those windows with more than two line-specific mutations were excluded from the analysis (95).

EMS mutagenesis

We combined long-term environmental induction to induce heritable mutations in *P. pacificus* and incubated a mixture of RSC011 J4 larvae grown on *Novosphingobium* L76 for 14 generations in M9 buffer [KH₂PO₄ (3 g/liter), Na₂HPO₄ (6 g/liter), NaCl 5 (g/liter), and 1 mM MgSO₄] with 47 mM EMS for 4 hours (96). Subsequently, worms were allowed to recover on agar plates with *Novosphingobium*, and 100 actively moving J4 larvae were singled out. After the mutagenized P0 animals had laid ~30 eggs, they were killed, and F1 progeny was allowed to develop and reach maturity. A total of 500 “F1” animals were singled out to establish new populations for one more generation on *Novosphingobium* before reversal. During reversal to kanamycin plates, we established 3000 F2 lines (which contained potential homozygous mutants) and allowed to lay F3 populations for mouth-form screening. Screening was performed for the progenies of F2 individuals (during reversal) using Discovery V20 stereomicroscope (see above). We searched for lines with Tid phenotypes, which show an immediate drop of the Eu response to 10 to 45%, whereas wild-type RSC011 animals keep a mouth-form memory of around 80 to 100% Eu after reversal. Mutant candidates with a Tid phenotype and a low Eu mouth-form frequency were transferred to fresh *E. coli* OP50 plates, and their progeny was rescreened for additional generations. Of the 3000 mutagenized F2’s progeny, 75 Tid candidate lines were isolated.

Whole-genome sequencing analysis of mutant lines

Mutant worms were grown on standard NGM plates before DNA extraction. We rinsed plates with M9 buffer and collected worm pellets by slow centrifugation at 1300 rpm for 3 min at 4°C. DNA was extracted with the Monarch Genomic DNA Purification Kit following the manufacturer’s protocol. Library preparation and sequencing were done by Novogene. The program BWA mem program (version 0.7.17-r188) (93) was used to align raw Illumina reads against the reference genome of the *P. pacificus* RSC011 strain. Initial variant calls were made by the SAMtools mpileup, bcfutils, and varFilter.pl commands (version 0.1.18-r982:295) (97). For candidate mutations, we filtered for high-quality, homozygous single-nucleotide substitutions that are not shared with resequencing data of the parental strain RSC011. The effect of mutations on protein coding genes was assessed by comparison with the RSC011 gene annotations (98). Last, the number of independent alleles per gene was computed to prioritize candidate genes. Given that classical mapping experiments are impractical for such an experimental design, all mutant candidate lines were whole-genome sequenced. We considered genes as candidates that had at least three independent alleles.

CRISPR-Cas9-induced mutations

Mutations were induced in candidate genes via CRISPR-Cas9 following the protocol described previously (81, 99). Gene-specific sgRNAs and universal trans-activating CRISPR RNA were purchased from Integrated DNA Technologies, and 5 μ l of each 100 μ M stock was mixed and denatured at 95°C for 5 min. After allowing the mixture to anneal at RT, Cas9 endonuclease (New England Biolabs) was added to the hybridized product and incubated at RT for 5 min. Tris EDTA buffer (TE buffer) was subsequently added to a final concentration of 18.1 μ M for the sgRNA and 2.5 μ M for Cas9. This was injected into the germline of 40 to 50 *P. pacificus* RSC011 young adults. Eggs from injected P0s were recovered up to 16 hours postinjection. Post-hatching and 2 days’ growth, these F1 worms were segregated onto individual plates until they had also developed and laid eggs sufficiently. The genotypes of the F1 animals were further analyzed via Sanger sequencing, and mutations were identified before re-isolation of homozygous mutants. sgRNAs and associated primers used in this study can be found in table S2. Evidence for the generation of putative null mutants was based on frame shifts, leading to premature stop codons in the protein coding sequences.

CRISPR-Cas9-mediated *miR-2235* locus deletion

Large deletions in the *miR-2235a* locus were induced via CRISPR-Cas9 following the protocol indicated above. We used a sgRNA against the *miR-2235a* sequence and injected 60 *P. pacificus* RSC011 young adults. A total of 200 F1 progenies of injected animals were segregated onto individual plates. After egg laying, these F1 animals were used for PCR experiments with primers in the flanking regions of the 10-kb *miR-2235a* locus. Visualizing of PCR product was performed on agarose gel electrophoresis. Individuals with PCR products were considered potential heterozygous candidates for deletions at the locus. We found two potential heterozygous individuals with a ~500-bp PCR fragment. Given that heterozygous and homozygous worms would be indistinguishable based on PCR and Sanger sequencing, we maintained potential heterozygous line by single-worm transfer for several generations and followed sublines that gave off clean PCR products in all of the singled progeny. Note that heterozygous animals should segregate 25% wild-type animals without a deletion that should result in the absence of the PCR product. The two homozygous mutants were whole-genome sequenced (see protocol above), which confirmed original Sanger sequencing results and the deletion at the locus.

Transgenesis

We generated transcriptional reporter constructs to visualize tissues that actively express *Ppa-ebax-1*. We not only took the 1065-bp upstream sequence of the first methionine but also extend the first 515 bp of the gene sequence for optimal primer design. For the *miR-2235a* locus, the 1455-bp-long putative promoter of the first precursor *miR-2235a* sequence was taken. Each of the promoter sequences was PCR amplified and subsequently assembled into a codon-optimized pUC19 vector containing TurboRFP and the *Ppa-rpl-23* 3’UTR (81). Assembly and bacterial transformation were generally done using the NEBuilder HiFi DNA Assembly Kit and NEB-5- α *E. coli* cells. Plasmid extraction was done using the QIAGEN Plasmid Midi Kit. Linearization of the plasmids was carried out with Pst1 enzyme digestion according to the manufacturer’s protocol. Final concentration of the injection mix contained the linearized plasmids (10 ng/ μ l) with Pst1-linearized RSC011 genomic carrier DNA

(60 ng/μl). Fluorescent worms were immobilized on a 5% Noble agarose pad with 0.3% NaN₃ added as an anesthetic and were screened using a Leica SP8 confocal laser scanning microscope.

Fluorescence imaging and quantification

The transcriptional reporter construct of the heavily expanded *miR-2235a* cluster (see above) was grown on *Novosphingobium* for five generations before fluorescence imaging. Imaging of the *miR-2235ap::TurboRFP* animals was done by comparing J1 embryonic stages between the naïve/unexposed generation on *E. coli* and upon *Novosphingobium* exposure. For imaging experiments, *miR-2235ap::TurboRFP* animals were transferred on 5% Noble agarose pads and anesthetized with 0.3% NaN₃. Animals were imaged using Axiolmager.Z1 (Zeiss, Oberkochen, Germany) with 63×/1.4 objective at automatic exposure under RFP excitation fluorescence. Quantification of the RFP signal was performed using Fiji/ImageJ by tracing the regions of J1 animals. Statistical analysis was performed using Wilcoxon rank test as described in the figure legends. The corrected total fluorescence was calculated using the formula:

Total fluorescence = Integrated density – (Area of selected cell × Mean fluorescence of background readings).

miRNA target site prediction

The pipeline that searched for TDMD triggers was implemented following previously established in silico approach in *Drosophila* (57). A file annotation for all RSC011 3'UTRs was loaded as input to start the search for miRNA targets. 3'UTR sequences were derived from RSC011 transcriptome assemblies or from protein homology with the *P. pacificus* PS312 annotations (El Paco V3) by taking the region downstream of the coding sequence within the assembled transcript or 100 bp downstream of the annotated gene. Filtering with the raw RNA-seq data from *ebax-1* animals was implemented, retaining only those reads with more than 10 counts. The input file was queried for binding complementarity toward the 3' region (starting from 13th nucleotide until the last *miR* sequence) of the *miR-2235a* (22 nt long) and *miR-8286b* (extended to 22 nt long). Search for complementarity sites to the seed sequence (2 to 8 nt of the *miR*) was implemented within 30 nt upstream of the 3' region. Both queries at the seed and 3' end tolerate a maximum of two mismatches.

To search for canonical miRNA-target pairing sites, the query was restricted to the seed sequence of the miRNA. Different types of *miR*-target searches were performed: 8-nt, 7-nt, A1-7-nt, and 6-nt types of pairing. The search was done independent of any expression dataset. All targets are listed in data S3.

Phylogenetic analyses

For *P. pacificus* genes retrieved from the Tid screen, one-to-one orthologous gene prediction between *P. pacificus* and *C. elegans* was identified from BLASTP searches. One-to-one orthology was further confirmed by phylogenetic analyses. Proteins were then aligned by Clustal Omega (100) and output in FASTA format. FASTA files were uploaded to the IQ-TREE tool (101, 102) using default settings with the auto substitution model, and 1000 bootstrap alignments were calculated under the ultrafast setting. The resulting phylogenetic tree files were visualized using FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>).

Radical treatment

Radical treatment was done as previously described (59, 103). In brief, Radical (Sigma-Aldrich) was dissolved in dimethyl

sulfoxide (DMSO) and then added to NGM agar to the final concentration of 19 μg/ml. Solution containing the same amount of DMSO was used as control. Supplementation was done two generations after reversal to *E. coli*.

RNA-seq and data analysis

Mixed-staged RSC011 worms grown on *Novosphingobium* and *E. coli* OP50 were collected from three NGM plates. Specifically, worms were pelleted for RNA certain generations of dietary exposure. Total RNA was extracted using a Direct-Zol RNA Mini prep kit (Zymo Research) according to the manufacturer's guidelines. The RNA-seq library preparation and sequencing were done by the company Novogene. Software Hisat2 (version 2.1.0) (104) was used to map raw reads to the *P. pacificus* RSC011 reference genome, and featureCounts (105) was used to quantify the counts of reads mapped to the genomic feature based on the RSC011 gene annotations. Tests for differential expression were performed in R (version 4.0.3) using DESeq2 (version 1.18.1) (106). Genes with a false discovery rate (FDR)-corrected *P* value < 0.01 and fold change cutoff of 2 were considered as significantly differentially expressed. Tests for overrepresentation of protein domains in sets of differentially expressed genes were performed using a Fisher's exact test in R. We used the FDR method as implemented in the *p.adjust* function in R to correct for multiple testing and only retained results with adjusted *P* value < 0.01.

sRNA extraction

Total RNA was extracted as described above. After RNA extraction, TAP (*Cap-Clip* Acid Pyrophosphatase) was performed by adding a total of 25 U of TAP to <4 μg of total RNA, 5 μl of reaction buffer (10× *Cap-Clip* Acid Pyrophosphatase Reaction Buffer), and PCR water to a total reaction of 50 μl per sample. The reaction was then incubated for 2 hours at 37°C. RNA clean-up step was done post-incubation using RNA Clean and Concentrator (Zymo Research) as per manufacturer's recommendations. sRNA-seq library preparation and sequencing were done by the company Novogene with 50-nt single-end reads.

Analysis of sRNA-seq data

We trimmed adapters from raw sequencing reads with the software Cutadapt (version 3.5, options: -a AGATCGGAAGA -m 14) (107). For each comparison, we counted the number of occurrences for each unique read across the samples (at least two biological replicates per condition). During this step, we discarded reads that were present in only a single sample. DE-seq2 was run to identify significantly differentially expressed sequences between (corrected *P* value < 0.05). To focus on nematode sequences, we removed differentially expressed sequences that mapped to the genomes of *Novosphingobium* or *E. coli* OP50 by bowtie (version 2.2.3) (108). To visualize the distribution of miRNAs across the *P. pacificus* genome, we downloaded 38,589 hairpin sequences from miRBase (release 2023-10-23), which contained 354 sequences from *P. pacificus* (65–70, 109). These sequences were first clustered on the basis of a 90% nucleotide identity cutoff using the cdhit-est program (version 4.8.1, option: -c 0.90) (110). We then ran BLASTN (version 2.10.1; option, -evalue 0.001) to search for matches of these sequences in the *P. pacificus* genome (version El Paco) (111). BLASTN matches with minimum length of 50 nt and a minimum percentage identity of 90% were then visualized in nonoverlapping 100-kb windows.

For combining all miRNA isoforms into single counts, we used string-matching to the first 18 nt of the mature miRNA sequence while also binning reads by lengths from 19 to 30 nt (75). Trimmed reads were all reads shorter than the mature miRNA length, and tailed reads were all reads longer than the mature length (as indicated in miRBase). In this manner, we were able to visualize the aggregate number of potential trimmed and tailed reads for each miRNA in the heavily expanded *miR-2235a* locus.

Genome assembly and annotation

Raw PacBio reads of *P. pacificus* RSC011 were assembled by the software Canu (version 1.4; options, genomeSize = 200 m -pacbio-corrected) (112). For strain RSC011, this resulted in an initial assembly of 54 contigs. This assembly was scaffolded using whole-genome sequencing data from recombinant inbred lines of the cross between *P. pacificus* strains RSC011 and RSA076 (113). Specifically, whole-genome sequencing data were aligned to the raw assembly by BWA aln and sampe programs (version 0.7.17-r188) (93). Subsequently, the SAMtools (version 0.1.18-r982:295) (97) mpileup, bcfutils, and varFilter.pl commands were run to genotype single-nucleotide variant markers between the parental strains RSC011 and RSA076 (114). The resulting genotype maps were plotted in R and manually scaffolded after visual inspection. At this step, one contig spanning 4.6 Mb was identified as *E. coli* on the basis of a BLASTN search and was discarded. The resulting genome assembly spans 159.0 Mb of which 156.4 Mb (98.3%) could be placed onto the six chromosomes. To annotate protein-coding genes in the RSC011 assembly, we used the PPCAC pipeline (version 1) (115), which generated evidence-based gene annotations from existing gene annotations for the *P. pacificus* reference strain PS312 (El Paco gene annotations, version 3) (116) as well as from RNA-seq data that were generated for this study. We refined the RSC011 gene annotations by removing species-specific orphan genes that overlapped in antisense direction with conserved genes as we previously observed that coding potential on the antisense strand can result in gene annotation errors (116, 117). Evaluating the completeness of the final RSC011 gene annotations using the BUSCO approach (version 3.1, nematode odb9 dataset) (118) revealed a completeness level of 89.5%, which demonstrates a comparable degree of completeness with regard to previously assembled *P. pacificus* strain genomes (117). Protein domains were annotated by the hmmsearch program with the Pfam-A database of the HMMER package (version 3.3; options, -E 0.001) (119).

Supplementary Materials

The PDF file includes:

Figs. S1 to S5
Tables S1 and S2
Legends for data S1 to S3

Other Supplementary Material for this manuscript includes the following:

Data S1 to S3

REFERENCES AND NOTES

- R. C. Painter, C. Osmond, P. Gluckman, M. Hanson, D. I. W. Phillips, T. J. Roseboom, Transgenerational effects of prenatal exposure to the Dutch famine on neonatal adiposity and health in later life. *BJOG* **115**, 1243–1249 (2008).
- S. Song, W. Wang, P. Hu, Famine, death, and madness: Schizophrenia in early adulthood after prenatal exposure to the Chinese Great Leap Forward Famine. *Soc. Sci. Med.* **68**, 1315–1321 (2009).
- L. R. Baugh, T. Day, Nongenetic inheritance and multigenerational plasticity in the nematode *C. elegans*. *eLife* **9**, 1–13 (2020).
- N. Frolows, A. Ashe, Small RNAs and chromatin in the multigenerational epigenetic landscape of *Caenorhabditis elegans*. *Philos. Trans. R. Soc. B Biol. Sci.* **376**, 20200112 (2021).
- X. Chen, O. Rechavi, Plant and animal small RNA communications between cells and organisms. *Nat. Rev. Mol. Cell Biol.* **23**, 185–203 (2022).
- F. Santilli, A. Boskovic, Mechanisms of transgenerational epigenetic inheritance: Lessons from animal model organisms. *Curr. Opin. Genet. Dev.* **79**, 102024 (2023).
- A. Ashe, A. Sapetschnig, E. M. Weick, J. Mitchell, M. P. Bagijn, A. C. Cording, A. L. Doebley, L. D. Goldstein, N. J. Lehrbach, J. Le Pen, G. Pintacuda, A. Sakaguchi, P. Sarkies, S. Ahmed, E. A. Miska, PiRNAs can trigger a multigenerational epigenetic memory in the germline of *C. elegans*. *Cell* **150**, 88–99 (2012).
- B. A. Buckley, K. B. Burkhart, S. G. Gu, G. Spracklin, A. Kershner, H. Fritz, J. Kimble, A. Fire, S. Kennedy, A nuclear Argonaute promotes multigenerational epigenetic inheritance and germline immortality. *Nature* **489**, 447–451 (2012).
- S. G. Gu, J. Pak, S. Guang, J. M. Maniar, S. Kennedy, A. Fire, Amplification of siRNA in *Caenorhabditis elegans* generates a transgenerational sequence-targeted histone H3 lysine 9 methylation footprint. *Nat. Genet.* **44**, 157–164 (2012).
- B. G. Dias, K. J. Ressler, Parental olfactory experience influences behavior and neural structure in subsequent generations. *Nat. Neurosci.* **17**, 89–96 (2014).
- J. Bozler, B. Z. Kacsoh, G. Bosco, Transgenerational inheritance of ethanol preference is caused by maternal NPF repression. *eLife* **8**, 1–18 (2019).
- R. S. Moore, R. Kaletsky, C. T. Murphy, Piwi/PRG-1 Argonaute and TGF- β mediate transgenerational learned pathogenic avoidance. *Cell* **177**, 1827–1841.e12 (2019).
- L. Hourt-Zeevi, Y. Korem Kohanim, O. Antonova, O. Rechavi, Three rules explain transgenerational small RNA inheritance in *C. elegans*. *Cell* **182**, 1186–1197.e12 (2020).
- M. F. Perez, B. Lehner, Intergenerational and transgenerational epigenetic inheritance in animals. *Nat. Cell Biol.* **21**, 143–151 (2019).
- O. Rechavi, L. Hourt-Zeevi, S. Anava, W. S. S. Goh, S. Y. Kerk, G. J. Hannon, O. Hobert, Starvation-induced transgenerational inheritance of small RNAs in *C. elegans*. *Cell* **158**, 277–287 (2014).
- A. Klosin, E. Casas, C. Hidalgo-Carcedo, T. Vavouri, B. Lehner, Transgenerational transmission of environmental information in *C. elegans*. *Science* **356**, 320–323 (2017).
- N. O. Burton, C. Riccio, A. Dallaire, J. Price, B. Jenkins, A. Koulman, E. A. Miska, Cysteine synthases CYSL-1 and CYSL-2 mediate *C. elegans* heritable adaptation to *P. vranovensis* infection. *Nat. Commun.* **11**, 1741 (2020).
- M. F. Perez, M. Shamalnasab, A. Mata-Cabana, S. Della Valle, M. Olmedo, M. Francesconi, B. Lehner, Neuronal perception of the social environment generates an inherited memory that controls the development and generation time of *C. elegans*. *Curr. Biol.* **31**, 4256–4268.e7 (2021).
- R. F. Ketting, L. Cochella, Concepts and functions of small RNA pathways in *C. elegans*. *Curr. Top. Dev. Biol.* **144**, 45–89 (2021).
- L. Hourt-Zeevi, G. Teichman, H. Gingold, O. Rechavi, Stress resets ancestral heritable small RNA responses. *eLife* **10**, e65797 (2021).
- A. K. Webster, J. M. Jordan, J. D. Hibshman, R. Chitrakar, L. Ryan Baugh, Transgenerational effects of extended Dauer diapause on starvation survival and gene expression plasticity in *Caenorhabditis elegans*. *Genetics* **210**, 263–274 (2018).
- R. Kaletsky, R. Moore, T. Sengupta, R. Seto, C. T. Murphy, Absence of evidence is not evidence of absence: The many flaws in the case against transgenerational epigenetic inheritance of pathogen avoidance in *C. elegans*. *bioRxiv* 597568 [Preprint] (2024). <https://doi.org/10.1101/2024.06.07.597568>.
- D. P. Gainey, A. V. Shubin, C. P. Hunter, Irreproducibility of transgenerational learned pathogen-aversion response in *C. elegans*. *bioRxiv* 596941 [Preprint] (2024). <https://doi.org/10.1101/2024.06.01.596941>.
- R. E. Lenski, Experimental evolution and the dynamics of adaptation and genome evolution in microbial populations. *ISME J.* **11**, 2181–2194 (2017).
- R. J. Sommer, L. Carta, S.-Y. Kim, P. W. Sternberg, Morphological, genetic and molecular description of *Pristionchus pacificus* sp. n. (Nematoda: Neodiplogastriidae). *Fundam. Apply. Nematol.* **19**, 511–521 (1996).
- N. E. Schroeder, Introduction to *Pristionchus pacificus* anatomy. *J. Nematol.* **53**, e2021-91 (2021).
- G. Bento, A. Ogawa, R. J. Sommer, Co-option of the hormone-signalling module daifchronic acid-DAF-12 in nematode evolution. *Nature* **466**, 494–497 (2010).
- E. J. Ragsdale, M. R. Müller, C. Rödelberger, R. J. Sommer, A developmental switch coupled to the evolution of plasticity acts through a sulfatase. *Cell* **155**, 922–933 (2013).
- J. W. Lightfoot, M. Wilecki, C. Rödelberger, E. Moreno, V. Susoy, H. Witte, R. J. Sommer, Small peptide-mediated self-recognition prevents cannibalism in predatory nematodes. *Science* **364**, 86–89 (2019).
- R. J. Sommer, Phenotypic plasticity: From theory and genetics to current and future challenges. *Genetics* **215**, 1–13 (2020).
- M. Wilecki, J. W. Lightfoot, V. Susoy, R. J. Sommer, Predatory feeding behaviour in *Pristionchus* nematodes is dependent on phenotypic plasticity and induced by serotonin. *J. Exp. Biol.* **218**, 1306–1313 (2015).

32. T. Renahan, R. J. Sommer, Nematode interactions on beetle hosts indicate a role of mouth-form plasticity in resource competition. *Front. Ecol. Evol.* **9**, 1–9 (2021).
33. M. Ptashne, A genetic switch: Gene control and phage. *J. Med. Genet.* **24**, 789–790 (1986).
34. G. Balázs, A. Van Oudenaarden, J. J. Collins, Cellular decision making and biological noise: From microbes to mammals. *Cell* **144**, 910–925 (2011).
35. P. M. Brakefield, J. Gatest, D. Keyst, F. Kesbeke, P. J. Wijngaarden, A. Monteiro, V. French, S. B. Carroll, Development, plasticity and evolution of butterfly eyespot patterns. *Nature* **384**, 236–242 (1996).
36. M. R. Kieninger, N. A. Ivers, C. Rödelberger, G. V. Markov, R. J. Sommer, E. J. Ragsdale, The nuclear hormone receptor NHR-40 acts downstream of the sulfatase EUD-1 as part of a developmental plasticity switch in *Pristionchus*. *Curr. Biol.* **26**, 2174–2179 (2016).
37. L. T. Bui, N. A. Ivers, E. J. Ragsdale, A sulfotransferase dosage-dependently regulates mouthpart polyphenism in the nematode *Pristionchus pacificus*. *Nat. Commun.* **9**, 4119 (2018).
38. B. Sieriebriennikov, N. Prabh, M. Dardiry, H. Witte, W. Röseler, M. R. Kieninger, C. Rödelberger, R. J. Sommer, A developmental switch generating phenotypic plasticity is part of a conserved multi-gene locus. *Cell Rep.* **23**, 2835–2843.e4 (2018).
39. B. Sieriebriennikov, S. Sun, J. W. Lightfoot, H. Witte, E. Moreno, C. Rödelberger, R. J. Sommer, Conserved nuclear hormone receptors controlling a novel plastic trait target fast-evolving genes expressed in a single cell. *PLOS Genet.* **16**, e1008687 (2020).
40. S. Sun, H. Witte, R. J. Sommer, Chitin contributes to the formation of a feeding structure in a predatory nematode. *Curr. Biol.* **33**, 15–27.e6 (2023).
41. S. Casasa, E. Katsougia, E. J. Ragsdale, A Mediator subunit imparts robustness to a polyphenism decision. *Proc. Natl. Acad. Sci. U.S.A.* **120**, e2308816120 (2023).
42. N. A. Levis, E. J. Ragsdale, A histone demethylase links the loss of plasticity to nongenetic inheritance and morphological change. *Nat. Commun.* **14**, 8439 (2023).
43. M. S. Werner, T. Loschko, T. King, S. Reich, T. Theska, M. Franz-Wachtel, B. Macek, R. J. Sommer, Histone 4 lysine 5/12 acetylation enables developmental plasticity of *Pristionchus* mouth form. *Nat. Commun.* **14**, 2095 (2023).
44. T. Theska, R. J. Sommer, Feeding-structure morphogenesis in “rhabditid” and diplogastrid nematodes is not controlled by a conserved genetic module. *Evol. Dev.* **26**, e12471 (2024).
45. M. S. Werner, B. Sieriebriennikov, T. Loschko, S. Namdeo, M. Lenuzzi, M. Dardiry, T. Renahan, D. R. Sharma, R. J. Sommer, Environmental influence on *Pristionchus pacificus* mouth form through different culture methods. *Sci. Rep.* **7**, 7207 (2017).
46. M. Lenuzzi, H. Witte, M. Riebesell, C. Rödelberger, R. L. Hong, R. J. Sommer, Influence of environmental temperature on mouth-form plasticity in *Pristionchus pacificus* acts through daf-11-dependent cGMP signaling. *J. Exp. Zool. Part B Mol. Dev. Evol.* **340**, 214–224 (2023).
47. N. Bose, A. Ogawa, S. H. Von Reuss, J. J. Yim, E. J. Ragsdale, R. J. Sommer, F. C. Schroeder, Complex small-molecule architectures regulate phenotypic plasticity in a nematode. *Angew. Chemie Int. Ed. Engl.* **51**, 12438–12443 (2012).
48. M. Dardiry, V. Piskobulu, A. Kalirad, R. J. Sommer, Experimental and theoretical support for costs of plasticity and phenotype in a nematode cannibalistic trait. *Evol. Lett.* **7**, 48–57 (2023).
49. N. Akduman, J. W. Lightfoot, W. Röseler, H. Witte, W. S. Lo, C. Rödelberger, R. J. Sommer, Bacterial vitamin B12 production enhances nematode predatory behavior. *ISME J.* **14**, 1494–1507 (2020).
50. T. Beltran, V. Shahrezaei, V. Katju, P. Sarkies, Epimutations driven by small RNAs arise frequently but most have limited duration in *Caenorhabditis elegans*. *Nat. Ecol. Evol.* **4**, 1539–1548 (2020).
51. A. M. Weller, C. Rödelberger, G. Eberhardt, R. I. Molnar, R. J. Sommer, Opposing forces of A/T-biased mutations and G/C-biased gene conversions shape the genome of the nematode *Pristionchus pacificus*. *Genetics* **196**, 1145–1152 (2014).
52. R. Kaletsky, R. S. Moore, G. D. Vrla, L. R. Parsons, Z. Gitai, C. T. Murphy, C. *elegans* interprets bacterial non-coding RNAs to learn pathogenic avoidance. *Nature* **586**, 445–451 (2020).
53. M. Legüe, M. Caneo, B. Aguila, B. Pollak, A. Calixto, Interspecies effectors of a transgenerational memory of bacterial infection in *Caenorhabditis elegans*. *iScience* **25**, 104627 (2022).
54. E. Moreno, B. Sieriebriennikov, H. Witte, C. Rödelberger, J. W. Lightfoot, R. J. Sommer, Regulation of hyperoxia-induced social behaviour in *Pristionchus pacificus* nematodes requires a novel cilia-mediated environmental input. *Sci. Rep.* **7**, 17550 (2017).
55. C. Y. Shi, E. R. Kingston, B. Kleaveland, D. H. Lin, M. W. Stubna, D. P. Bartel, The ZSWIM8 ubiquitin ligase mediates target-directed microRNA degradation. *Science* **370**, eabc9359 (2020).
56. J. Han, C. A. Lavigne, B. T. Jones, H. Zhang, F. Gillett, J. T. Mendell, A ubiquitin ligase mediates target-directed microRNA decay independently of tailing and trimming. *Science* **370**, eabc9546 (2020).
57. E. R. Kingston, L. W. Blodgett, D. P. Bartel, Endogenous transcripts direct microRNA degradation in *Drosophila*, and this targeted degradation is required for proper embryonic development. *Mol. Cell* **82**, 3872–3884.e9 (2022).
58. Z. Wang, Y. Hou, X. Guo, M. van der Voet, M. Boxem, J. E. Dixon, A. D. Chisholm, Y. Jin, The EBAX-type Cullin-RING E3 ligase and Hsp90 guard the protein quality of the SAX-3/robo receptor in developing neurons. *Neuron* **79**, 903–916 (2013).
59. B. Sieriebriennikov, G. V. Markov, H. Witte, R. J. Sommer, The role of DAF-21/Hsp90 in mouth-form plasticity in *Pristionchus pacificus*. *Mol. Biol. Evol.* **34**, 1644–1653 (2017).
60. C. Y. Shi, L. E. Elcavage, R. R. Chivukula, J. Stefano, B. Kleaveland, D. P. Bartel, ZSWIM8 destabilizes many murine microRNAs and is required for proper embryonic growth and development. *Genome Res.* **33**, 1482–1496 (2023).
61. P. H. Wu, P. D. Zamore, To degrade a MicroRNA, destroy its argonaute protein. *Mol. Cell* **81**, 223–225 (2021).
62. J. Han, J. T. Mendell, MicroRNA turnover: A tale of tailing, trimming, and targets. *Trends Biochem. Sci.* **48**, 26–39 (2023).
63. S. Iwasaki, M. Kobayashi, M. Yoda, Y. Sakaguchi, S. Katsuma, T. Suzuki, Y. Tomari, Hsc70/Hsp90 chaperone machinery mediates ATP-dependent RISC loading of small RNA duplexes. *Mol. Cell* **39**, 292–299 (2010).
64. X. Liu, Y. Y. Yang, Y. Wang, HSP90 and Aha1 modulate microRNA maturation through promoting the folding of Dicer1. *Nucleic Acids Res.* **50**, 6990–7001 (2022).
65. R. Ahmed, Z. Chang, A. E. Younis, C. Langnick, N. Li, W. Chen, N. Brattig, C. Dieterich, Conserved miRNAs are candidate post-transcriptional regulators of developmental arrest in free-living and parasitic nematodes. *Genome Biol. Evol.* **5**, 1246–1260 (2013).
66. E. De Wit, S. E. V. Linsen, E. Cuppen, E. Berezikov, Repertoire and evolution of miRNA genes in four divergent nematode species. *Genome Res.* **19**, 2064–2074 (2009).
67. A. Kozomara, M. Birgaoanu, S. Griffiths-Jones, miRBase: From microRNA sequences to function. *Nucleic Acids Res.* **47**, D155–D162 (2019).
68. A. Kozomara, S. Griffiths-Jones, miRBase: Annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res.* **42**, D68–D73 (2014).
69. S. Griffiths-Jones, R. J. Grocock, S. van Dongen, A. Bateman, A. J. Enright, miRBase: MicroRNA sequences, targets and gene nomenclature. *Nucleic Acids Res.* **34**, 140–144 (2006).
70. S. Griffiths-Jones, H. K. Saini, S. Van Dongen, A. J. Enright, miRBase: Tools for microRNA genomics. *Nucleic Acids Res.* **36**, 154–158 (2008).
71. B. F. Donnelly, B. Yang, A. L. Grimme, K. F. Vieux, C. Y. Liu, L. Zhou, K. McJunkin, The developmentally timed decay of an essential microRNA family is seed-sequence dependent. *Cell Rep.* **40**, 111154 (2022).
72. E. Alvarez-Saavedra, H. R. Horvitz, Many families of *C. elegans* microRNAs are not essential for development or viability. *Curr. Biol.* **20**, 367–373 (2010).
73. M. Liu, P. Liu, L. Zhang, Q. Cai, G. Gao, W. Zhang, Z. Zhu, D. Liu, Q. Fan, Mir-35 is involved in intestine cell G1/S transition and germ cell proliferation in *C. elegans*. *Cell Res.* **21**, 1605–1618 (2011).
74. D. R. Sharma, W. Roeseler, H. Witte, M. S. Werner, R. J. Sommer, Developmental small RNA transcriptomics reveals divergent evolution of the conserved microRNA *miR-100* and the *let-7*-complex in nematodes. bioRxiv 604269 [Preprint] (2024). <https://doi.org/10.1101/2024.07.19.604269>.
75. B. Kleaveland, C. Y. Shi, J. Stefano, D. P. Bartel, A network of noncoding regulatory RNAs acts in the mammalian brain. *Cell* **174**, 350–362.e17 (2018).
76. C. T. Neilsen, G. J. Goodall, C. P. Bracken, IsoMiRs - The overlooked repertoire in the dynamic microRNAome. *Trends Genet.* **28**, 544–549 (2012).
77. P. Y. Wang, D. P. Bartel, A statistical approach for identifying primary substrates of ZSWIM8-mediated microRNA degradation in small-RNA sequencing data. *BMC Bioinformatics* **24**, 195 (2023).
78. A. Bitetti, A. C. Mallory, E. Golini, C. Carrieri, H. Carreño Gutiérrez, E. Perlas, Y. A. Pérez-Rico, G. P. Tocchini-Valentini, A. J. Enright, W. H. J. Norton, S. Mandillo, D. O’Carroll, A. Shkumatava, MicroRNA degradation by a conserved target RNA regulates animal behavior. *Nat. Struct. Mol. Biol.* **25**, 244–251 (2018).
79. F. Ghini, C. Rubolino, M. Climent, I. Simeone, M. J. Marzi, F. Nicassio, Endogenous transcripts control miRNA levels and activity in mammalian cells by target-directed miRNA degradation. *Nat. Commun.* **9**, 3119 (2018).
80. L. Li, P. Sheng, T. Li, C. J. Fields, N. M. Hiers, Y. Wang, J. Li, C. M. Guardia, J. D. Licht, M. Xie, Widespread microRNA degradation elements in target mRNAs can assist the encoded proteins. *Genes Dev.* **35**, 1595–1609 (2021).
81. Z. Han, W. S. Lo, J. W. Lightfoot, H. Witte, S. Sun, R. J. Sommer, Improving transgenesis efficiency and CRISPR-associated tools through codon optimization and native intron addition in *Pristionchus* nematodes. *Genetics* **216**, 947–956 (2020).
82. K. Kagias, R. Pocock, MicroRNA regulation of the embryonic hypoxic response in *Caenorhabditis elegans*. *Sci. Rep.* **5**, 11284 (2015).
83. V. Susoy, E. J. Ragsdale, N. Kanzaki, R. J. Sommer, Rapid diversification associated with a macroevolutionary pulse of developmental plasticity. *eLife* **4**, 7–17 (2015).
84. V. Susoy, M. Herrmann, N. Kanzaki, M. Kruger, C. N. Nguyen, C. Rödelberger, W. Röseler, C. Weiler, R. M. Giblin-Davis, E. J. Ragsdale, R. J. Sommer, Large-scale diversification without genetic isolation in nematode symbionts of figs. *Sci. Adv.* **2**, e1501031 (2016).
85. S. Wighard, H. Witte, R. J. Sommer, Conserved switch genes that arose via whole-genome duplication regulate a cannibalistic nematode morph. *Sci. Adv.* **10**, eadk6062 (2024).

86. A. Holz, A. Streit, Gain and loss of small RNA classes-characterization of small RNAs in the parasitic nematode family Strongyloididae. *Genome Biol. Evol.* **9**, 2826–2843 (2017).
87. A. Grishok, H. Tabara, C. C. Mello, Genetic requirements for inheritance of RNAi in *C. elegans*. *Science* **287**, 2494–2497 (2000).
88. S. Devanapally, P. Raman, M. Chey, S. Allgood, F. Ettefa, M. Diop, Y. Lin, Y. E. Cho, A. M. Jose, Mating can initiate stable RNA silencing that overcomes epigenetic recovery. *Nat. Commun.* **12**, 4239 (2021).
89. J. K. Kruschke, *Doing Bayesian Data Analysis: A Tutorial With R, JAGS, and Stan* (Elsevier Science, ed. 2, 2015).
90. O. Abril-Pla, V. Andreani, C. Carroll, L. Dong, C. J. Fonnesbeck, M. Kochurov, R. Kumar, J. Lao, C. C. Luhmann, O. A. Martin, M. Osthege, R. Vieira, T. Wiecki, R. Zinkov, PyMC: A modern, and comprehensive probabilistic programming framework in Python. *PeerJ Comput. Sci.* **9**, e1516 (2023).
91. C. R. Harris, K. J. Millman, S. J. van der Walt, R. Gommers, P. Virtanen, D. Cournapeau, E. Wieser, J. Taylor, S. Berg, N. J. Smith, R. Kern, M. Picus, S. Hoyer, M. H. van Kerkwijk, M. Brett, A. Haldane, J. F. del Río, M. Wiebe, P. Peterson, P. Gérard-Marchant, K. Sheppard, T. Reddy, W. Weckesser, H. Abbasi, C. Gohlke, T. E. Oliphant, Array programming with NumPy. *Nature* **585**, 357–362 (2020).
92. J. K. Kruschke, Bayesian analysis reporting guidelines. *Nat. Hum. Behav.* **5**, 1282–1291 (2021).
93. H. Li, R. Durbin, Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* **25**, 1754–1760 (2009).
94. R. Poplin, V. Ruano-Rubio, M. A. DePristo, T. J. Fennell, M. O. Carneiro, G. A. Van der Auwera, D. E. Kling, L. D. Gauthier, A. Levy-Moonshine, D. Roazen, K. Shakir, J. Thibault, S. Chandran, C. Whelan, M. Lek, S. Gabriel, M. J. Daly, B. Neale, D. G. MacArthur, E. Banks, Scaling accurate genetic variant discovery to tens of thousands of samples. *bioRxiv* 2011178 [Preprint] (2017). <https://doi.org/10.1101/2011178>.
95. P. Danecek, J. K. Bonfield, J. Liddle, J. Marshall, V. Ohan, M. O. Pollard, A. Whitwham, T. Keane, S. A. McCarthy, R. M. Davies, H. Li, Twelve years of SAMtools and BCFtools. *Gigascience* **10**, giab008 (2021).
96. A. Pires da Silva, “*Pristionchus pacificus* protocols” in *WormBook* (2013), pp. 1–20; www.wormbook.org/chapters/www_ppageneticprotocols.2/ppageneticprotocols.html.
97. H. Li, B. Handsaker, A. Wysoker, T. Fennell, J. Ruan, N. Homer, G. Marth, G. Abecasis, R. Durbin, The sequence alignment/map format and SAMtools. *Bioinformatics* **25**, 2078–2079 (2009).
98. R. Rae, H. Witte, C. Rödelberger, R. J. Sommer, The importance of being regular: *Caenorhabditis elegans* and *Pristionchus pacificus* defecation mutants are hypersusceptible to bacterial pathogens. *Int. J. Parasitol.* **42**, 747–753 (2012).
99. H. Witte, E. Moreno, C. Rödelberger, J. Kim, J. S. Kim, A. Streit, R. J. Sommer, Gene inactivation using the CRISPR/Cas9 system in the nematode *Pristionchus pacificus*. *Dev. Genes Evol.* **225**, 55–62 (2014).
100. F. Gabler, S. Nam, S. Till, M. Mirdita, M. Steinegger, J. Söding, A. N. Lupas, V. Alva, Protein sequence analysis using the MPI bioinformatics toolkit. *Curr. Protoc. Bioinformatics* **72**, e108 (2020).
101. J. Trifunopoulos, L. T. Nguyen, A. von Haeseler, B. Q. Minh, W-IQ-TREE: A fast online phylogenetic tool for maximum likelihood analysis. *Nucleic Acids Res.* **44**, W232–W235 (2016).
102. L. T. Nguyen, H. A. Schmidt, A. Von Haeseler, B. Q. Minh, IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol. Biol. Evol.* **32**, 268–274 (2015).
103. G. E. Janssens, X. X. Lin, L. Millan-Ariño, A. Kavšek, I. Sen, R. I. Seinstra, N. Stroustrup, E. A. A. Nollen, C. G. Riedel, Transcriptomics-based screening identifies pharmacological inhibition of Hsp90 as a means to defer aging. *Cell Rep.* **27**, 467–480.e6 (2019).
104. D. Kim, B. Langmead, S. L. Salzberg, HISAT: A fast spliced aligner with low memory requirements. *Nat. Methods* **12**, 357–360 (2015).
105. Y. Liao, G. K. Smyth, W. Shi, FeatureCounts: An efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* **30**, 923–930 (2014).
106. M. I. Love, W. Huber, S. Anders, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550 (2014).
107. M. Marcel, Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* **17**, 10–12 (2011).
108. B. Langmead, C. Wilks, V. Antonescu, R. Charles, Scaling read aligners to hundreds of threads on general-purpose processors. *Bioinformatics* **35**, 421–432 (2019).
109. A. Kozomara, S. Griffiths-Jones, MiRBase: Integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Res.* **39**, D152–D157 (2011).
110. W. Li, A. Godzik, Cd-hit: A fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics* **22**, 1658–1659 (2006).
111. C. Rödelberger, J. M. Meyer, N. Prabh, C. Lanz, F. Bemm, R. J. Sommer, Single-molecule sequencing reveals the chromosome-scale genomic architecture of the nematode model organism *Pristionchus pacificus*. *Cell Rep.* **21**, 834–844 (2017).
112. S. Koren, B. P. Walenz, K. Berlin, J. R. Miller, N. H. Bergman, A. M. Phillippy, Canu: Scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. *Genome Res.* **27**, 722–736 (2017).
113. M. Dardiry, G. Eberhard, H. Witte, C. Rödelberger, J. W. Lightfoot, R. J. Sommer, Divergent combinations of cis-regulatory elements control the evolution of phenotypic plasticity. *PLoS Biol.* **21**, e3002270 (2023).
114. A. McGaughan, C. Rödelberger, D. G. Grimm, J. M. Meyer, E. Moreno, K. Morgan, M. Leaver, V. Seroby, B. Rakitsch, K. M. Borgwardt, R. J. Sommer, Genomic profiles of diversification and genotype-phenotype association in island nematode lineages. *Mol. Biol. Evol.* **33**, 2257–2272 (2016).
115. C. Rödelberger, The community-curated *Pristionchus pacificus* genome facilitates automated gene annotation improvement in related nematodes. *BMC Genomics* **22**, 216 (2021).
116. M. Athanasoulis, H. Witte, C. Weiler, T. Loschko, G. Eberhardt, R. J. Sommer, C. Rödelberger, Comparative genomics and community curation further improve gene annotations in the nematode *Pristionchus pacificus*. *BMC Genomics* **21**, 708 (2020).
117. N. Prabh, C. Rödelberger, Multiple *Pristionchus pacificus* genomes reveal distinct evolutionary dynamics between de novo candidates and duplicated genes. *Genome Res.* **32**, 1315–1327 (2022).
118. F. A. Simão, R. M. Waterhouse, P. Ioannidis, E. V. Kriventseva, E. M. Zdobnov, BUSCO: Assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics* **31**, 3210–3212 (2015).
119. S. C. Potter, A. Luciani, S. R. Eddy, Y. Park, R. Lopez, R. D. Finn, HMMER web server: 2018 Update. *Nucleic Acids Res.* **46**, W200–W204 (2018).

Acknowledgments: We would like to thank H. Hausmann for assisting in freezing nematode cultures. We are also grateful to A. Streit and C. Igreja for discussions and helpful comments on the manuscript and M. Dardiry for conceptual discussions at the beginning of this long-term project. **Funding:** The work was funded by the Max Planck Society. **Author contributions:** Conceptualization: S.P.Q. and R.J.S. Investigation: S.P.Q. Software: S.P.Q. and A.K. Data curation: S.P.Q. and A.K. Formal analysis: S.P.Q., C.R., and A.K. Methodology: S.P.Q., A.K., C.R., and R.J.S. Writing—original draft: S.P.Q. and R.J.S. Writing—review and editing: S.P.Q., A.K., C.R., and R.J.S. Visualization: S.P.Q. and A.K. Resources: S.P.Q., A.K., W.R., H.W., and Y.W. Validation: S.P.Q. and R.J.S. Funding acquisition: R.J.S. Supervision: R.J.S. Project administration: R.J.S. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. *P. pacificus* strains and bacterial isolates generated in this work are freely available through the corresponding author. All the code and data for Bayesian analysis are archived on Dryad: 10.5061/dryad.2rbnzs80v. Sequencing data that were generated for the current study have been submitted to the European Nucleotide Archive under the project accession PRJEB74486.

Submitted 23 October 2024

Accepted 6 February 2025

Published 12 March 2025

10.1126/sciadv.adu0875

EBAX-1/ZSWIM8 destabilizes miRNAs, resulting in transgenerational inheritance of a predatory trait

Shiela Pearl Quiobe, Ata Kalirad, Waltraud Röseler, Hanh Witte, Yinan Wang, Christian Rödelberger, and Ralf J. Sommer

Sci. Adv. **11** (11), eadu0875. DOI: 10.1126/sciadv.adu0875

View the article online

<https://www.science.org/doi/10.1126/sciadv.adu0875>

Permissions

<https://www.science.org/help/reprints-and-permissions>

Use of this article is subject to the [Terms of service](#)

Science Advances (ISSN 2375-2548) is published by the American Association for the Advancement of Science. 1200 New York Avenue NW, Washington, DC 20005. The title *Science Advances* is a registered trademark of AAAS.

Copyright © 2025 The Authors, some rights reserved; exclusive licensee American Association for the Advancement of Science. No claim to original U.S. Government Works. Distributed under a Creative Commons Attribution NonCommercial License 4.0 (CC BY-NC).

Supplementary Materials for
EBAX-1/ZSWIM8 destabilizes miRNAs, resulting in transgenerational inheritance of a predatory trait

Shiela Pearl Quiobe *et al.*

Corresponding author: Ralf J. Sommer, ralf.sommer@tuebingen.mpg.de

Sci. Adv. **11**, eadu0875 (2025)
DOI: 10.1126/sciadv.adu0875

The PDF file includes:

Figs. S1 to S5
Tables S1 and S2
Legends for data S1 to S3

Other Supplementary Material for this manuscript includes the following:

Data S1 to S3

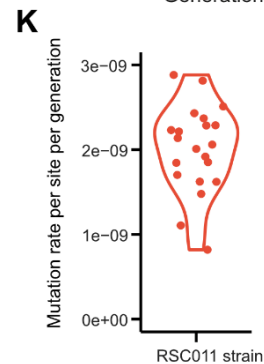
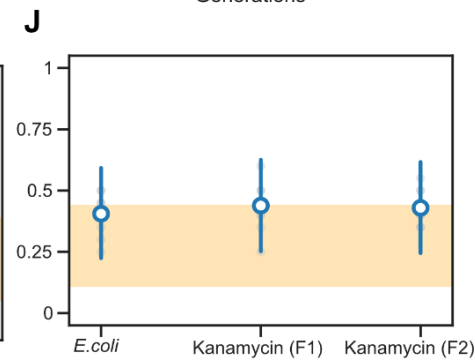
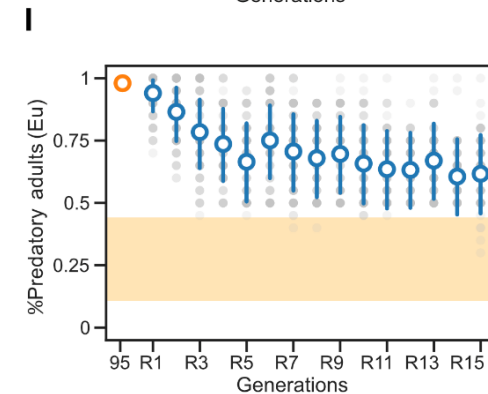
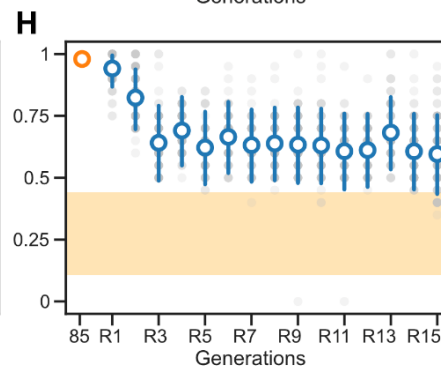
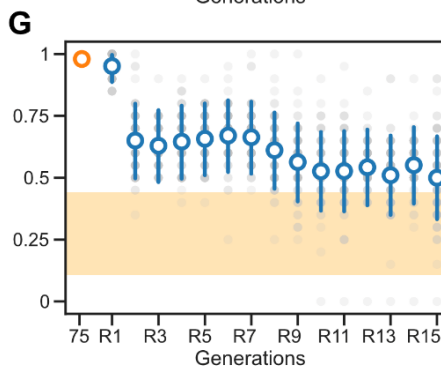
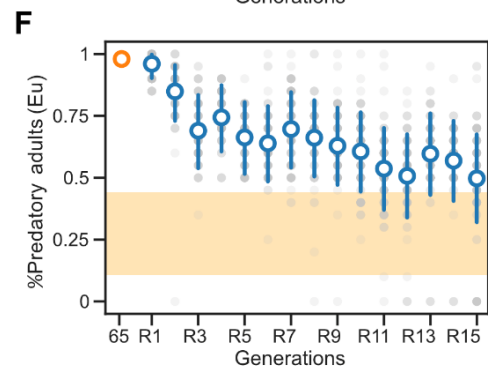
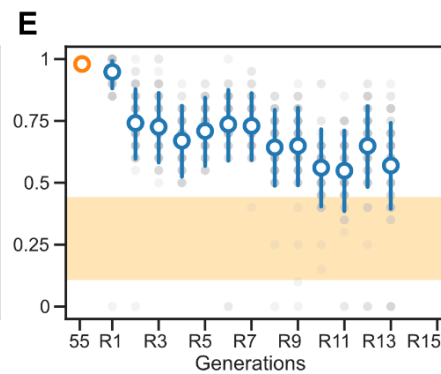
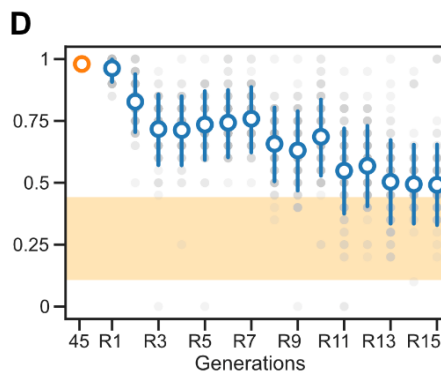
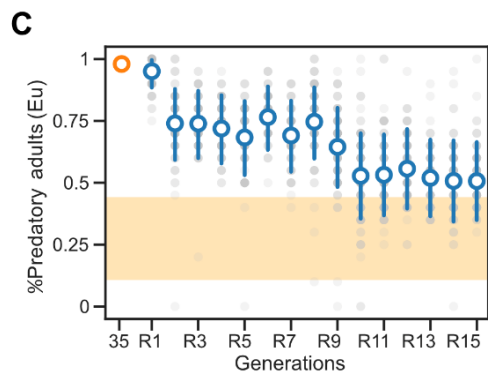
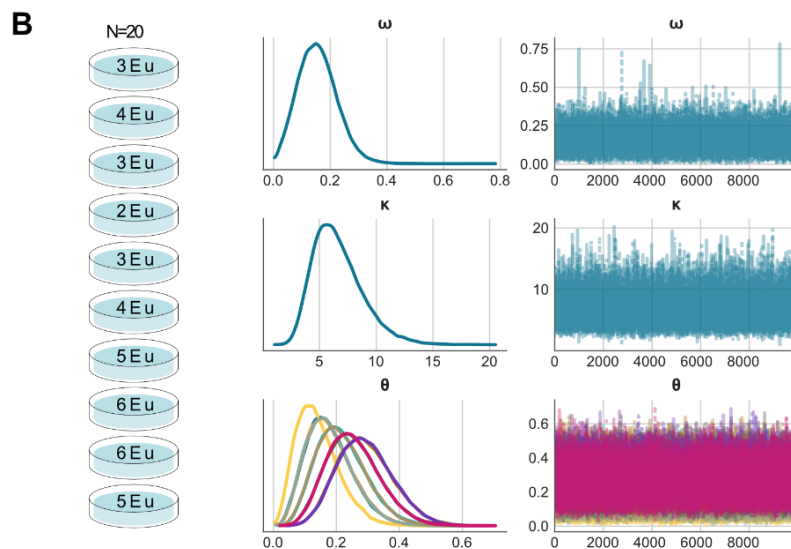
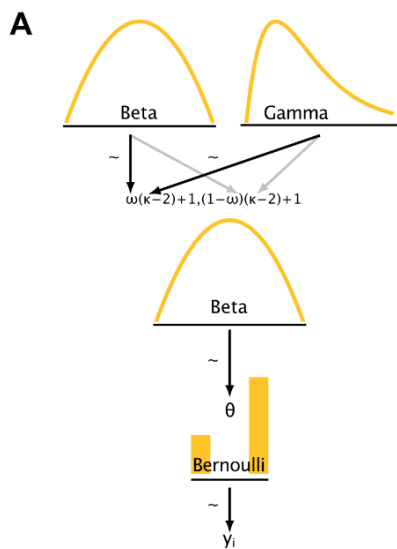


Fig. S1. Diet reversal experiments and mutation rate of RSC011 after 100 generations of single worm transfer.

(A) The Hierarchical Bayesian model used to infer mean probability estimates of the predatory mouth form. Graphical representation of the model to infer the mean highest density interval (HDI). (B) Plates represent independent replicates of the actual Eu counts. The probability of developing the Eu mouth form (θ) for each replicate was derived by linking all replicates using the hyperparameters, ω and κ . The prior distribution on the given parameters is described as $\omega \sim \text{Beta}(\alpha, \beta)$ and $\kappa \sim \text{Gamma}(3, 1)$. The stability of the estimated θ values for a given generation was validated using common convergence diagnostics (see Materials and Methods for details). (C-I) Mean probability of the predatory mouth form during food reversal experiments back to *E. coli* after original exposure to *Novosphingobium* for 35, 45, 55, 65, 75, 85 and 95 generations. (J) Mean probability of the predatory mouth form after exposure to kanamycin-resistant *E. coli* strain for two generations. The 95% HDI for the means was estimated using a Bayesian hierarchical model and indicates the probability of expressing the Eu morph inferred from the experimental data. The yellow area displays the upper and lower limit of the baseline Eu response of RSC011 ($0.115 \leq \text{HDI}(\bar{\theta}_{\text{Control}}) \leq 0.436$). (K) Mutation rate per site per generation for *P. pacificus* RSC011. Each dot represents the mutation rate for independent lines sequenced after 100 generations.

A

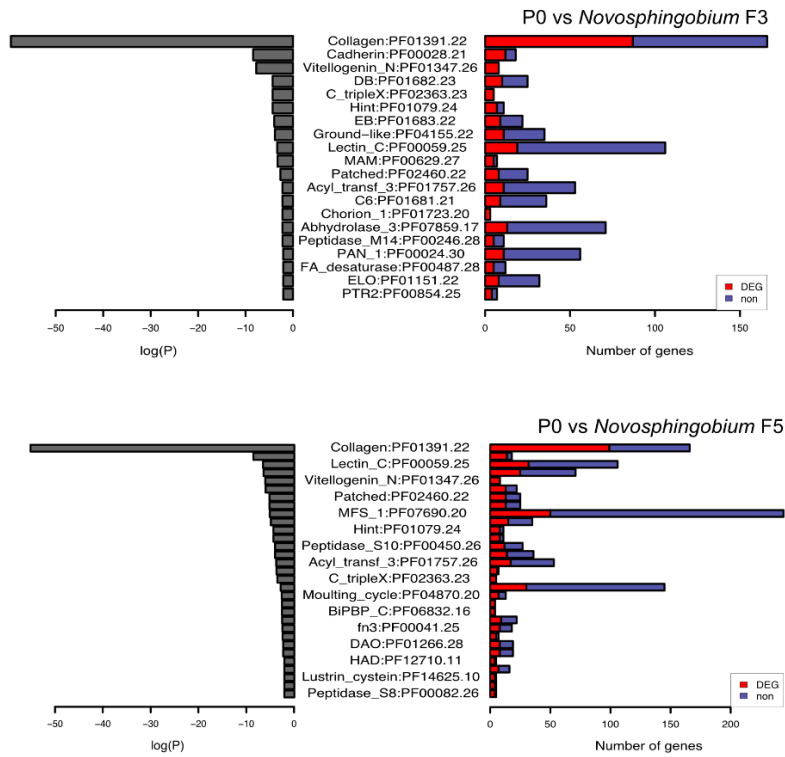


Fig. S2. Gene enrichment analysis upon *Novosphingobium* exposure.

(A) Gene-enrichment analysis on *Novosphingobium* for three and five generations. The complete gene lists are listed in Data S1.

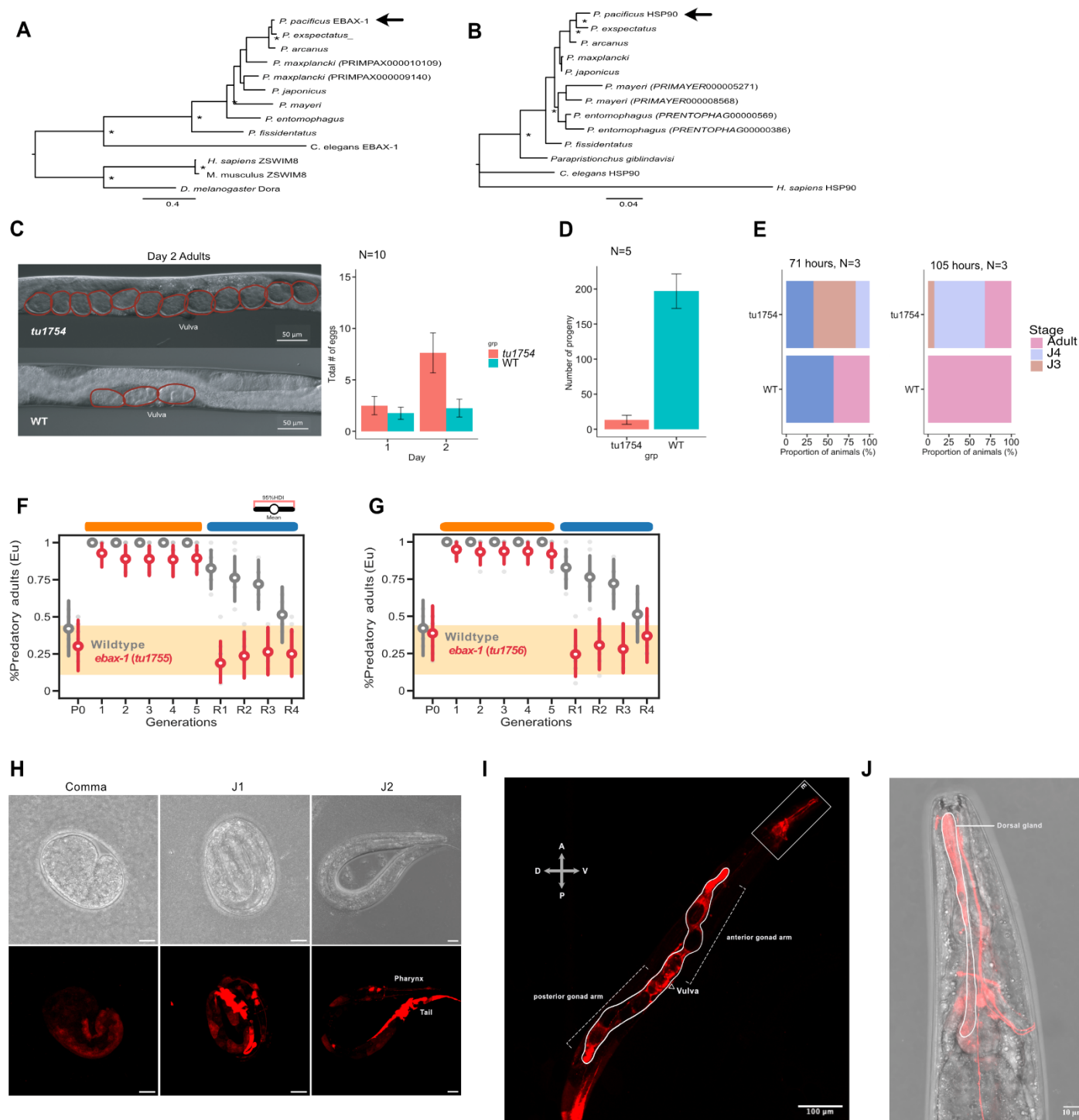


Fig. S3. Pairwise orthology relationships of the candidate genes and involvement of *ebax-1* in transgenerational inheritance of the predatory morph.

(A) One-to-one orthology relationships for EBAX-1 and (B) DAF-21. Both maximum likelihood phylogenies are based on the genus *Pristionchus* (*P. pacificus*, *P. expectatus*, *P. arcanus*, *P. maxplancki*, *P. japonicus*, *P. mayeri*, *P. entomophagus*, *P. fissidentatus*) (some includes its ‘basal’ relative *Paraprisionchus*), *C. elegans*, mammals, and insects. Nodes with bootstrap support values of ≥ 90 are labelled with asterisks (*) and arrows indicate *P. pacificus* genes targeted for functional

analyses. *P. maxplancki* has two proteins encoding a zinc-finger SWIM-type profile (PRIMPAX000009140) and a ZSWIM4-8- C-terminal (PRIMPAX000010109), both are included in the EBAX-1 phylogeny. The top two protein BLAST hits of *P. mayeri* and *P. entomophagus* species against *C. elegans* DAF-21 are also included. (C) Egg-laying-defective phenotype in *Ppa-ebax-1(tu1754)* (left). Eggs inside the worm body are outlined in red circles. Average number of accumulated eggs on day-2 *Ppa-ebax-1(tu1754)* adults compared to wild type (right). (D) Average number of progeny counts in *Ppa-ebax-1(tu1754)* and wild type animals. Error bars are represented in standard deviation. Orange, *Ppa-ebax-1(tu1754)*; green, wild type. (E) Developmental staging of *Ppa-ebax-1(tu1754)* and wild type showing the percentage of J3, J4 and young adults after 71 and 105 hours on *E. coli*. (F-G) Mean probability of the predatory morph in other *Ppa-ebax-1* in-frame alleles for 5 generations of *Novopshingobium* exposure and reversal to *E. coli*. Red, *Ppa-ebax-1(tu1755* and *tu1756)*; grey, wild type. The 95% highest density interval (HDI) was used to visualize the probability of observing the Eu morph. The yellow area displays the upper and lower limit of the RSC011 baseline Eu response. More details about the molecular lesions are listed in Table S2. (H-J) Transcriptional reporter construct of *Ppa-ebax-1*. (H) *Ppa-ebax-1p::TurboRFP* during embryonic development, starting from the comma stage, and J2 stage. Upper panels are DIC images and bottom panels are fluorescent images of the same embryos. Scale bars represent 10 μ m. (I) RFP expression on the pharyngeal neurons, vulval region and gonad tissue of adults. The double-arrows in a cross indicate anatomical worm orientation: A-anterior, P-posterior, D-dorsal, V-ventral. (J) Zoom-in on the head region depicted in (I), previous image.

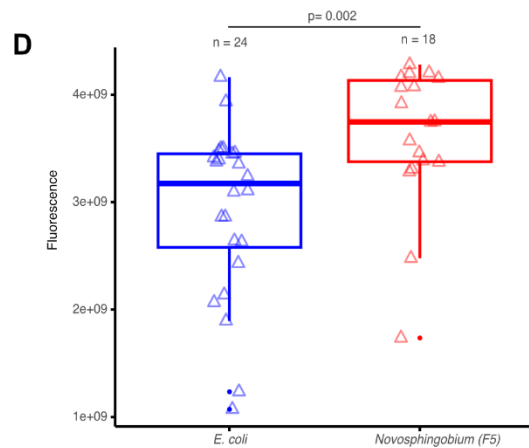
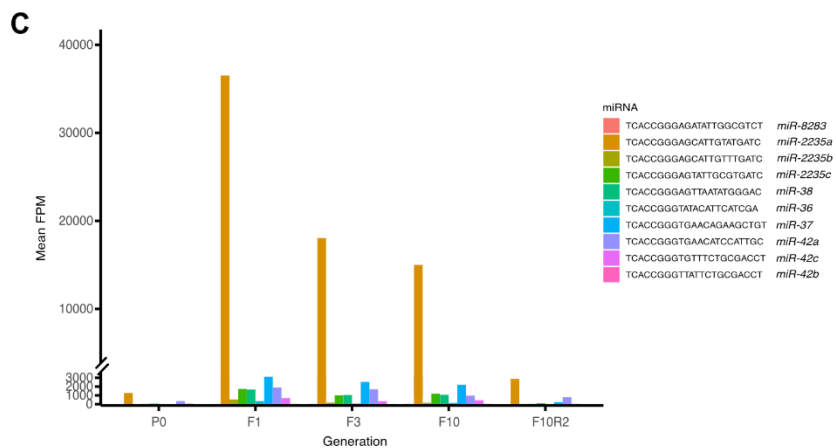
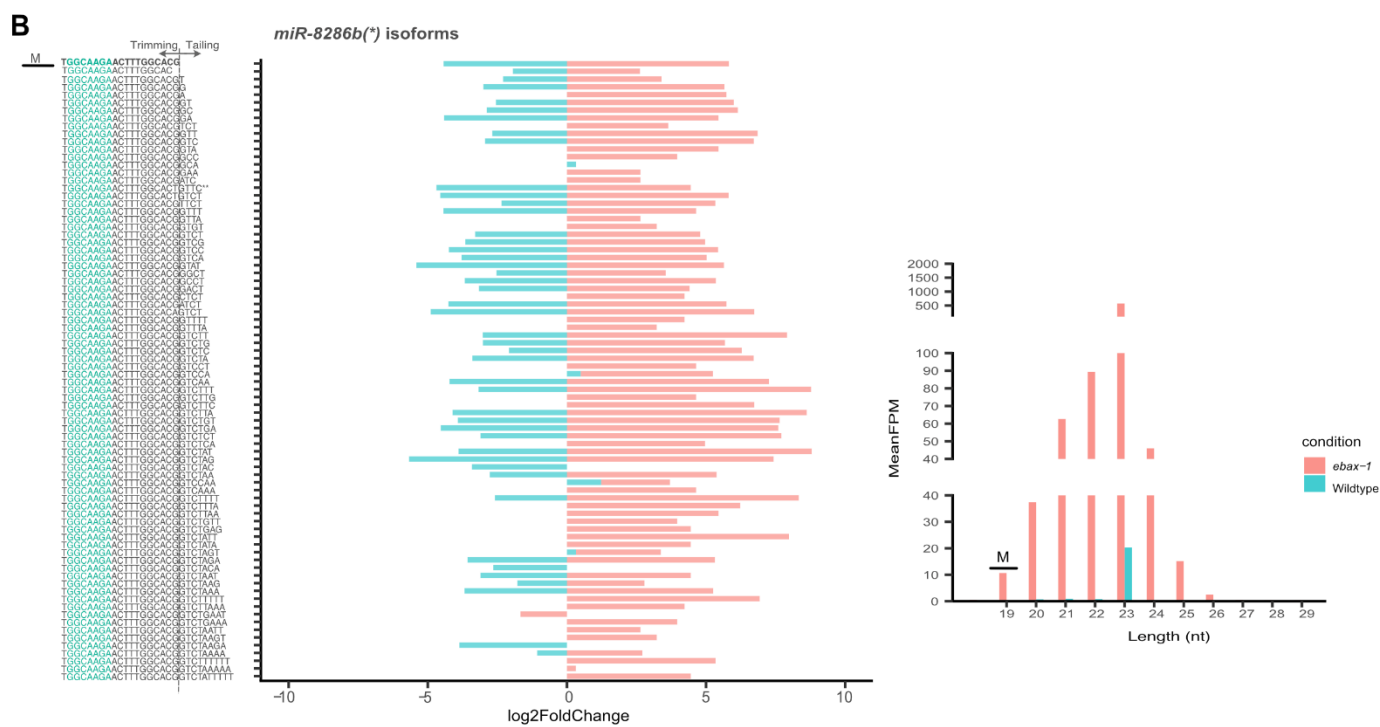
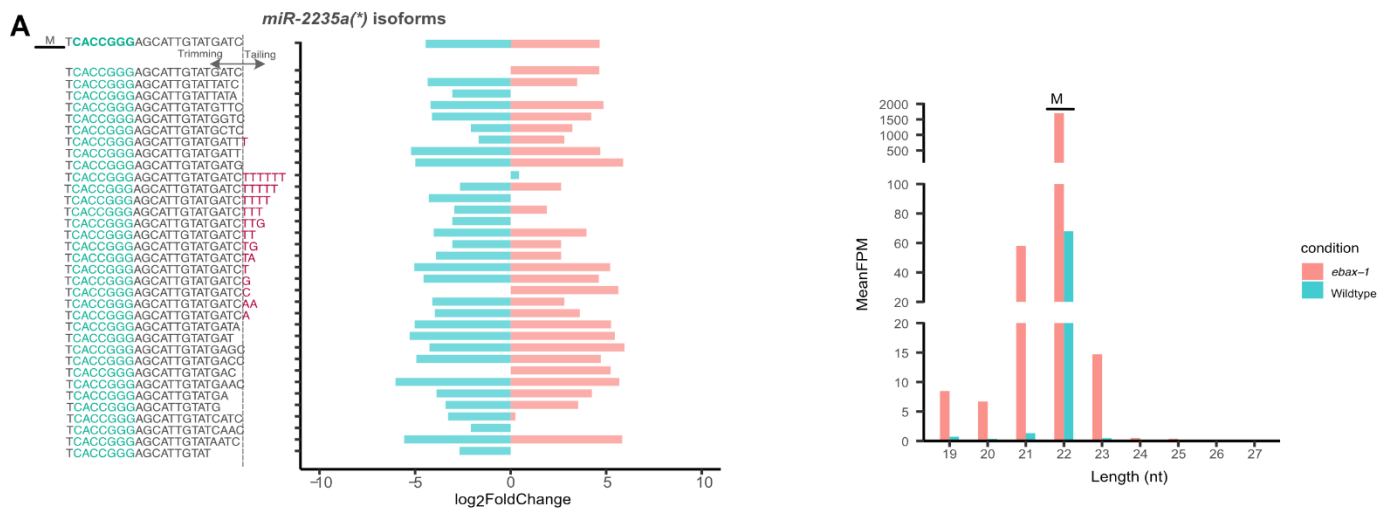
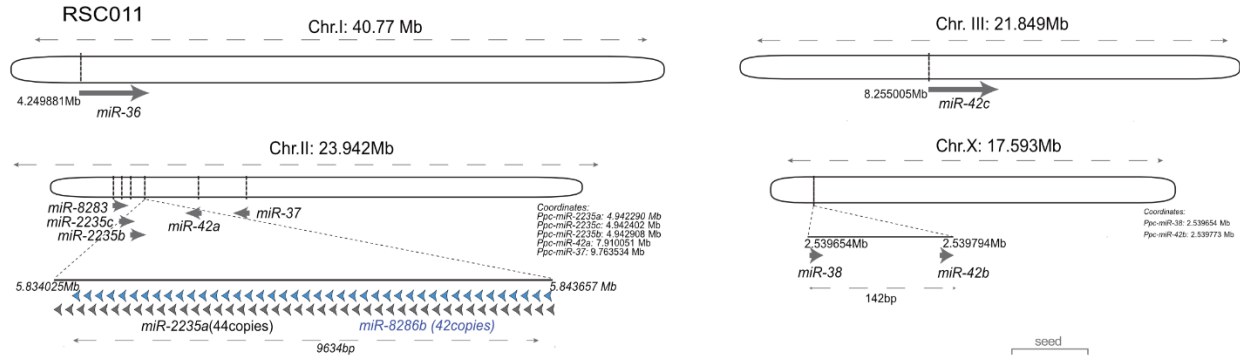


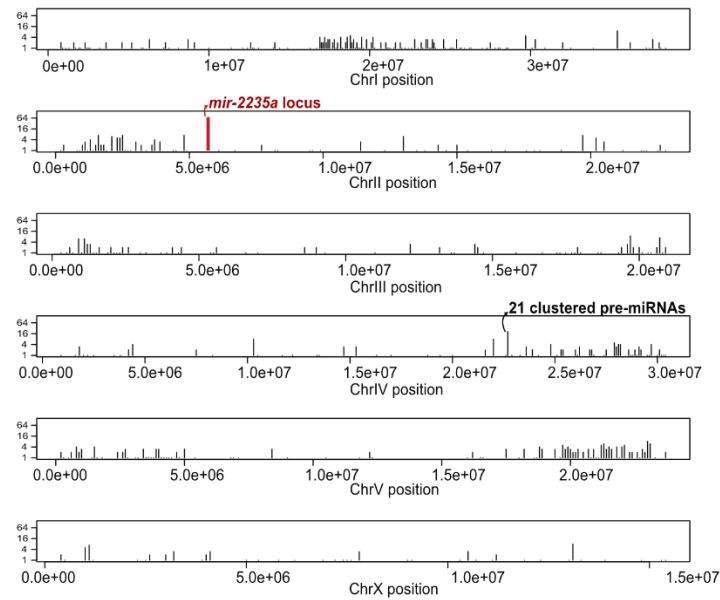
Figure S4. Influence of *ebax-1* on the distribution and patterns of *miR-8286b* and *miR-2235a* isoforms.

(A-B) Tailed and trimmed versions of *miR-2235a* and *miR-8286b* during reversal to *E. coli* (left). Plotted are the log₂ fold change from wild type (blue) and *ebax-1(tu1754)* animals (red). Also plotted are the length distribution and normalized Fragments per Million (FPM) counts of *miR-2235a* and *miR-8286b* reads (left). M represents the designated mature miRNA lengths for each strand are shown. (C) Mean normalized FPM counts of *miR-35* family members before (P0), during (F1, F3, F10) and after (F10R2) *Novosphingobium* exposure. (D) Fluorescence level of *Ppa-miR-2235p::TurboRFP* on F5 generation on *Novosphingobium*. Wilcoxon signed-rank test between dietary conditions was performed.

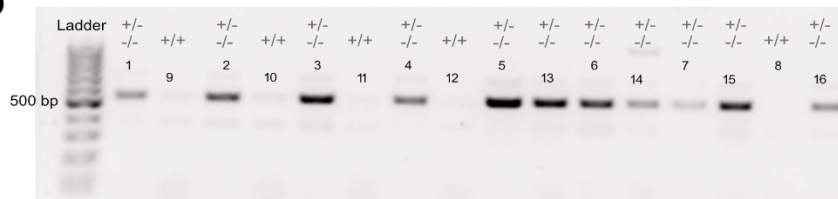
A *P. pacificus*



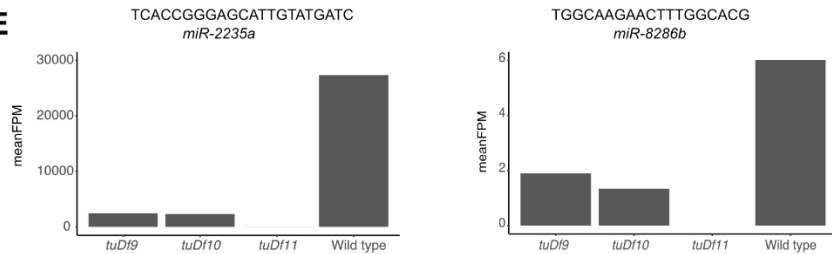
B



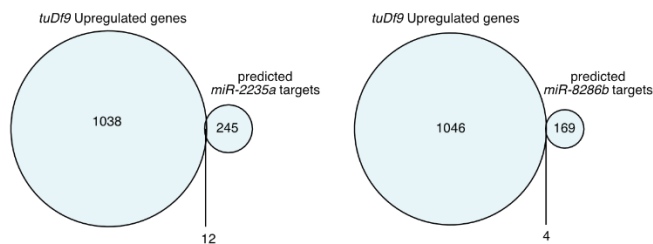
D



E



F



C

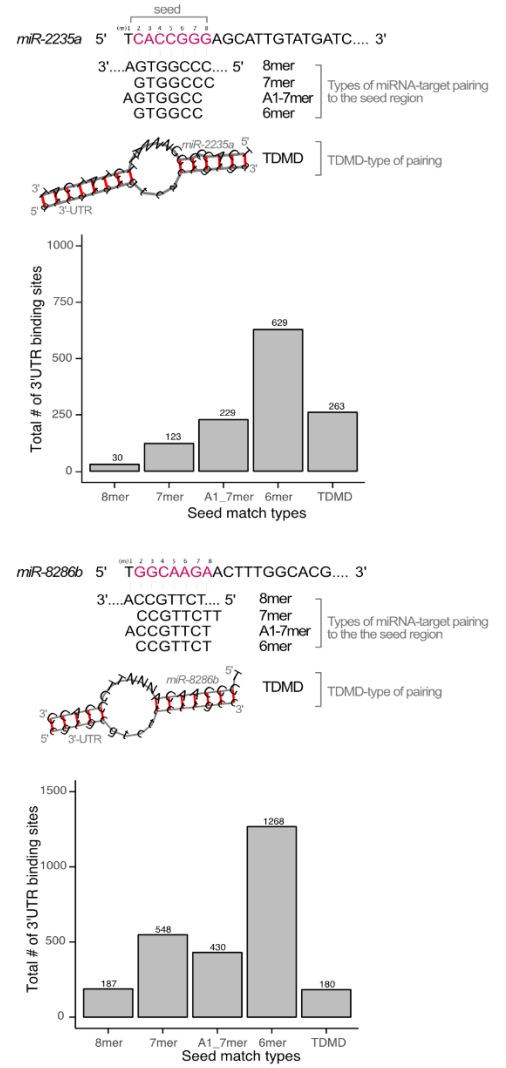


Figure S5. Earlier and extended predatory mouth form memory of *miR-2235a* deletion alleles

(A). *miR-35/miR-2235a* family members distributed in different chromosomes. Grey arrows, *miR-2235a* members. Blue arrows, *miR-8286b*. (B) Graphical representation of precursor miRNA in clusters across the *P. pacificus* genome. *miR-2235a* (red) is identified to be the largest clustered miRNAs in chromosome II, followed by the 21 clustered miRNAs in chromosome IV. (C) 3'-UTR prediction of *miR-2235a* and *miR-8286b* targets. Various types of miRNA target sites are shown with pairings restricted to the seed region and the TDMD-type of pairing with complementarity extending to the 3' region of the miRNA. The complete list of predicted targets are compiled in Data S3. (D) Agarose gel documentation after PCR of progeny individuals from a heterozygous animal for the *miR-2235a* cluster deletion. Lanes without bands are wild type (+/+), without the deletion), with bands of <500 bp are either heterozygous (+/-) or homozygous (-/-) lines for the deletion (see Materials and Methods for details). More details about the molecular lesions are listed in Table S2. (E) Mean normalized FPM counts of *miR-2235a* and *miR-8286b* upon *Novosphingobium* exposure. (F) Overlapping gene sets between the upregulated transcripts on *tuDf9* and the predicted genes whose 3'UTRs contain extensive (TDMD) pairing with *miR-2235a* and *miR-8286b*.

Table S1. *Ppa-ebax-1* and *Ppa-daf-21* alleles isolated from the genetic screen by EMS mutagenesis.

<i>C. elegans</i> 1:1							
Allele	Gene ID	ortholog	Position	Reference	Variant	Mutation type	nt substitution
<i>tu803</i>	RSC011000001067	<i>daf-21</i>	35424586	C	A	nonsynonym [E>D]	GAG>GAT
<i>tu797</i>	RSC011000001067	<i>daf-21</i>	35425940	C	T	nonsynonym [G>S]	GGC>AGC
<i>tu770</i>	RSC011000001067	<i>daf-21</i>	35425073	C	T	nonsynonym [E>K]	GAG>AAG
<i>tu776</i>	RSC011000009259	<i>ebax-1</i>	7625936	C	T	nonsynonym [S>F]	TCC>TTC
<i>tu780</i>	RSC011000009259	<i>ebax-1</i>	17621261	C	T	nonsynonym [T>I]	ACT>ATT
<i>tu759</i>	RSC011000009259	<i>ebax-1</i>	17626295	T	A	nonsynonym [V>D]	GTT>GAT

Table S2. Molecular lesions of *Ppa-ebax-1* and the *miR-2235a* locus. *tid*, transgenerational inheritance defective. TEI, transgenerational epigenetic inheritance.

Alleles	Gene ID; <i>C. elegans</i> best hit	Molecular lesions	Phenotype	sgRNA(PAM)	Forward primer	Reverse primer
<i>tul754</i>	RSC011000009259; <i>ebax-1</i>	10 bp insertion	Egg-laying defective, slow growth, low progeny, <i>tid</i>	AAGGAGTAGTAAAAGGATAT (GGG)	GGTGCAAAACAAAAGTGACCT	CTGTTGGGATAGACAGCCAG
<i>tul755</i>		60 bp deletion	<i>tid</i>			
<i>tul756</i>		T1231I + 12 bp deletion	<i>tid</i>			
<i>tulDf9</i>	<i>miR-2235a</i> (ChrII:5,834,025-5,843,657)	9,408 bp deletion	Earlier and extended TEI	GCCCCGATCATACAATGCTCC (CGG)	CCCTGCGTACTGACTATCTG	GGTTGGCTGGATAGCGTTTG
<i>tulDf10</i>		9,632 bp deletion	Earlier and extended TEI			
<i>tulDf11</i>		9,856 bp deletion	Earlier and extended TEI			

Data S1. Differential gene expression analysis and enriched gene sets after exposure to *Novosphingobium* for three and five generations.

(A and B) Upregulated and downregulated gene lists after three and five generations on *Novosphingobium*. (C and D) Lists of Pfam-domains enriched in the differentially expressed gene sets after three and five generations on *Novosphingobium*.

Data S2. Small RNA sequencing analysis of wildtype and *ebax-1* mutant animals. (A) List of differentially-expressed sRNAs between P0 on *E. coli* and F10 on *Novosphingobium*. (B) List of differentially-expressed sRNAs between F10 on *Novosphingobium* and two generations on *E. coli* (R2). (C) List of differentially-expressed sRNAs between wild type and *ebax-1* on F10 *Novosphingobium*. (D) List of differentially-expressed sRNAs between wild type and *ebax-1* after reversal to *E. coli*. (E-F) List of *miR-2235a* and *miR-8286a* isoforms during reversal upon *ebax-1* knockout.

Data S3. Genome-wide 3'-UTR prediction of miRNA targets in RSC011.

Vitamin B12 induces memory of predation through vitellogenin provisioning

Shiela Pearl Quiobe, Ata Kalirad, Raphaela Zurheide, Hanh Witte,
Christian Rödelsperger, Ralf J. Sommer

Published in:

Nature Communications (2026)17:3408. DOI: 10.1038/s41467-026-71494-w

1
2
3
4
5
6 **Vitamin B12 induces memory of predation through vitellogenin**
7 **provisioning**
8
9

10
11 **Shiela Pearl Quiobe¹, Ata Kalirad¹, Raphaela Zurheide¹, Hanh Witte¹,**
12 **Christian Rödelisperger¹ & Ralf J. Sommer^{1*}**
13
14
15

16 ¹Max Planck Institute for Biology Tübingen, Department for Integrative Evolutionary
17 Biology, Max-Planck Ring 9, 72076 Tübingen, Germany
18
19

- 20 • Author for correspondence at: ralf.sommer@tuebingen.mpg.de
21

SUMMARY

Dietary and, more generally, nutritional effects on trait inheritance are widespread in animals including human famines. Such effects represent some of the most severe environmental influences on organismal phenotypes and can even be transmitted over multiple generations^{1,2}. However, the chemical nature of the stimuli and the downstream events inducing such memory remain largely elusive. The nematode *Pristionchus pacificus* exhibits mouth-form plasticity including predation and was recently shown to respond to a multigenerational *Novosphingobium* diet with the induction and subsequent transgenerational inheritance of the predatory morph³. Here, we show that bacteria-derived vitamin B12 is both necessary and sufficient for transgenerational memory. Specifically, *Novosphingobium* mutants defective in vitamin B12 production do not induce transgenerational inheritance, but vitamin B12 supplementation can rescue the memory phenotype. Different vitamin B12 concentrations are required for the original induction and subsequent transgenerational inheritance of the predatory morph. Mutant analysis revealed that vitamin B12 functions through methionine-synthase and involves methionine but not folate. The inherited effect acts through increased multigenerational vitellogenin transcription suggesting elevated nutrient provisioning. Consistently, mutants in the vitellogenin receptor *Ppa-rme-2* are memory-defective indicating that a vitamin acts through maternal provisioning to progeny. Thus, vitamin B12 induces vitellogenin provisioning to control organismal physiology and behavior.

Keywords: Transgenerational epigenetic inheritance, developmental plasticity, *Pristionchus pacificus*, *Caenorhabditis elegans*, vitamin B12, vitellogenin

Dietary and nutritional effects can alter organismal phenotypes and influence trait inheritance over generations, so-called transgenerational epigenetic inheritance (TEI)^{1,2}. A large body of evidence indicates that dietary factors can induce various morphological, physiological and behavioral changes which are often transmitted for three generations or more. However, the chemical nature of the inducing agents and downstream events following the original dietary stimuli are largely unknown. One major obstacle in identifying inducing stimuli from the diet and the genetic machinery underlying TEI in the receiving organism is the lack of model systems with robust phenotypes to be analyzed under laboratory conditions. However, nematodes such as *Caenorhabditis elegans* and *Pristionchus pacificus* with their short generation time, isogenic propagation and simple husbandry have started to provide mechanistic insight (for review see⁴⁻⁷).

For instance, *P. pacificus* exhibits a mouth-form dimorphism that is an example of developmental plasticity with morphological and behavioral implications and therefore, provides a robust system to analyze dietary effects and TEI⁸⁻¹⁰. During postembryonic development, genetically identical *P. pacificus* worms adopt either the narrow-mouthed 'stenostomatous' (St) morph with a single dorsal tooth, or the wide-mouthed 'eurystomatous' (Eu) form with a claw-like dorsal and an opposing sub-ventral tooth (Fig. 1a). Importantly, only the Eu morph allows omnivorous feeding including predation on other nematodes (Fig. 1a). Mouth-form plasticity is characterized as a bi-stable developmental switch allowing the underlying gene regulatory network (GRN) to be elucidated in detail¹⁰⁻¹³. Additionally, multiple environmental cues influence mouth-form development including changes in diet, establishing an easy study system for experimental manipulation¹⁴.

A recent study introduced a long-term environmental induction experiment with distinct diets by propagating 110 *P. pacificus* isogenic lines for 101 generations with associated food-reversal experiments (Fig. 1b)³. In addition to the standard nematode laboratory food *Escherichia coli* OP50, this study used the *Pristionchus* environment-derived bacterium *Novosphingobium* L76 that was originally shown to increase killing efficiency of *P. pacificus*¹⁵. The long-term environmental induction experiment study revealed diet-derived induction of the Eu mouth form during *Novosphingobium* L76 exposure and subsequent TEI of this predatory phenotype when worms were reverted to an *E. coli* diet (Fig. 1b). Interestingly, TEI of the Eu morph requires a minimal exposure of five generations on the inducing

Novosphingobium L76 diet, which is phenomenologically different from most other examples of TEI that require only a short-term stimulus. Subsequent genetic studies identified a previously unknown mechanism in the regulation of TEI³. Forward genetic screens for mutants defective in transgenerational inheritance indicated an essential role of the ubiquitin ligase EBAX-1/ZSWIM8 and the destabilization of clustered microRNAs of the *miR-2235/miR-35* family for transgenerational memory. Specifically, *Ppa-ebax-1* mutants show no memory whereas deletions of the *miR-35* cluster result in precocious and extended TEI of the predatory mouth form (Fig. 1b). While these studies start to provide molecular mechanisms underlying TEI, nothing is known about the factors from *Novosphingobium* that induce the predatory mouth form and its subsequent TEI. In this work, we studied bacterial metabolites and found that vitamin B12 is both necessary and sufficient to induce the predatory mouth form and its subsequent memory in a concentration-dependent manner. This effect is inherited through increased vitellogenin transcription and consistent with this finding, mutations in the single vitellogenin germline uptake receptor *Ppa-rme-2* are memory-defective. Thus, vitamin B12 induces vitellogenin provisioning in a multigenerational manner to control organismal physiology and behavior.

RESULTS

Vitamin B12 supplementation induces the predatory mouth form and its subsequent transgenerational memory

Long-term environmental induction is performed by switching naïve *P. pacificus* RSC011 cultures that were always grown on the standard laboratory food source *E. coli* OP50 to *Novosphingobium* L76 (Fig. 1b). This *P. pacificus* strain from La Réunion Island is preferentially non-predatory (30%Eu:70%St) when grown on *E. coli*. The original long-term environmental induction experiment with 110 isogenic lines derived from the same ancestral RSC011 individual revealed dietary induction of the predatory mouth form (Fig. 1b). This dietary effect is i) immediate (in generation 1 on *Novosphingobium*), ii) complete (100% Eu progeny), iii) systemic (in all 110 lines), and iv) permanent (throughout the entire experiment of 101 generations). Following the reversal from *Novosphingobium* back to the standard *E. coli* OP50 diet, we observed TEI of the predatory mouth form (Fig.1b). Importantly, such TEI is only seen after a minimal exposure to *Novosphingobium* for five generations, whereas a shorter exposure will not result in memory of the predatory morph.

We wanted to determine the molecular nature of the dietary stimulus inducing the predatory mouth form and its transgenerational inheritance in *P. pacificus* RSC011. The *Novosphingobium* L76 strain used in this experimental setup was originally isolated from a *Pristionchus*-associated environment and was shown to enhance killing efficiency of the *P. pacificus* wild type strain PS312, which is naturally 100% Eu¹⁵. This study also identified bacterial vitamin B12 production to cause enhanced predation, to accelerate worm development and increase brood size¹⁵. Therefore, we wanted to know if vitamin B12 would also play a role in dietary induction of the predatory mouth form and TEI in *P. pacificus* RSC011 animals. For that, we transferred single naïve J4 animals to vitamin B12-supplemented agar plates with *E. coli* OP50 as food source (Fig. 2a). It is important to note that *E. coli* OP50 does not produce vitamin B12 but rather obtains its vitamin B12 from the tryptone in the agar^{15–17}. We originally used final concentrations of 500 nM, 1,000 nM and 1,500 nM of one of the two active forms of vitamin B12, methyl-Cobalamin (Me-Cbl), for supplementation, similar to previous studies in *P. pacificus*¹⁵. Note that

these concentrations are substantially higher than those used in previous work in *C. elegans*¹⁷. Strikingly, we observed an immediate induction of the Eu mouth form after vitamin B12 supplementation at all three concentrations used (Fig. 2b-d; Supplementary Fig. 1b-d). However, vitamin B12 supplementation results in a strong but not a complete induction of the Eu mouth form. Specifically, in three independent biological replicates with a total of 60 assay plates we found high but incomplete induction in the first generation after vitamin B12 supplementation ($0.878 \leq \text{HDI}(\theta) \leq 0.994$, where θ is the inferred probability of developing the predatory mouth form). This effect was seen over a period of 5 and 10 generations and was observed in all lines (Fig. 2b-d; Supplementary Fig. 1b-d). Thus, vitamin B12 mimics *Novosphingobium*-based induction of the predatory mouth form in *P. pacificus*.

To determine if vitamin B12 would also induce TEI of the predatory mouth form, we established a mini-assay with a 5-generation or 10-generation exposure of worms to a vitamin B12-supplemented *E. coli* diet, followed by 5 generations on an un-supplemented diet (Fig. 2a-d; Supplementary Fig. 1a-d). Indeed, we observed TEI of the predatory mouth form (Fig. 2b-d; Supplementary Fig. 1b-d). Animals supplemented with 1,000 nM and 1,500 nM vitamin B12 showed TEI of the Eu mouth form similar to animals that were exposed to *Novosphingobium* for 5 or 10 generations (Fig. 2b, c; Supplementary Fig. 1b, c). In contrast, a supplementation with 500 nM of vitamin B12 only caused the induction of the predatory mouth form but no full TEI of the Eu morph (Fig. 2d). Specifically, the response during reversal only lasted for two generations, with all following generations being down to baseline Eu frequencies ($0.841 \leq \text{HDI}(\theta_{R1}) \leq 0.987$, $0.52 \leq \text{HDI}(\theta_{R2}) \leq 0.842$, and $0.305 \leq \text{HDI}(\theta_{R3}) \leq 0.637$) (Fig. 2d and Supplementary Fig. 1d). Such a response is referred to as ‘intergenerational’, but not transgenerational inheritance⁴. These results allow two major conclusions. First, vitamin B12 supplementation mimics diet-derived induction of the predatory mouth form and its subsequent transgenerational inheritance. Second, the effect of vitamin B12 is concentration dependent with only 1,500 and 1,000 nM but not 500 nM of vitamin B12 causing TEI. To our knowledge, this is the first example of vitamin-induced transgenerational memory.

***Novosphingobium* and vitamin B12 supplementation induce limited overlapping transcriptomic changes**

Next, we wanted to know whether vitamin B12 supplementation can elicit similar effects on gene expression as those caused by the *Novosphingobium* diet. In principle, the *Novosphingobium* diet and vitamin B12 supplementation might induce similar transcriptomic responses or alternatively, have limited overlap in individual genes only. We examined the overlap of differential gene expression (DGE) found between vitamin B12 supplementation on *E. coli* OP50 with *Novosphingobium*-induced gene expression changes in RSC011 animals by using single worm transcriptomics of day 1 adult individuals (Fig. 2g). After three generations of exposure, *Novosphingobium*-upregulated genes were in parts also upregulated upon vitamin B12 supplementation (Fig. 2g; Supplementary Data 1). Specifically, of the 530 upregulated genes after a three-generation vitamin B12 supplementation, 103 (12.2% of all uniquely upregulated genes) were also upregulated on *Novosphingobium*. Notably, these shared genes correspond to 19.4% of the vitamin B12-induced and 24.6% of the *Novosphingobium*-induced transcriptional responses. Analysis of PFAM enrichment among these shared genes revealed that Lipase GDSL (PF00657.26) and Lectin_C (PF00059.25) were enriched in both conditions, suggesting that lipid metabolism and carbohydrate-binding/cell-surface signaling form a core transcriptional response. Beyond this overlap, vitamin B12-specific genes were uniquely enriched for CUB (PF00431.24), associated with extracellular protein interactions, whereas *Novosphingobium*-specific genes were enriched for Yae1_N (PF09811.13) and EryCIII-like_C (PF06722.16), reflecting ribosome-associated functions and secondary metabolite biosynthesis, respectively. These results highlight both conserved and condition-specific molecular programs activated in response to vitamin B12 supplementation and *Novosphingobium* exposure. (Fig. 2g; Supplementary Data 1). Thus, vitamin B12 supplementation to the *E. coli* OP50 diet exhibits limited overlap with *Novosphingobium*-induced gene expression changes.

Vitamin B12 is both necessary and sufficient to induce the predatory mouth form and subsequent TEI

Previous studies that established a role of dietary vitamin B12 on life history traits in *P. pacificus* and *C. elegans* were based on screening Tn5-based bacterial mutant libraries^{15,17}. We used a *Novosphingobium* vitamin B12-deficient mutant to study the effect of *Novosphingobium*-derived vitamin B12 on mouth-form plasticity and TEI of the predatory morph. Using the 5-generation mini-assay described above, we found that a *Novosphingobium* vitamin B12-deficient diet would still induce the Eu mouth form (Fig. 2e). However, in contrast to a wild type *Novosphingobium* diet, the induction is not complete and plateaus $\sim 0.95\%$ Eu ($0.818 \leq \text{HDI}(\theta_{F1}) \leq 0.98$ and $0.883 \leq \text{HDI}(\theta_{F5}) \leq 0.999$). These results indicate that additional factor(s) besides vitamin B12 are able to induce the predatory mouth form. Thus, the complete (100%) induction of the Eu morph of *P. pacificus* RSC011 on *Novosphingobium* is due to multiple factors.

In contrary, we found that the *Novosphingobium* vitamin B12-deficient diet would not cause TEI of the predatory mouth form. When we reverted worm lines that were exposed to the *Novosphingobium* vitamin B12-deficient diet for 5 generations back to *E. coli*, we observed an immediate return to the base line response level ($0.135 \leq \text{HDI}(\theta_{R1}) \leq 0.47$) (Fig. 2e). Similarly, when we reverted RSC011 lines that had been exposed to this diet for 10 generations, we also did not observe TEI of the predatory mouth form (Supplementary Fig. 1e). In contrast, vitamin B12 supplementation for 5 or 10 generations to the *Novosphingobium* vitamin B12-deficient diet restored the normal TEI response after reversal to *E. coli* OP50 ($0.772 \leq \text{HDI}(\theta_{R1}) \leq 0.971$, $0.65 \leq \text{HDI}(\theta_{R2}) \leq 0.919$, and $0.47 \leq \text{HDI}(\theta_{R3}) \leq 0.832$) (Fig. 2f; Supplementary Fig. 1f). These results indicate that vitamin B12 is both necessary and sufficient to induce the TEI of the predatory mouth form.

Vitamin B12-induced TEI of the predatory mouth form requires *metr-1*

In animals including humans, vitamin B12 acts as a cofactor of two enzymes, methionine-synthase in the cytoplasm and methylmalonyl coenzyme A (CoA) mutase in mitochondria (Fig. 3a)¹⁸. In *P. pacificus* as in *C. elegans*, the methionine-synthase is encoded by *Ppa-metr-1*. To manipulate the methylmalonyl coenzyme A (CoA) mutase pathway, we targeted the upstream factor *Ppa-mce-1*, that is one-to-one orthologous to *Cel-mce-1*. We targeted *Ppa-metr-1* and *Ppa-mce-1* in *P. pacificus*

RSC011 by CRISPR to study a potential role in dietary induction and TEI of the predatory mouth form (Fig. 3b). We obtained two frameshift alleles of *Ppa-metr-1* and *Ppa-mce-1* each, and tested all of them in our standard assay (Fig. 3b). First, we studied the response of *Ppa-metr-1* mutants to an *E. coli* diet supplemented with 500 nM, 1,000 nM and 1,500 nM vitamin B12. *Ppa-metr-1* mutant animals failed to respond to vitamin B12 indicating that the role of vitamin B12 in inducing the Eu mouth form requires *Ppa-metr-1* (Fig. 3c). Second, we grew *Ppa-metr-1* mutants on *Novosphingobium* and observed a mean induction of $\bar{\theta}=0.96$ throughout the exposure of 5-10 generations (Fig. 3d; Supplementary Fig. 2a, b). This result is likely due to the other *Novosphingobium* factor(s) that can induce the Eu mouth form as indicated in the experiments using the *Novosphingobium* vitamin B12-deficient diet (Fig. 2e; Supplementary Fig. 2e). Importantly, when we reverted *Ppa-metr-1* mutants from *Novosphingobium* back to *E. coli*, we observed no TEI of the predatory mouth form (Fig. 3d; Supplementary Fig. 2a, b). These findings indicate that vitamin B12 acts as a cofactor of methionine-synthase in the transgenerational inheritance of the Eu mouth form. Third, we tested *Ppa-mce-1* mutants and observed an induction of the Eu mouth form on *Novosphingobium* similar to wild type animals (Fig. 3e; Supplementary Fig. 2d, e). After reversal, *Ppa-mce-1* mutant animals responded similar to wild type controls indicating that *Ppa-MCE-1* is not involved in the TEI of the predatory mouth form (Fig. 3e; Supplementary Fig. 2d, e). Note however, that the response of *Ppa-mce-1* mutants to vitamin B12-supplemented *E. coli* diet only resulted in a partial Eu induction ($0.256 \leq \text{HDI}(\theta) \leq 0.616$ for the highest vitamin B12 concentration) (Supplementary Fig. 2c). Together, our mutant analysis identifies a requirement of *Ppa-metr-1* for the vitamin B12-mediated induction of the predatory mouth form and its subsequent TEI.

The Eu morph is already induced by 0.1 nM vitamin B12 supplementation

Given that the induction of the Eu morph and the transgenerational memory of the predatory mouth form require different concentrations of vitamin B12, we next wanted to know the minimal amount of vitamin B12 necessary to induce the Eu morph. While our original experiments used a final concentration between 500 and 1,500 nM Me-Cbl (Fig. 2), we now reduced the concentration of vitamin B12 for

several orders of magnitude (Fig. 4). Supplementation experiments using 250 nM and 125 nM vitamin B12, resulted in an immediate Eu mouth-form induction, but reversal experiments after 5 or 10 generations resulted in an even shorter memory than supplementation with 500 nM vitamin B12. Specifically, the response seen after 250 nM, 125 nM and 50 nM vitamin B12 lasted for only one generation (Fig. 4a-c; Supplementary Fig. 3a, b). When we reduced the concentration of vitamin B12 even further and supplemented with 10 nM, 5 nM, 2 nM and 0.1 nM vitamin B12, all four of these concentrations still resulted in a strong induction of the Eu mouth-form (Supplementary Fig. 3c-f). In contrast, worms had lost their memory already in the F5R2 generation (for 50nM, $0.276 \leq \text{HDI}(\theta_{R2}) \leq 0.655$, for 10 nM, $0.238 \leq \text{HDI}(\theta_{R2}) \leq 0.612$, and for 5 nM, $0.27 \leq \text{HDI}(\theta_{R2}) \leq 0.645$) (Supplementary Fig. 3c-f). Thus, the reduction of vitamin B12 concentration in supplementation experiments results in the loss of TEI of the predatory mouth form, while the induction of the Eu morph remains high. When we reduced the vitamin B12 concentration even further, we found no induction of the predatory mouth form at a concentration of 0.01 nM vitamin B12 (Supplementary Fig. 3g). Instead, worms treated with this amount of vitamin B12 stayed at the baseline level of Eu mouth-form similar to un-supplemented growth conditions. Therefore, TEI of the predatory mouth form requires a substantially higher vitamin B12 concentration than the initial induction of the Eu mouth form.

Transgenerational inheritance of the predatory morph requires methionine but not folate

Next, we used supplementation experiments with two major metabolites in the one-carbon cycle (Fig. 3a). First, we used 10 μM , 50 μM and 100 μM of folate (vitamin B9) for supplementation of standard *E. coli* plates using similar range of concentrations as a previous study in *C. elegans*⁴¹. However, we found no credible induction of the predatory mouth form (e.g., for 100 μM , $0.384 \leq \text{HDI}(\theta_{F2}) \leq 0.755$, Fig. 4d-f). Note that this might be due to the micromolar doses of folate that had to be used in these experiments due to solubility. In contrast, methionine supplementation resulted in the induction of the predatory mouth form and subsequent TEI; however, with different dynamics than what is observed after

vitamin B12 supplementation. Specifically, supplementation with 5 mM, 10 mM and 20 mM methionine resulted in the induction of the Eu mouth form after several generations of exposure (Fig. 4g-i). Note that this supplementation does not result in 100% Eu animals similar to supplementation with lower concentrations of vitamin B12 (Fig. 4). However, reversal after five generations of exposure to methionine indicates a TEI of the predatory mouth form that lasts for three generations (Fig. 4g-i). Given that methionine significantly alters osmolarity, we controlled for this effect using sorbitol¹⁹. However, sorbitol supplementation did not induce the Eu morph, indicating that the observed response is specific to methionine rather than osmotic changes (Supplementary Fig. 3h). Overall, these results suggest that vitamin B12 might exert its function through methionine in the one-carbon cycle.

Given that vitamin B12 and methionine supplementations can both cause TEI of the predatory morph, we performed single worm transcriptomic analysis of methionine supplemented worms of day 1 adult individuals. The overlap of upregulated genes between methionine supplementation and *Novosphingobium* exposure was limited (6.8%), whereas vitamin B12 supplementation showed a larger overlap with *Novosphingobium* (12.2%), indicating that vitamin B12 recapitulates a larger subset of the bacterial-induced transcriptional program (Fig. 4j). Notably, the overlap between significantly upregulated genes after vitamin B12 and methionine supplementation represents the largest fraction of 19% (Fig. 4j). Together, these patterns suggest that vitamin B12 accounts for a larger fraction of the *Novosphingobium*-induced transcriptional responses,

Vitamin B12 causes elevated nutrient provisioning

It is important to determine what type of changes act downstream of the original stimulus to cause the inherited effect. This is of particular importance given the different vitamin B12 concentrations necessary to induce the predatory mouth form and its TEI. One possibility would be that vitamin B12 is directly transmitted from the mother to the offspring. Alternatively, TEI might be a consequence of factors acting downstream of vitamin B12. To test for the former possibility, we generated mutants in *mrp-5*, a gene that was shown in *C. elegans* to be responsible for the transport of vitamin B12 from mother to the offspring²⁰. Indeed, *P. pacificus*

has a 1:1 ortholog of *mrp-5* and we were able to create two mutations in *Ppa-mrp-5* that result in frameshifts (Fig. 5a). However, *Ppa-mrp-5* mutants show a normal TEI of the predatory mouth form (Fig. 5b,c). These findings suggest that TEI is unlikely to be driven by direct vitamin B12 transmission and instead depends on downstream factors or alternative transport mechanisms.

In principle, a multigenerational vitamin B12-rich diet could induce a plethora of transcriptional changes in the worm. Therefore, we extended our gene expression analysis to cultures after reversal and compared transcriptional profiles of single naïve, F3 and F5 generation worms on *Novosphingobium* with F3R2, F4R2, F5R2 and F10R2 generations after reversal to *E. coli* (Fig. 5d). We used FnR2 rather than FnR1 generations to rule out any effect of the kanamycin treatments in the FnR1 generation. Note that we could not use single worm transcriptomic analyses for this large set of conditions and instead, performed these experiments by harvesting cultures with preferentially adult individuals. We found a consistently strong increase in the expression of vitellogenin genes, encoding the nematode yolk proteins, after *Novosphingobium* exposure with multiple vitellogenin genes remaining at higher expression levels in comparison to naïve animals (FDR-corrected p value <0.01) (Fig. 5e). Note that vitellogenin genes were also upregulated in our single worm transcriptomic dataset described above (Fig. 2g), but several of them were not significant. As vitellogenins are transmitted from mothers to offspring, they represent potential candidates for a function in TEI of the predatory mouth form.

P. pacificus shows a more complex vitellogenin composition when compared with *C. elegans*. While the *C. elegans* N2 genome contains six vitellogenin genes, *P. pacificus* RSC011 has 9 genes all of which are most closely related to *Cel-vit-6* (Fig. 5f, g). We therefore named these genes *Ppa-vit-6-A* to *Ppa-vit-6-I* based on their location along the chromosome (Fig. 5f, g). While more similar to one another in sequence than the *Cel-vit* genes, the divergence between the *Ppa-vit* genes still allows the distinction of individual genes in gene expression analysis (Fig. 5e). We found that 8 of the 9 vitellogenin genes are upregulated upon *Novosphingobium* exposure, four of which show an increased expression of at least eightfold in the F10 generation (Fig. 5e; Supplementary Data 2). A similar increase in expression was seen in all other tested generations on *Novosphingobium*. After reversal to *E. coli* the majority of vitellogenin genes were less expressed than on *Novosphingobium*; however, expression remained substantially higher than in naïve animals that had

never been exposed to *Novosphingobium* (Fig. 5e). Thus, exposure to a multigenerational *Novosphingobium* diet and subsequent reversal to *E. coli* cause elevated vitellogenin transcription resulting in increased nutritional provisioning.

Receptor-mediated endocytosis (*rme-2*) mutants have no memory

The correlation between elevated vitellogenin expression and the formation of the predatory mouth-form during and after *Novosphingobium* exposure suggests a role of nutritional provisioning in transgenerational memory. To provide mechanistic evidence for this hypothesis, we investigated the receptor involved in vitellogenin uptake into the germline. In nematodes, vitellogenin is produced in the intestine and the low-density lipoprotein receptor (LDLR) encoded by *rme-2* (receptor-mediated endocytosis protein) was shown to represent the single protein involved in vitellogenin uptake into the germline^{21,22}. In *C. elegans*, *rme-2* mutant embryos contain no detectable yolk and the brood size of homozygous mutant animals is strongly reduced²¹. In *P. pacificus*, there is a 1:1 ortholog *Ppa-RME-2* with a 55% amino acid sequence similarity (Fig. 6a). Strikingly, we had isolated three mutant alleles of *Ppa-rme-2* in our EMS mutagenesis screen for predatory mouth-form Transgenerational-inheritance-defective (Tid) mutants reported previously (Fig. 6a; Supplementary Table 2)³. This finding would be consistent with a role of vitellogenin in memory formation.

To rule out the possibility that EMS-induced mutations in genes other than *Ppa-rme-2* have resulted in the observed Tid phenotype, we generated an additional knock-out mutation by CRISPR engineering. The *Ppa-rme-2(tu2155)* allele has a 16 bp insertion that results in a premature stop codon (Fig. 6a). When we tested the *Ppa-rme-2(tu2155)* allele in a 5 generation *Novosphingobium* assay, we observed an incomplete induction of the Eu morph for all five generations of *Novosphingobium* exposures ($0.64 \leq \text{HDI}(\theta_{F1-tu2155}) \leq 0.928$, $0.664 \leq \text{HDI}(\theta_{F2}) \leq 0.94$, and $0.628 \leq \text{HDI}(\theta_{F5}) \leq 0.921$) (Fig. 6b). This result indicates that in the absence of RME-2 and vitellogenin uptake into the germline, the predatory mouth-form can still be induced, which is likely due to other *Novosphingobium*-derived factors as previously indicated (Fig. 2c). In contrast, after the reversal to *E. coli*, *Ppa-rme-2(tu2155)* did not show transgenerational memory of the predatory

405 morph ($0.12 \leq \text{HDI}(\theta_{\text{F5R1-tu2115}}) \leq 0.431$, $0.147 \leq \text{HDI}(\theta_{\text{F5R2}}) \leq 0.484$) (Fig. 6b). Thus,
406 vitellogenin is involved in the formation of transgenerational memory of the predatory
407 mouth-form in *P. pacificus* indicating a role for nutrient provisioning in this process.
408

DISCUSSION

This work demonstrates that a food-derived vitamin can cause transgenerational memory of a morphological trait and its associated behavior over multiple generations. The identification of vitamin B12 as inducing stimulus for the TEI of the predatory mouth form in *P. pacificus* after multigenerational exposure to *Novosphingobium* indicates the significance of bacterial metabolites for animal development, growth and behavior and provides links to vitamin B12 deficiency in humans. Vitamin B12 (cobalamin) is the largest and most complex vitamin^{18,23}. Exclusively synthesized by bacteria and some archaea, vitamin B12 is essential for animals including humans. However, not all bacteria produce vitamin B12 and the standard *C. elegans*/*P. pacificus* food source, *E. coli* OP50, is a non-vitamin B12 producer. Therefore, under standard laboratory growth conditions, vitamin B12 is one of the growth-limiting factors for *E. coli* bacteria and worms, and both obtain vitamin B12 exclusively from the tryptone in the agar plates¹⁶. Consistently, previous work indicated that vitamin B12 supplementation or a vitamin B12-rich *Comamonas* or *Novosphingobium* diet affect several *C. elegans* life history traits and killing efficiency in *P. pacificus*^{15,17,24,25}.

Bacterial and animal requirement for vitamin B12 is low. For example, for *E. coli* it was estimated that only 20 cobalamin molecules per cell are sufficient to support growth²⁶. In humans, the total body content of vitamin B12 is estimated to be 1-5 mg with a daily requirement of 2-3 μg ^{27,28}. In *C. elegans*, studies by Bito and co-worker have shown that culture of worms under strict vitamin B12-deficient conditions will result in a gradual decrease of the vitamin B12 content over five generations¹⁶. In the fifth generation, *C. elegans* had only 4% of vitamin B12 relative to worms growing under standard conditions and started to show a loss of fertility and reduced lifespan at these reduced concentrations¹⁶. These findings are consistent with three of our observations; i) the multigenerational exposure to a *Novosphingobium* diet being necessary to induce TEI of the predatory mouth form, ii) the capability of vitamin B12 supplementation to induce TEI of this phenotype, and most importantly iii) the concentration-dependent effect of vitamin B12 supplementation. It is important to note that most studies in nematodes used unphysiological amounts of vitamin B12 supplementation and in *P. pacificus*, we have to use even higher concentrations than in *C. elegans* to see TEI of the predatory

morph. However, vitamin B12 is not directly taken up by the worms from the agar and instead is ingested through the *E. coli* diet. Given that the direct measurement of vitamin B12 still represents a major challenge, the exact amount of vitamin B12 in *C. elegans* or *P. pacificus* after a *Comamonas* or *Novosphingobium* diet is currently unknown, a limitation that is common to all studies on vitamin B12²⁹.

The experiments with *Ppa-mrp-5* mutants show a normal TEI suggesting that it is not the direct transmission of vitamin B12 but a downstream factor causing the observed memory. However, the transgenerational effect is slightly decreased compared to wild type and one possibility that cannot be completely ruled out is that multiple transporters in addition to *Ppa-mrp-5* are involved in vitamin B12 transmission. Nonetheless, *Ppa-mrp-5* is one-to-one orthologous to *Cel-mrp-5*; thus, there are no additional copies of this transporter in the *P. pacificus* genome.

In *P. pacificus*, the inherited effect of vitamin B12 acts through vitellogenin. Vitellogenins are a family of yolk proteins representing the most abundant proteins in oviparous animals. Previous work in *C. elegans* had indicated a role of vitellogenins for post-embryonic development and fertility²⁰. Increased vitellogenin provisioning is associated with several post-embryonic phenotypic alterations, i.e. advanced maternal age. Specifically, young *C. elegans* mothers provide less embryonic vitellogenin resulting in early-hatched larvae being smaller and slower to reach adulthood. In contrast, older mothers provide more vitellogenin resulting in larger larvae that are also more starvation resistant, indicating an intergenerational signal that mediates the influence of parental physiology on progeny^{22,30}. In addition, it is important to note that vitellogenins are part of a lipoprotein complex which also carries lipids. These lipids are also dependent on RME-2 and recent studies in *C. elegans* have indeed indicated that they are involved in intergenerational inheritance^{31,32}.

The work described in this study has to be seen in the context of the ecology of *P. pacificus* nematodes. The majority of *Pristionchus* species is found in association with scarab beetles, where nematodes attach as dauer larvae to adult beetles³³. After the death of the insect in the soil, *Pristionchus* and other nematodes compete for the limited food source on the beetle carcass. Through the ability to consume bacteria, fungi and other nematodes, *Pristionchus* has an advantage in this heterogeneous environment, a phenomenon referred to as intraguild predation³⁴. Therefore, the observed transgenerational memory might represent an advantage for

477 subsequent generations. Interestingly, we have previously shown that *P. pacificus*
478 can selectively feed on vitamin B12-producing bacteria in a semi-natural beetle
479 carcass habitat³⁵. In conclusion, our work extends recent findings indicating that
480 nutrient provisioning affects organismal physiology and behavior involving
481 transgenerational inheritance and requires epigenetic factors. This multigenerational
482 effect highlights the significance of exceptions to the Weissmann barrier and
483 suggests the transmission of vitellogenin and potentially other lipids from somatic
484 tissues into the germline to have important consequences for development and
485 evolution.

Methods

Data and code availability

All the code and data for Bayesian analysis are available at <https://github.com/shielapearl18/Bayesian-estimates-of-mouth-form-responses-in-vitamin-B12-enriched-environment>.

Sequencing data that was generated for the current study has been submitted to the European Nucleotide Archive under the project accession PRJEB74486.

Experimental model and subject details

The ancestral *P. pacificus* RSC011 isolate used in this study was frozen within the first 5-10 generations after original isolation from Coteau Kerveguen on La Réunion to minimize domestication and thereby, facilitate the investigation of diet-induced plasticity and transgenerational epigenetic inheritance. Nematodes were grown under standard nematode growth conditions on NGM plates seeded with either *Escherichia coli* OP50 or *Novosphingobium* L76 and maintained at 20°C. The RSC011 stock was not subjected to bleaching, starvation, extreme temperature fluctuations as they are known to influence mouth-form ratios.

Bacterial strains conditions

All bacterial strains and mutants were grown overnight in LB (Lysogeny broth) supplemented with 50 µg/ml kanamycin where required to initiate food reversal. Bacteria were grown at 30 °C or 37 °C depending on the species and 6 cm nematode growth medium (NGM) plates were seeded with 300 µl bacterial overnight cultures and were incubated for 2 days.

511 Nematode culture and dietary reversal experiments

512 Overnight cultures of *E. coli* OP50 and *Novosphingobium* L76 were spread to NGM
513 plates and incubated at room temperature for 2 days. The *P. pacificus* RSC011
514 strain is preferentially St (20-40% Eu) on a standard *E. coli* OP50 diet.
515 *Novosphingobium* exposure is performed by picking single J4 larvae onto a
516 *Novosphingobium* L76 diet. J4 worms were initially left for at least an hour on an
517 initial *Novosphingobium* diet to reduce traces of *E. coli* OP50 before transferring to
518 final F1 *Novosphingobium* plates. Reversal experiments to an *E. coli* OP50 diet were
519 performed after exposing of RSC011 worms for various numbers of generations, i.e.
520 1, 3, 5, 10 or 15 generations on *Novosphingobium* L76. Worms were initially
521 transferred from *Novosphingobium* L76 to NGM plates supplemented with 50 µg/ml
522 of kanamycin for one generation, then back to *E. coli* OP50-seeded NGM plates for
523 subsequent generations. Exposure to kanamycin for a single generation to eliminate
524 traces of *Novosphingobium* did not affect mouth-form response³.

525 Mouth-form phenotyping

526 Mouth-form phenotyping was performed using Zeiss Discovery V.20 stereo
527 microscope (X150 magnification) by observing the nematode buccal cavity based on
528 mouth-form identities previously described⁹. Final mouth-form frequencies are the
529 mean of at least 20 replicates, each assaying 20 animals from a single plate.

530 Statistical analysis

531 To estimate the probability of developing the probability of developing the Eu mouth
532 form in *P. pacificus* (θ), we constructed a hierarchical Bayesian model as previously
533 described³

$$\begin{aligned}y_i &\sim \text{Bernoulli}(\theta) \\ \theta &\sim \text{Beta}(\omega(\kappa-2)+1, (1-\omega)(\kappa-2)+1) \\ \omega &\sim \text{Beta}(\alpha, \beta) \\ \kappa &\sim \text{Gamma}(3, 1)\end{aligned}$$

534 where θ is calculated for each replicate and hyperparameters ω and κ link the
535 biological replicates for a given generation under a given experimental condition³⁶.
536 The model was fitted to the laboratory measurements using PyMC³⁷ in Python 3.11

with Numpy 1.25.2³⁸. The mean highest density interval (HDI) for θ for a group of observations was used to visualize the inferred probability of developing the Eu mouth form. We ensured that the estimated θ values were stable using common convergence diagnostics with effective sample size (ESS) ≥ 10000 ³⁹.

CRISPR/Cas9-Induced Mutations

Mutations in candidate genes were generated using CRISPR/Cas9 following the previously described protocols⁴⁰. Gene-specific crRNAs and universal trans-activating CRISPR RNA (tracrRNA) were obtained from Integrated DNA Technologies. Equal volumes of (5 μ l) of each 100 μ M stock were mixed and denatured at 95 °C for 5 min, then allowed to anneal at room temperature. Cas9 endonuclease (New England Biolab) was added to the hybridized product and incubated at room temperature for 5 min. TE buffer was used to adjust to final concentrations of 18.1 μ M for the sgRNA and 2.5 μ M for Cas9. The resulting mixture was microinjected into the germline of 40-50 *P. pacificus* RSC011 young adults. Eggs from injected P0s were collected up to 16 hours post-injection. After hatching and two days of growth, the F1 progeny was segregated onto individual plates until they had fully-developed and laid eggs sufficiently. F1 genotypes were screened using Sanger sequencing and mutations were identified prior to isolation of homozygous mutants. Details of sgRNAs and primers used in this study are provided in the supplementary table. Evidence for null alleles was based on frameshift mutations causing premature stop codons in protein coding sequences.

Metabolite supplementation assays

Methylcobalamin (Vitamin B12 CAS No. 13422–55–4), L-methionine (CAS No. 63–68–3) and folate (CAS No. 59-30-3) were purchased from Sigma and dissolved in water at the highest possible soluble concentrations to prepare stock solution. To quantify real phenotypic effects and control for altered NGM osmolarity, D-sorbitol (CAS No.) was added¹⁹ at a final working concentration of 5 mM, 10 mM and 20 mM. All stocks were prepared fresh before use in each experiment. Metabolite solutions were mixed with NGM agar at the required concentration just before pouring to 6 cm plates. Plates were allowed to dry at room temperature for two days and then spotted with *E. coli* OP50. We first tested different concentrations of vitamin B12 and

found the strongest and most reliable transgenerational epigenetic memory effect with a concentration of 1000 nM and 1500 nM, which is most likely un-physiological. Similarly in *C. elegans*, dose-dependent effects have been seen for vitamin B₁₂.¹⁷ Different concentrations for methionine and folate supplementation were based on previous studies^{15,41}.

RNA sequencing and data analysis

We used single worm transcriptomics (SWT) of naïve, and RSC011 worms reared on *Novosphingobium* for three generations using five independent replicates, similar to previously established protocols⁴². *E. coli* OP50 supplemented with vitamin B12 and methionine were also processed with the SWT protocol. In contrast, we used mixed cultures for assaying independent generational datapoints during *Novosphingobium* induction and reversal experiments in F3R2, F4R2, F5R2 and F10R2 generations. For that, worms were harvested from three NGM plates. In general, worms were pelleted for RNA extraction and total RNA was isolated using Direct-Zol RNA Mini prep kit (Zymo Research) following the manufacturer's instructions. The RNA-seq library preparation and sequencing were performed by the company Novogene. Raw reads were aligned to the *P. pacificus* RSC011 reference genome using Hisat2⁴³ (version 2.1.0), and read counts were quantified with featureCounts⁴⁴ based on the RSC011 gene annotations. For the analysis of vitellogenin expression, we combined raw counts of the three transcripts (RSC011000007769, RSC011000004509 and RSC011000017258) into a single *vit-6-B* count. Differential gene expression analysis was performed in R (version 4.0.3) using DESeq2 (version 1.18.1)⁴⁵. For single-worm RNA-seq samples, genes with $\text{padj} < 0.05$ were considered differentially expressed to account for higher inter-individual variability. For mixed-worm RNA-seq samples, a more stringent cutoff of $\text{padj} < 0.01$ was applied, reflecting the lower variability and increased confidence in expression differences. Tests for overrepresentation of protein domains in sets of differentially expressed genes were performed using a Fisher's exact test in R. We used the FDR method as implemented in the `p.adjust` function in R to correct for multiple testing and only retained results with adjusted P-value < 0.01 .

Phylogenetic analysis

600 One- to- one orthologous genes between *P. pacificus* and *C. elegans* was identified
601 from BLASTP searches and were validated by phylogenetic analysis. Proteins were
602 aligned using Clustal Omega⁴⁶ and the output FASTA file was uploaded to the IQ-
603 TREE tool^{47,48}. Analysis was performed under default settings using the auto
604 substitution model, and 1000 bootstrap alignments were calculated with ultrafast
605 setting. FigTree was used to visualized the resulting phylogenetic tree
606 (<http://tree.bio.ed.ac.uk/software/figtree/>).

References

1. Painter, R. C. *et al.* Transgenerational effects of prenatal exposure to the Dutch famine on neonatal adiposity and health in later life. *BJOG* **115**, 1243–1249 (2008).
2. Song, S., Wang, W. & Hu, P. Famine, death, and madness: Schizophrenia in early adulthood after prenatal exposure to the Chinese Great Leap Forward Famine. *Soc. Sci. Med.* **68**, 1315–1321 (2009).
3. Quiobe, S. P. *et al.* EBAX-1/ZSWIM8 destabilizes miRNAs resulting in transgenerational memory of a predatory trait. *Sci. Adv.* **11**, 1–17 (2025).
4. Baugh, L. R. & Day, T. Nongenetic inheritance and multigenerational plasticity in the nematode *C. elegans*. *Elife* **9**, 1–13 (2020).
5. Frolows, N. & Ashe, A. Small RNAs and chromatin in the multigenerational epigenetic landscape of *Caenorhabditis elegans*. *Philos. Trans. R. Soc. B Biol. Sci.* **376**, 1–12 (2021).
6. Chen, X. & Rechavi, O. Plant and animal small RNA communications between cells and organisms. *Nat. Rev. Mol. Cell Biol.* **23**, 185–203 (2022).
7. Santilli, F. & Boskovic, A. Mechanisms of transgenerational epigenetic inheritance: lessons from animal model organisms. *Curr. Opin. Genet. Dev.* **79**, 1–8 (2023).
8. Bento, G., Ogawa, A. & Sommer, R. J. Co-option of the hormone-signalling module dafachronic acid-DAF-12 in nematode evolution. *Nature* **466**, 494–497 (2010).
9. Ragsdale, E. J., Müller, M. R., Rödelberger, C. & Sommer, R. J. A developmental switch coupled to the evolution of plasticity acts through a sulfatase. *Cell* **155**, 922–933 (2013).
10. Lightfoot, J. W. *et al.* Small peptide-mediated self-recognition prevents cannibalism in predatory nematodes. *Science* **364**, 86–89 (2019).
11. Ptashne, M. A genetic switch: Gene control and phage. *J. Mol. Biol.* **24**, 789–790 (1986).
12. Balázsi, G., Van Oudenaarden, A. & Collins, J. J. Cellular decision making and biological noise: From microbes to mammals. *Cell* **144**, 910–925 (2011).
13. Sommer, R. J. Phenotypic plasticity: From theory and genetics to current and future challenges. *Genetics* **215**, 1–13 (2020).
14. Dardiry, M., Piskobulu, V., Kalirad, A. & Sommer, R. J. Experimental and theoretical support for costs of plasticity and phenotype in a nematode cannibalistic trait. *Evol. Lett.* **7**, 48–57 (2023).
15. Akduman, N. *et al.* Bacterial vitamin B12 production enhances nematode predatory behavior. *ISME J.* **14**, 1494–1507 (2020).

- 642 16. Bito, T., Matsunaga, Y., Yabuta, Y., Kawano, T. & Watanabe, F. Vitamin B12
643 deficiency in *Caenorhabditis elegans* results in loss of fertility, extended life cycle,
644 and reduced lifespan. *FEBS Open Bio* **3**, 112–117 (2013).
- 645 17. Watson, E. *et al.* Interspecies systems biology uncovers metabolites affecting *C.*
646 *elegans* gene expression and life history traits. *Cell* **156**, 759–770 (2014).
- 647 18. Litwack, G. *Vitamins and Hormones*. **119**, (Academic Press: Cambridge, MA, 2022).
- 648 19. Chandler-Brown, D. *et al.* Sorbitol treatment extends lifespan and induces the osmotic
649 stress response in *Caenorhabditis elegans*. *Front. Genet.* **6**, 1–9 (2015).
- 650 20. Na, H., Ponomarova, O., Giese, G. E. & Walhout, A. J. M. *C. elegans* MRP-5 Exports
651 Vitamin B12 from Mother to Offspring to Support Embryonic Development. *Cell Rep.*
652 **22**, 3126–3133 (2018).
- 653 21. Grant, B. & Hirsh, D. Receptor-mediated endocytosis in the *Caenorhabditis elegans*
654 oocyte. *Mol. Biol. Cell* **10**, 4311–4326 (1999).
- 655 22. Perez, M. F. & Lehner, B. Vitellogenins - Yolk Gene Function and Regulation in
656 *Caenorhabditis elegans*. *Front. Physiol.* **10**, 1–17 (2019).
- 657 23. Eschenmoser, A. Etiology of potentially primordial biomolecular structures: From
658 vitamin B12 to the nucleic acids and an inquiry into the chemistry of life's origin: A
659 retrospective. *Angew. Chemie - Int. Ed.* **50**, 12412–12472 (2011).
- 660 24. Watson, E., MacNeil, L. T., Arda, H. E., Zhu, L. J. & Walhout, A. J. M. Integration of
661 metabolic and gene regulatory networks modulates the *C. elegans* dietary response.
662 *Cell* **153**, 253–266 (2013).
- 663 25. MacNeil, L. T., Watson, E., Arda, H. E., Zhu, L. J. & Walhout, A. J. M. Diet-induced
664 developmental acceleration independent of TOR and insulin in *C. elegans*. *Cell* **153**,
665 240–252 (2013).
- 666 26. Di Girolamo, P. M., Kadner, R. J. & Bradbeer, C. Isolation of Vitamin B12 Transport
667 Mutants of *Escherichia coli*. *J. Bacteriol.* **106**, 751–757 (1971).
- 668 27. Doets, E. L. *et al.* Systematic review on daily Vitamin B12 losses and bioavailability
669 for deriving recommendations on Vitamin B12 intake with the factorial approach. *Ann.*
670 *Nutr. Metab.* **62**, 311–322 (2013).
- 671 28. Institute of Medicine. Vitamin B12. in *Dietary Reference Intakes for Thiamin,*
672 *Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin and*
673 *Choline*. 306–348 (Washington DC:National Academies Press, 1998).
- 674 29. Lawrence, A. D. *et al.* Construction of Fluorescent Analogs to Follow the Uptake and
675 Distribution of Cobalamin (Vitamin B12) in Bacteria, Worms, and Plants. *Cell Chem.*
676 *Biol.* **25**, 941-951.e6 (2018).
- 677 30. Perez, M. F., Francesconi, M., Hidalgo-Carcedo, C. & Lehner, B. Maternal age

678 generates phenotypic variation in *Caenorhabditis elegans*. *Nature* **552**, 106–109
679 (2017).

680 31. Wang, W. *et al.* An intestinal sphingolipid confers intergenerational neuroprotection.
681 *Nat. Cell Biol.* **25**, 1196–1207 (2023).

682 32. Wilhelm, T., Chen, W.-W., Wenzel, A., Tang, W. & Cicerone, M. T. An
683 intergenerational lipid memory of the social environment in *C. elegans*. *bioRxiv*
684 2025.06.03.657568 (2025).

685 33. Sommer, R. J. *Pristionchus* – Beetle associations: Towards a new natural history. *J.*
686 *Invertebr. Pathol.* **209**, 108243 (2025).

687 34. Quach, K. T. & Chalasani, S. H. Intraguild predation between *Pristionchus pacificus*
688 and *Caenorhabditis elegans*: a complex interaction with the potential for aggressive
689 behaviour. *J. Neurogenet.* **34**, 404–419 (2020).

690 35. Lo, W. S., Sommer, R. J. & Han, Z. Microbiota succession influences nematode
691 physiology in a beetle microcosm ecosystem. *Nat. Commun.* **15**, 1–11 (2024).

692 36. Kruschke, J. K. *Doing Bayesian data analysis: A tutorial with R, JAGS, and Stan*,
693 *second edition. Doing Bayesian Data Analysis: A Tutorial with R, JAGS, and Stan*,
694 *Second Edition* (Elsevier Science, 2014). doi:10.1016/B978-0-12-405888-0.09999-2

695 37. Abril-Pla, O. *et al.* PyMC: a modern, and comprehensive probabilistic programming
696 framework in Python. *PeerJ Comput. Sci.* **9**, 1–35 (2023).

697 38. Harris, C. R. *et al.* Array programming with NumPy. *Nature* **585**, 357–362 (2020).

698 39. Kruschke, J. K. Bayesian Analysis Reporting Guidelines. *Nat. Hum. Behav.* **5**, 1282–
699 1291 (2021).

700 40. Witte, H. *et al.* Gene inactivation using the CRISPR/Cas9 system in the nematode
701 *Pristionchus pacificus*. *Dev. Genes Evol.* **225**, 55–62 (2014).

702 41. Rathor L. *et al.* Folic acid supplementation at lower doses increases oxidative stress
703 resistance and longevity in *Caenorhabditis elegans*. *Age* **37**, 1-15 (2015).

704 42. Sun, S., Rödelberger, C. & Sommer, R. J. Single worm transcriptomics identifies a
705 developmental core network of oscillating genes with deep conservation across
706 nematodes. *Genome Res.* **31**, 1590–1601 (2021).

707 43. Kim, D., Langmead, B. & Salzberg, S. L. HISAT: A fast spliced aligner with low
708 memory requirements. *Nat. Methods* **12**, 357–360 (2015).

709 44. Liao, Y., Smyth, G. K. & Shi, W. FeatureCounts: An efficient general purpose
710 program for assigning sequence reads to genomic features. *Bioinformatics* **30**, 923–
711 930 (2014).

712 45. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and

- 713 dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 1–21 (2014).
- 714 46. Gabler, F. *et al.* Protein Sequence Analysis Using the MPI Bioinformatics Toolkit.
715 *Curr. Protoc. Bioinforma.* **72**, 1–30 (2020).
- 716 47. Nguyen, L. T., Schmidt, H. A., Von Haeseler, A. & Minh, B. Q. IQ-TREE: A fast and
717 effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol.*
718 *Biol. Evol.* **32**, 268–274 (2015).
- 719 48. Trifinopoulos, J., Nguyen, L. T., von Haeseler, A. & Minh, B. Q. W-IQ-TREE: a fast
720 online phylogenetic tool for maximum likelihood analysis. *Nucleic Acids Res.* **44**,
721 W232–W235 (2016).

722

723

724

Acknowledgments

We would like to thank the entire support team and technicians of the Sommer lab in particular Heike Hausmann for assisting in freezing nematode cultures. We are also grateful to Drs. Adrian Streit and Catia Igreja for discussions and helpful comments on the manuscript. The work was funded by the Max Planck Society.

Author contributions: Conceptualization, S.P.Q. and R.J.S.; Methodology, S.P.Q., A.K., C.R. and R.J.S; Formal Analysis, S.P.Q., A.K.; Investigation, S.P.Q.; Resources, R.Z. and H.W.; Writing – Original Draft, S.P.Q. and R.J.S.; Writing – Review & Editing, S.P.Q., A.K., C.R. and R.J.S.; Visualization, S.P.Q. and A.K.; Funding Acquisition, R.J.S; Supervision, R.J.S.

Competing interests

The authors declare no competing interests.

Materials and Correspondence

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact Ralf J. Sommer.

ralf.sommer@tuebingen.mpg.de

Materials availability

P. pacificus strains and bacterial isolates generated in this work are freely available through the Lead Contact

Figure Legends

Figure 1. A role for the ubiquitin ligase EBAX-1/ZWSIM8 in dietary-induced transgenerational inheritance of the predatory mouth form in *Pristionchus pacificus*

a, Mouth dimorphism of *P. pacificus*. The eurystomatous (Eu) morph has a wide mouth with two teeth and feeds on bacteria and nematodes, whereas the stenostomatous (St) morph has a narrow mouth with a single tooth and feeds only on microbes. Right picture: Adult Eu *P. pacificus* devouring a *C. elegans* larval prey. **b**, Graphical representation derived from Quiobe et al., 2025. Using long-term dietary induction and food-reversal experiments across 110 isogenic *P. pacificus* lines propagated for 101 generations demonstrated dietary induction and transgenerational inheritance of the predatory mouth form. Further studies identified EBAX-1/ZSWIM8-mediated destabilization of the expanded *miR-2235a/miR-35* miRNA cluster as a key mechanism underlying this inheritance, consistent with a role for target-directed miRNA degradation.

Figure 2. Diet-derived vitamin B12 induces the predatory mouth form and its transgenerational inheritance

a, Schematic diagram for vitamin B12 supplementation showing five generations on vitamin B12-supplemented plates and subsequent exposure to non-supplemented *E. coli* plates. **b-d**, Vitamin B12 supplementation mimics induction and subsequent transgenerational inheritance of the Eu mouth form. Mean probability of predatory mouth form for five generations on 1,500 nM, 1,000 nM, and 500 nM vitamin B12-supplemented *E. coli* and five generations on non-supplemented *E. coli*. **e**, *Novosphingobium* vitamin B12-deficient mutant induces the predatory morph but not its transgenerational inheritance. Mean probability of the predatory mouth form on a *Novosphingobium* vitamin B12-deficient mutant and reversal to *E. coli*. **f**, Rescue of transgenerational inheritance of the predatory mouth form by supplementing exogenous vitamin B12 to *Novosphingobium* vitamin B12-deficient mutant. Mean probability of predatory mouth form for 5 generations on vitamin B12-supplemented *Novosphingobium* mutants and subsequent reversal to *E. coli*. **g**, Differential gene expression and enrichment analyses through single-worm transcriptomics. Exposure to *Novosphingobium* and vitamin B12 supplemented *E. coli* revealed overlapping

differentially-expressed transcripts relative to worm cultures grown on a standard *E. coli* diet. The pathways with most significant enrichment (FDR-corrected p value <0.01) for three generations on *Novosphingobium* and B12-supplemented *E. coli* are shown. See also Supplementary Fig.1.

Figure 3. Vitamin B12 functions as a cofactor for methionine synthase *metr-1* in the transgenerational inheritance of the Eu morph

a, Requirement for vitamin B12 as cofactor for two enzymes in the cytosol and mitochondria. In the cytoplasm, it acts as cofactor of methionine synthase (*metr-1*) as part of the one carbon cycle. In the mitochondria, vitamin B12 is a co-factor of methylmalonyl coenzyme A mutase. Both genes (*metr-1* and *mce-1*) encoding the metabolic enzymes in which vitamin B12 acts as a cofactor are shown. Red, vitamin B12; Blue, vitamin B6. **b**, CRISPR/Cas9-induced mutations in *Ppa-METR-1* and *Ppa-MCE-1* with target locations indicated in respective protein domains (sgRNA, arrow). Molecular lesions of isolated mutations via CRISPR/Cas9 are also shown. **c-d**, Requirement of *Ppa-metr-1* in transgenerational inheritance of vitamin B12-induced Eu mouth form. **c**, Mean probability of predatory mouth-form in *Ppa-metr-1* mutant animals on vitamin B12-supplemented *E. coli* compared to wild type. **d**, Mean probability of the predatory mouth form in *Ppa-metr-1* mutant animals on a *Novosphingobium* diet and reversal to *E. coli* compared to the wild type response. **e**, Mean probability of the predatory mouth form in *Ppa-mce-1* mutant animals exposed to *Novosphingobium* and reversal to *E. coli* compared to wild type response. See also Supplementary Fig. 2 and Supplementary Table 1.

Figure 4. Memory of the induced predatory mouth form after supplementation with different concentrations of vitamin B12, methionine and folate.

a-c, Concentration-dependent effect of vitamin B12 on memory transmission. **(a)** Mean probability of predatory mouth-form after a 5-generation exposure to 250 nM vitamin B12 shows Eu memory for two generations. Further reduction of vitamin B12 concentration to **(b)** 125 nM, **(c)** 50 nM, shows a single generation of Eu memory on un-supplemented *E. coli* diets. nM, nanomolar. **d-f**, Mean probability of the predatory mouth form on folate-supplemented *E. coli* plates with **(d)** 100 μ M, **(e)** 50 μ M and **(f)**

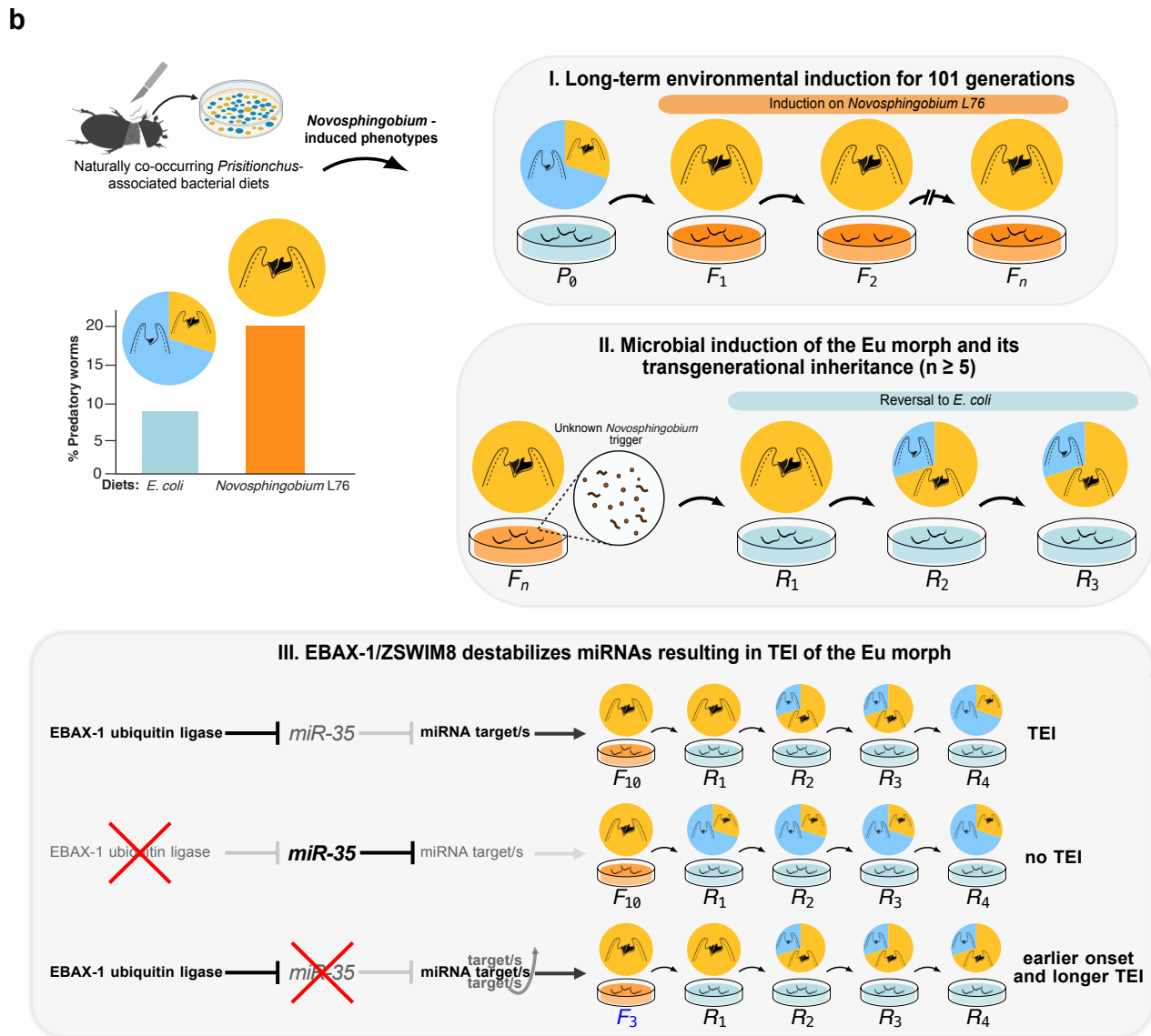
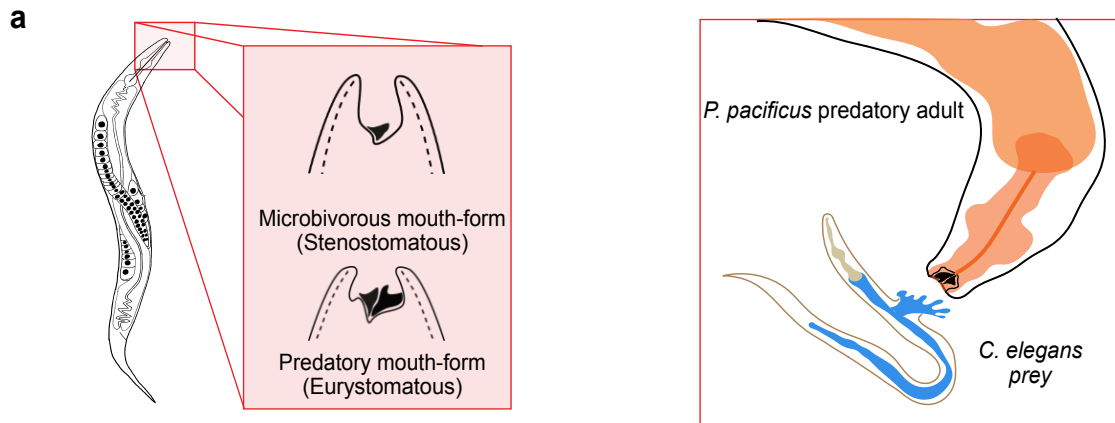
10 μ M working concentration of folate. **g-i**, Mean probability of the predatory mouth form on methionine-supplemented *E. coli* plates with **(g)** 20 mM, **(h)** 10 mM and **(i)** 5mM working concentration of methionine. mM, millimolar; μ M, micromolar. **j**, Differential gene expression and enrichment analyses. Exposure to methionine-supplemented *E. coli* showed upregulated overlapping transcripts with *Novosphingobium* and vitamin B12 supplemented *E. coli*. Differentially-induced genes are identified relative to worm cultures grown on a standard *E. coli* diet. The pathways with most significant enrichment (FDR-corrected p value <0.01) for three generations on methionine-supplemented *E. coli* are shown. See also Supplementary Fig.3.

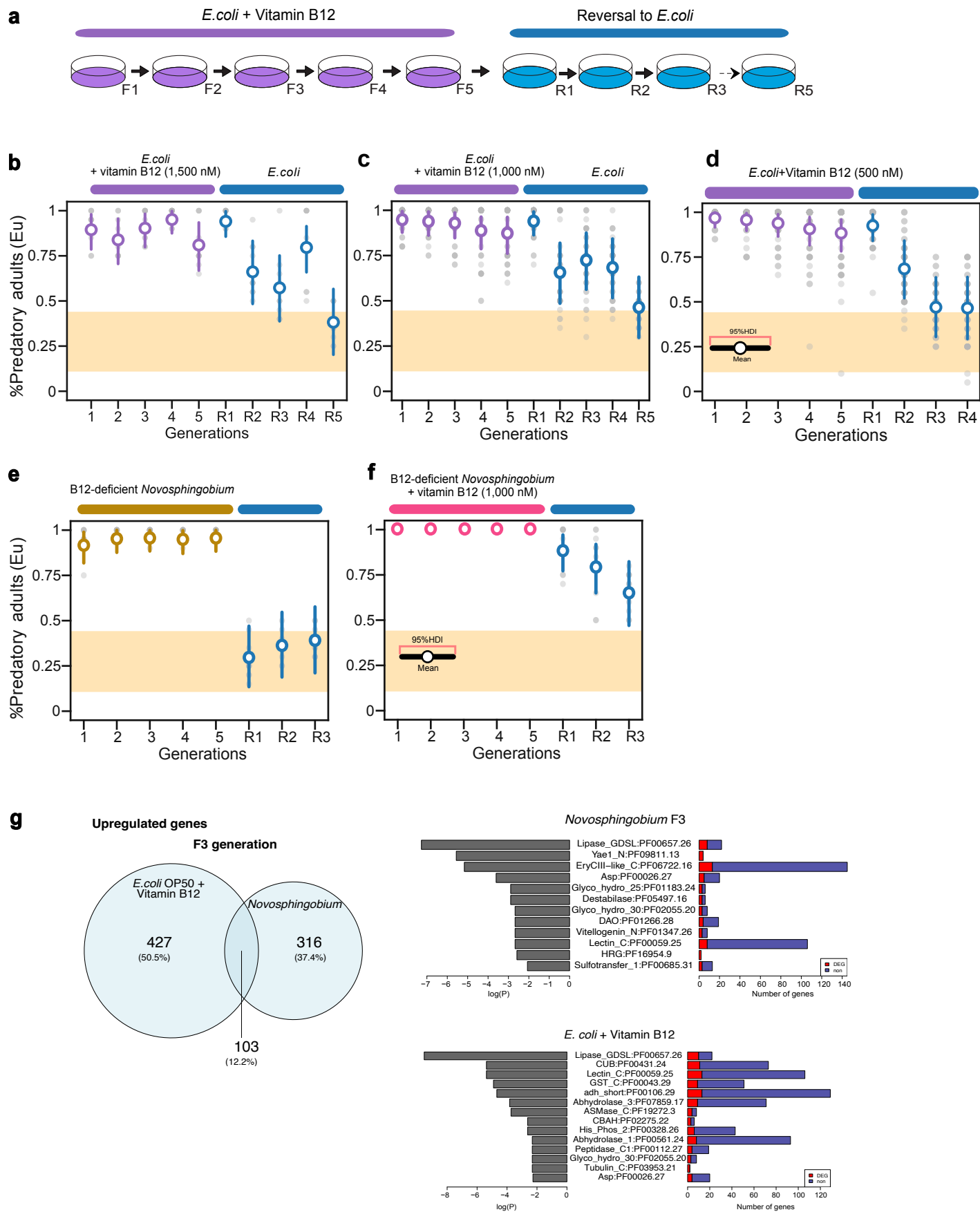
Figure 5. *Ppa*-MRP-5 is dispensable in TEI of the predatory morph and vitellogenin gene cluster organization.

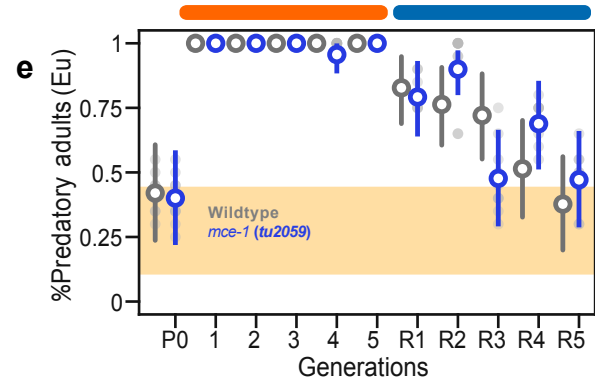
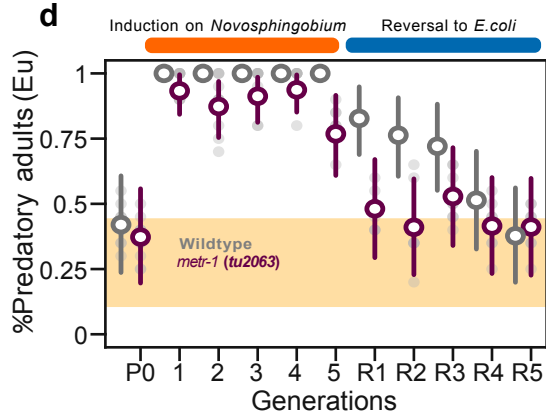
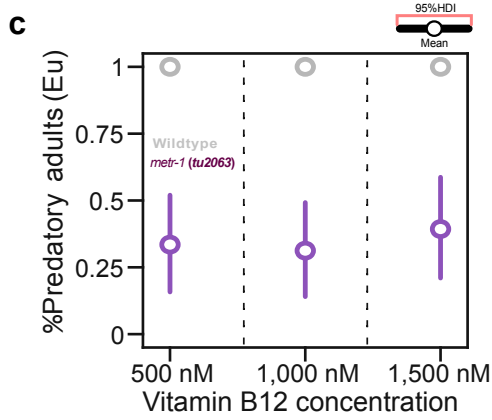
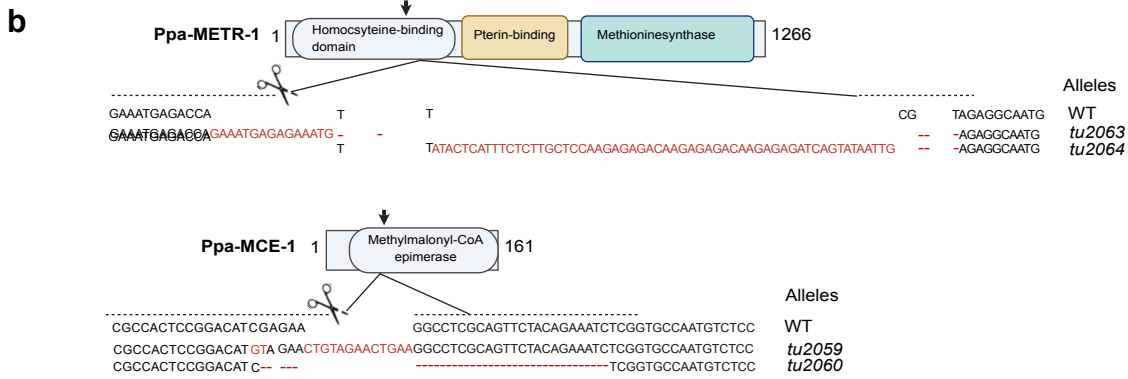
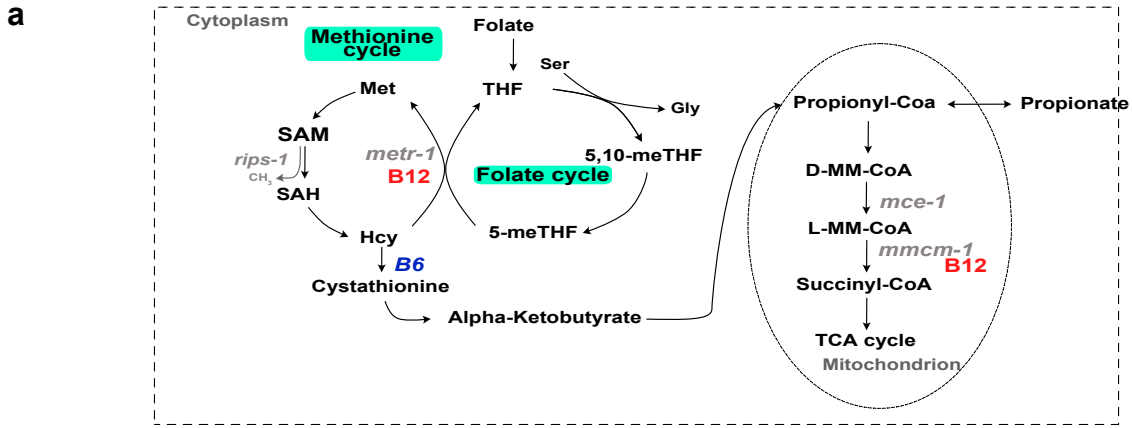
a, CRISPR/Cas9-induced mutations in *Ppa*-MRP-5 with target locations indicated in respective protein domains (sgRNA, arrow). Molecular lesions of isolated mutations via CRISPR/Cas9 are also shown. **b-c**, Mean probability of predatory mouth-form in *Ppa-mrp-5* mutant animals (*tu2061* and *tu2062*) on *Novosphingobium* for 10 generations compared to wild type. **d**, Graphical representation of the experiment. Generations highlighted in grey indicate worm populations sent for RNA sequencing. **e**, Vitellogenin expression of all 9 genes under different wildtype conditions: Log₂FoldChange (log₂FC) values are shown for worms on a *Novosphingobium* diet (F3, F4, F5, F10) and reversal to *E. coli* (F3R2, F4R2, F5R2, F10R2). Naïve animals on *E. coli* (P0) were used as reference to generate log₂FC values. **f**, *P. pacificus* RSC011 has 9 vitellogenin genes all located on the X chromosome distributed over a 17 Mb range. These genes are named *Ppa-vit-6-A* to *Ppa-vit-6-I* based on their location along the chromosome. Note that there are three transcripts that encodes for *Ppa-vit-6-B*. **g**, Phylogeny of the *P. pacificus* and *C. elegans* vitellogenin genes. In *C. elegans*, six vitellogenin genes show higher sequence divergence with the *Cel-vit-6* gene being the most diverse gene copy. Note that it is *Cel-vit-6* that is most similar to the individual genes in *P. pacificus* and also other nematodes. Nodes with bootstrap values of ≥ 90 are labelled with asterisks (*).

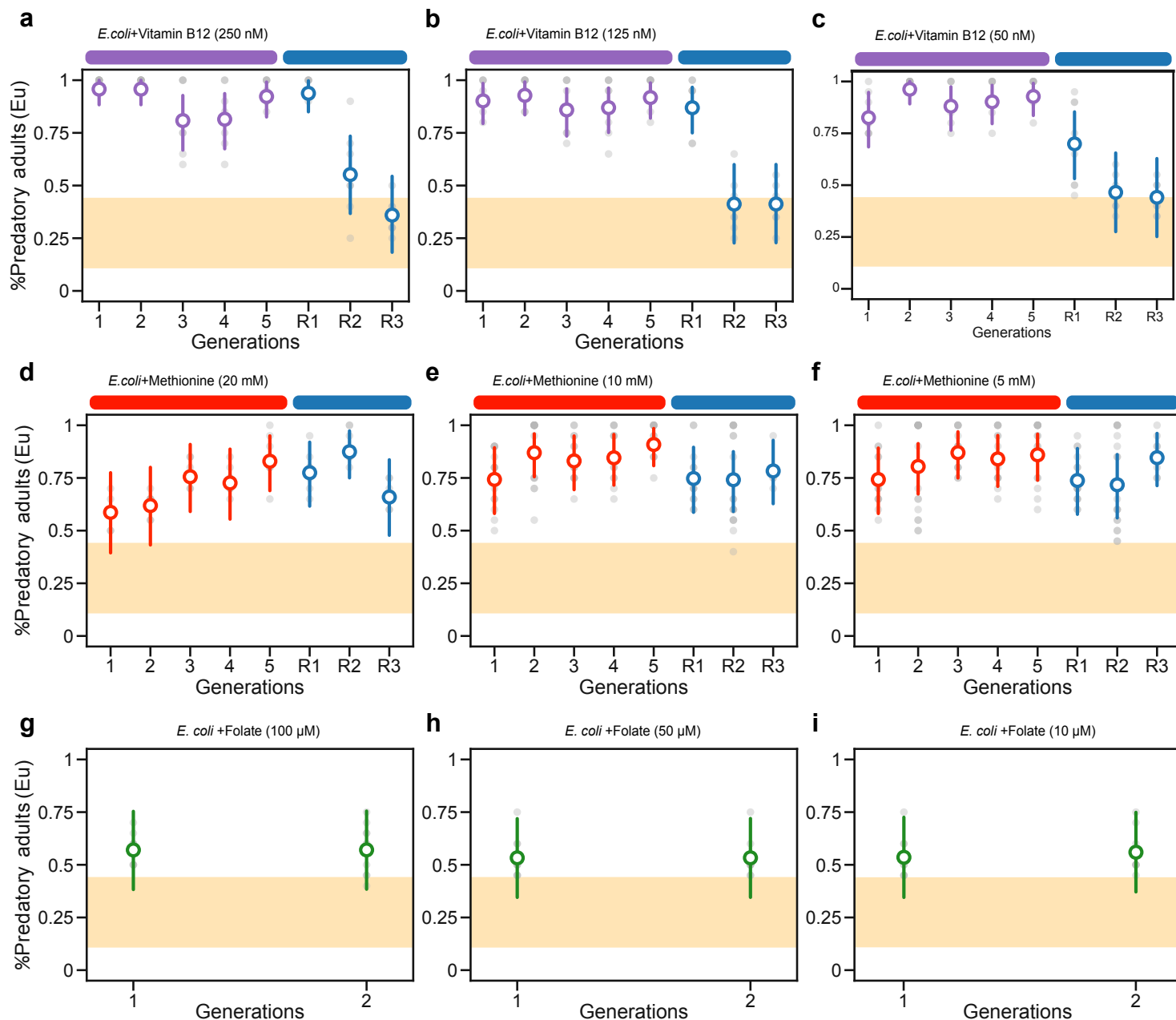
Figure 6. The low-density lipoprotein receptor related protein RME-2 is required for vitamin B12 transmission and transgenerational inheritance of the predatory morph

a, Domain architecture of *C. elegans* and *P. pacificus* RME-2, sequence similarity and position of molecular lesions in three *Ppa-rme-2* mutants are highlighted. Three alleles isolated in a forward genetic EMS mutagenesis screen carry point mutations resulting in amino acid changes as indicated. See also Supplementary Table 2 for the isolated alleles from the genetic screen. We designed a sgRNA in the C-terminal region where two of the original EMS alleles carry their mutation. The frameshift allele *tu2215* was used for further analysis. **b**, Mean probability of the predatory mouth form of *Ppa-rme-2(tu2215)* after a *Novosphingobium* exposure of 5 (left) and 10 (right) generations.

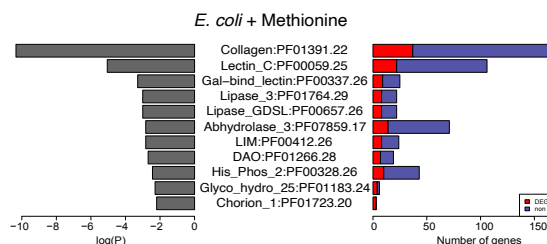
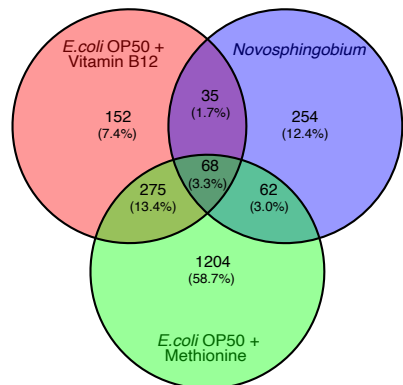


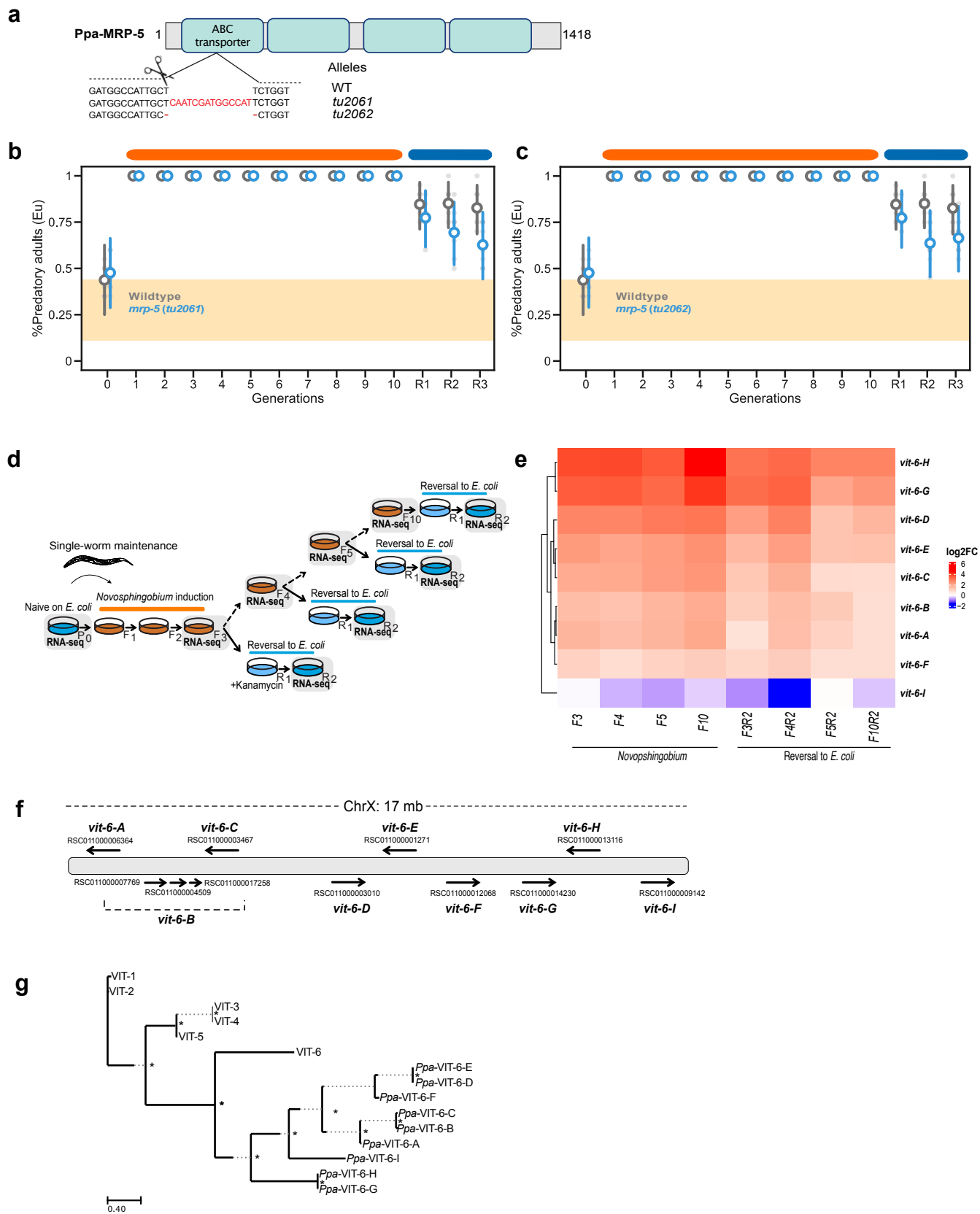


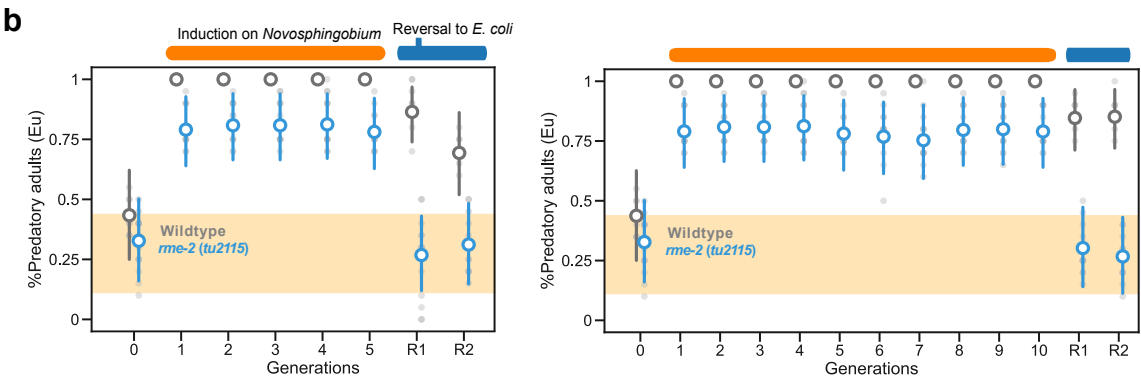
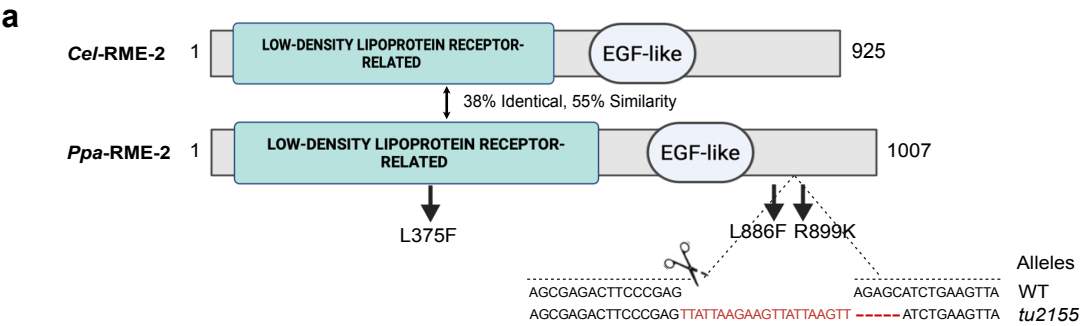




j







Supplementary information:

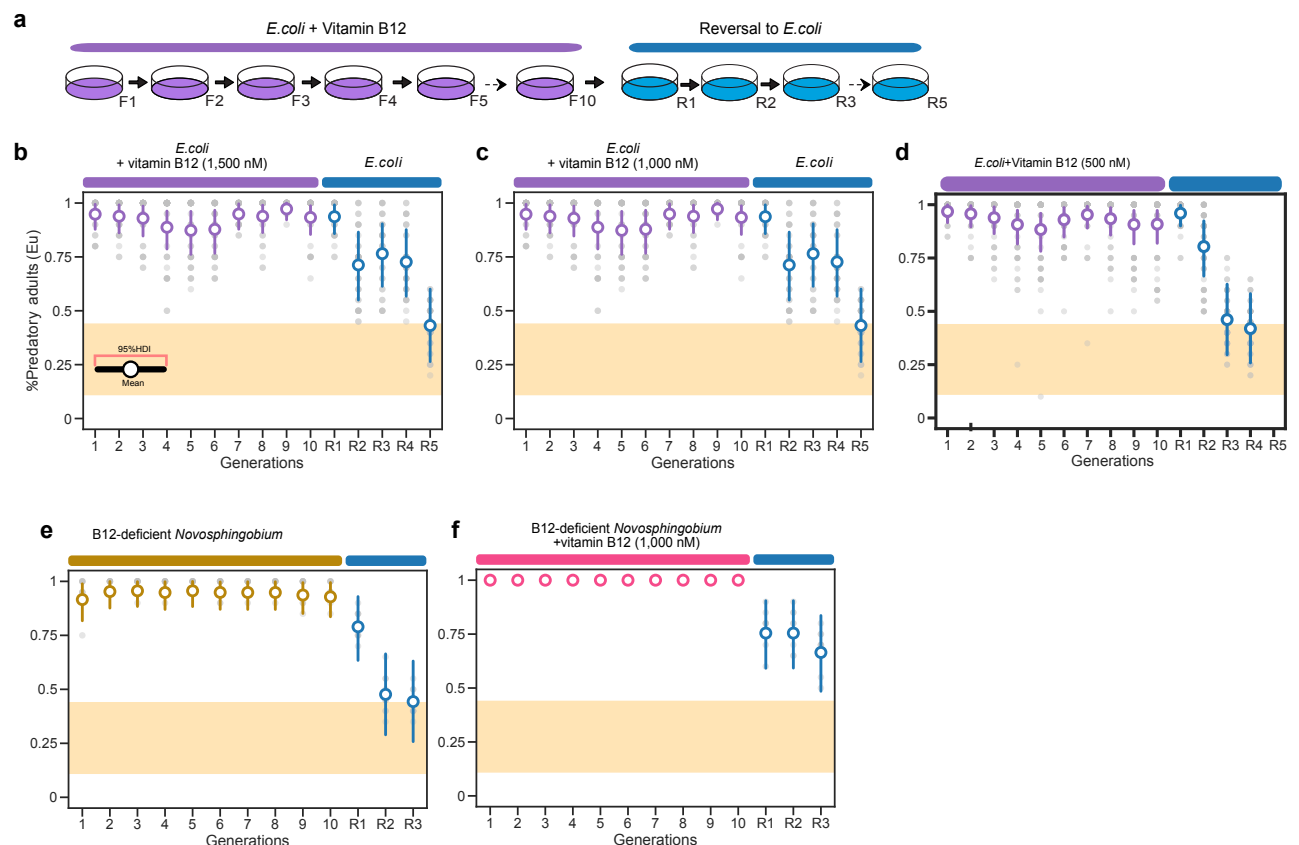
Vitamin B12 induces memory of predation through vitellogenin provisioning

Shiela Pearl Quiobe¹, Ata Kalirad¹, Raphaela Zurheide¹, Hanh Witte¹,

Christian Rödelisperger¹ & Ralf J. Sommer^{1*}

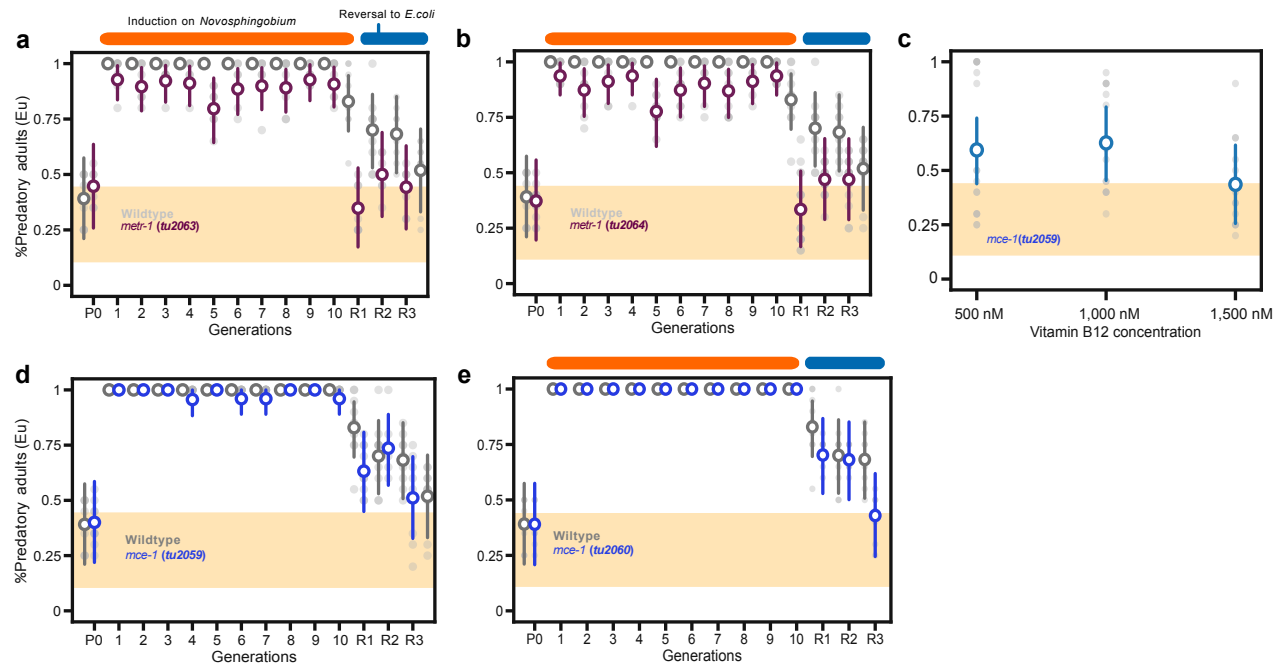
¹Max Planck Institute for Biology Tübingen, Department for Integrative Evolutionary Biology, Max-Planck Ring 9, 72076 Tübingen, Germany

- Author for correspondence at: ralf.sommer@tuebingen.mpg.de



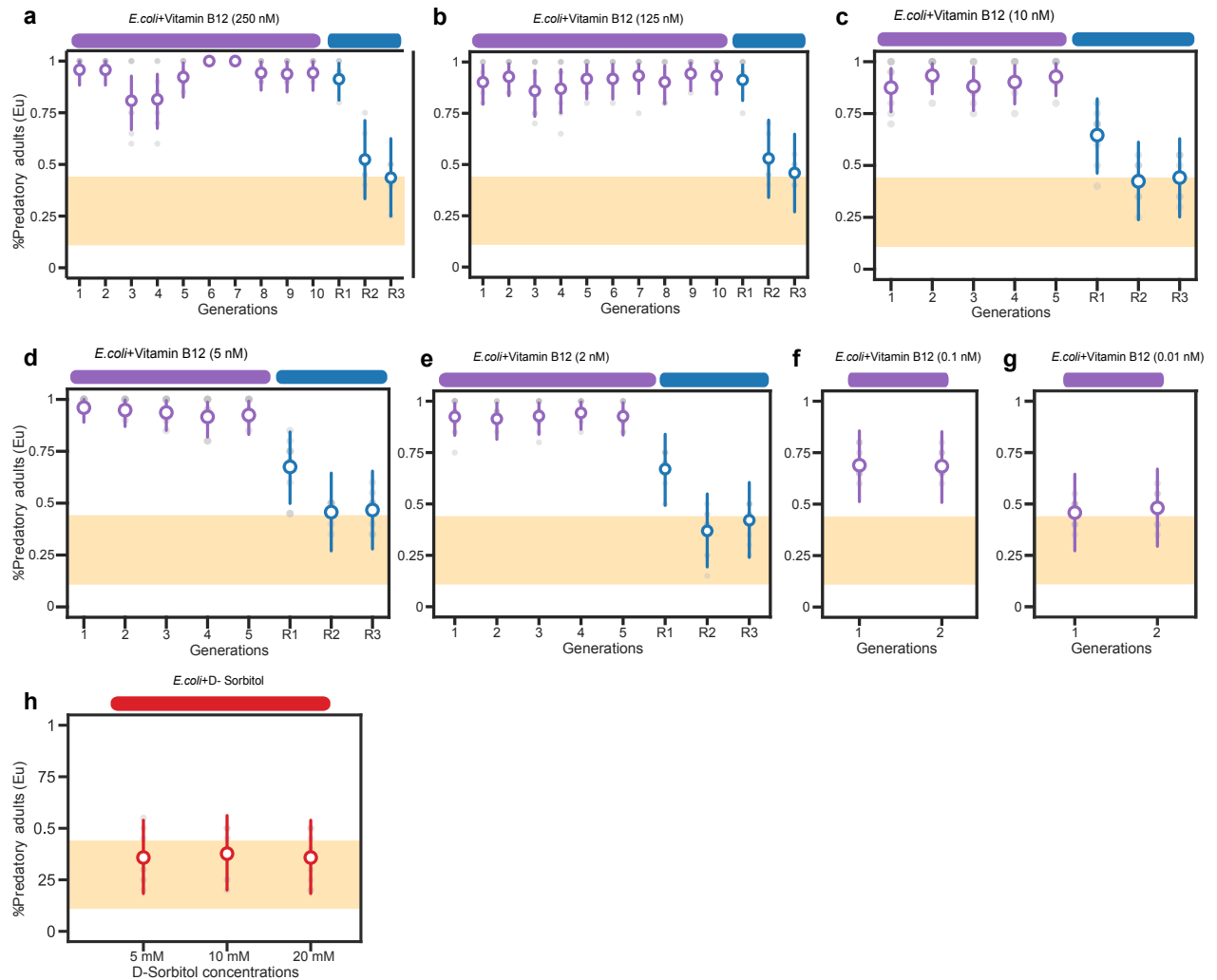
Supplementary Figure 1. Induction of the predatory mouth form by vitamin B12- supplemented *E. coli*, *Novosphingobium* vitamin B12-deficient mutant and vitamin B12 rescue.

a, Schematic diagram of worms grown on *E. coli* supplemented with vitamin B12 for 10 generations and reversal to un-supplemented *E. coli*. **b-d**, Mean probability of the predatory mouth form after 10 generations of vitamin B12 supplementation with 1,500 nM (b), 1000 nM (c) and 500 nM (d) vitamin B12 and reversal to un-supplemented *E. coli*. **e**, Mean probability of the predatory mouth form after 10 generations on a *Novosphingobium* vitamin B12-deficient mutant and reversal to *E. coli*. **f**, Mean probability of the predatory mouth form after 10 generations on a *Novosphingobium* vitamin B12-deficient mutant supplemented with exogenous vitamin B12 and reversal to *E. coli*. **g**, Overlap of downregulated transcripts during induction on *Novosphingobium* and vitamin B12-supplemented *E. coli* for three generations.



Supplementary Figure 2. *Ppa-metr-1* and *Ppa-mce-1* response on *Novosphingobium* and reversal to *E. coli*.

a,b, Mean probability of the predatory mouth form in *Ppa-metr-1* mutant animals on *Novosphingobium* after 10 generations of exposure and reversal to *E. coli*. **c**, Mean probability of the predatory mouth form in *Ppa-mce-1* mutant animals on vitamin B12-supplemented *E. coli* plates. **d,e**, Mean probability of the predatory mouth form in *Ppa-mce-1* mutant animals on *Novosphingobium* after 10 generations of exposure and reversal to *E. coli*. More details about the molecular lesions are listed in Supplementary Table 1.



Supplementary Figure 3. Vitamin B12 supplementation on the induced predatory mouth form and its maternal effect after reversal on *E. coli*.

a-b, Mean probability of predatory mouth-form on **(a)** 250, **(b)** 125 nanomolar (nM) of vitamin B12 supplementation for 10 generations and subsequent exposure to un-supplemented *E. coli*. **c-e**, Mean probability of predatory mouth-form on **(c)** 10, **(d)** 5, **(e)** 2 nM of vitamin B12 supplementation for 5 generations and subsequent exposure to un-supplemented *E. coli*. **f-g**, Mean probability of predatory mouth-form on **(f)** 0.1, **(g)** 0.01 nM. of vitamin B12 supplementation for 2 generations. **i**, Mean probability of predatory mouth-form on sorbitol-supplemented *E. coli* plates.

Alleles	Gene ID; <i>C. elegans</i> best hit	Molecular lesions	sgRNA(PAM)	Forward primer	Reverse primer
<i>tu2063</i>	RSC011000010081; <i>metr-1</i>	10 bp net insertion	AGAAATGAGACCATTCTAG(AGG)	GAGAACCACTCCGATACG	CAACTTGACTACGAGCTATTG
<i>tu2064</i>		55 bp net insertion			
<i>tu2059</i>	RSC011000034399; <i>mce-1</i>	13 bp insertion	CGCCACTCCGGACATCGAGA(AGG)	CGCCAATCTTACCAGAGTGA	CATTGATGTCCTTGACCTAC
<i>tu2060</i>		28 bp deletion			

Supplementary Table 1. Molecular lesions of *Ppa-metr-1* and *Ppa-mce-1*.

Allele	Gene ID	<i>C. elegans</i> 1:1 ortholog	Position	Reference	Variant	Mutation type	Nucleotide substitution
	RSC011000018058	<i>rme-2</i>	12582507	G	A	nonsynonym [R>K]	AGG>AAG
	RSC011000018058	<i>rme-2</i>	12582467	C	T	nonsynonym [L>F]	CTT>TTT
<i>tu797</i>	RSC011000018058	<i>rme-2</i>	12579999	C	T	nonsynonym [L>F]	CTC>TTC

Supplementary Table 2. *Ppa-rme-2* alleles isolated from the genetic screen by EMS mutagenesis.

Lessons learned from manual curation of thousands of gene models in the nematode *Pristionchus pacificus*

Christian Rödelberger, Neha Agyal, **Shiela Pearl Quiobe**, Hao Wu, Waltraud
Röseler, Dafne Ibarra, Ralf J. Sommer

Accepted for publication in *PLoS ONE*

Lessons learned from manual curation of thousands of gene models in the nematode *Pristionchus pacificus*

Christian Rödelberger^{1,*}, Neha Agyal¹, Shiela Pearl Quiobe¹, Hao Wu¹, Waltraud Röseler¹, Dafne Ibarra-Morales², Ralf J. Sommer¹

¹ Department for Integrative Evolutionary Biology, Max Planck Institute for Biology, Tübingen, Germany

² Genome center, Max Planck Institute for Biology, Tübingen, Germany

*Corresponding author:

Email: christian.roedelsperger@tuebingen.mpg.de

Short Title: Community-curated gene annotations for *P. pacificus* RSC011

33 Abstract

34 Continuous developments in sequencing technologies have led to the generation of
 35 chromosome-scale genome assemblies across the whole tree of life, but our ability to
 36 annotate genomes has lacked behind. One major problem consists in the fact that typically
 37 not all genes are expressed at detectable levels at any given life stage or environment.
 38 Therefore, available transcriptome data needs to be complemented by gene prediction
 39 programs and protein homology evidence. However, how to optimally combine these
 40 different data types is not well understood. Here, we present a case study, where we
 41 community curated gene annotations of the *Pristionchus pacificus* strain RSC011. By
 42 incorporation of new Iso-seq and RNA-seq data and genome-wide screening, we identified
 43 and corrected more than 7,500 (~24%) gene models. While the improved gene annotation
 44 for the RSC011 strain will be useful for the *P. pacificus* community, our study reveals several
 45 gene annotation problems that may affect data from other species. Among these, we
 46 identified assembly errors, artificial transcript fusions resulting from overlapping genes and
 47 polycistronic RNAs, falsely called open reading frames, and error propagation based on
 48 homology data as frequent sources of gene annotation errors. Thus, our findings may be
 49 helpful in guiding future efforts to annotate genomes across different taxonomic groups.

50 Introduction

51 The nematode *Pristionchus pacificus* is an established model for investigating
 52 developmental plasticity [1,2], host microbe interactions [3,4], and behavior [5,6]. It
 53 complements *Caenorhabditis elegans* by virtue of additional ecological and behavioral traits
 54 —most notably, a feeding-structure polyphenism in which individuals adopt either a narrow,
 55 bacteriovorous stenostomatous form or a wide, predatory eurystomatous form [7,8]. This
 56 discrete mouth form switch, conditioned by a combination of environmental cues and genetic

regulators, makes *P. pacificus* especially amenable for dissecting how organisms sense and integrate environmental signals resulting in developmental decisions [2,9].

P. pacificus is a cosmopolitan species and ongoing field trips have collected more than one thousand natural isolates of which hundreds have available whole genome resequencing data [10–12]. Among the diverse natural isolates of *P. pacificus*, the strain RSC011 is of particular interest. In contrast to many strains that are biased toward the eury stomatous morph under laboratory conditions, RSC011 maintains a relatively high baseline frequency of the stenostomatous form [7,13]. Previously, we exploited this feature in order to dissect the genetic basis of natural variation in the mouth form plasticity [13]. In that study, we crossed strains differing in mouth form frequencies, created recombinant inbred lines, and performed QTL mapping. This identified a multigene locus including the key regulator *eud-1* whose cis-regulatory architecture was found to modulate the frequency of the predatory morph [13]. More recently, we leveraged the *P. pacificus* RSC011 strain in an experimental evolution study to explore environmental influences on mouth form plasticity and associated transgenerational epigenetic inheritance [14]. Using long-term environmental induction and food-reversal experiments, we observed the dietary induction and subsequent transgenerational inheritance of the eury stomatous morph. By forward genetic screening, we identified a role of ubiquitin ligase EBAX-1/ZSWIM8 in this process. *Ppa-ebax-1* mutants are transgenerational inheritance defective, and *Ppa*-EBAX-1 regulates a microRNA cluster of the family *miR-2235a/miR-35*. One byproduct of that work was the assembly and annotation of the RSC011 genome, which provided a critical resource for identifying regulators of transgenerational inheritance [14]. The RSC011 genome could be assembled and scaffolded to the level of chromosomes and evidence-based gene models were generated by the PPCAC pipeline ('PPCAC1', Table 1) that uses transcriptomic and homology data to guide the annotation process [15]. However, the quality of these automatically generated gene annotations is likely inferior to the gene annotations of the commonly used *P. pacificus*

'wildtype' reference strain PS312, which had been subject to two rounds of extensive community curation [16,17]. In these two studies, we manually inspected thousands of suspicious candidate loci and could identify and correct artificial gene fusions and add missing genes [16,17]. Similar approaches were more recently adapted by different groups in order to improve the quality of gene annotations of other nematode species [18–21]. Here, we report a community curation effort to improve the quality of the RSC011 gene annotations. This will make the RSC011 genome a better resource for future experimental studies and at the same time, our findings may be used to guide and improve gene annotation processes for other genomes.

Results

Retained introns, overlapping genes, polycistronic reads, and assembly errors cause gene models with unusually long UTRs

In order to improve the gene annotations for the *P. pacificus* strain RSC011, we generated Iso-seq data of mixed-stage populations and RNA-seq data of different developmental stages (eggs, J2, J3, J4, and adult worms). All transcriptomic data were generated using nematode cultures that were grown on *E. coli* OP50 bacteria as diet. Overall, we obtained 1.37 million HiFi reads with a median length of 2.1 kb. These Iso-seq reads were aligned against the RSC011 genome assembly by the software GMAP and then assembled by the software stringtie [22,23]. Illumina RNA-seq data was first assembled into longer transcripts using the software trinity [24], and then processed in the same way as the Iso-seq data. We adapted the PPCAC annotation pipeline [15] to incorporate these transcriptomic gene models (Fig 1). Thereby, we noticed that 4113 gene models had more than three exons that were annotated as untranslated regions (UTR) either as 3' or 5'. Following previously established protocols for community curation, we inspected several hundreds of such cases

manually in the genome browser [16,17,25]. This showed that those unusually long UTRs were frequently caused by retained introns, overlapping genes, polycistronic reads, and assembly errors (Fig 2A-C). Note that nematodes have operon-like structures that produce polycistronic transcripts which are later processed into mature monocistronic transcripts by trans-splicing [19]. Most cases of polycistronic reads in the RSC011 genome appear to be derived from such immature operon transcripts. While in many cases, community curation was able to replace problematic cases with alternative gene models, this method could not easily correct misannotations resulting from assembly errors. Therefore, we decided to create a polished version of the RSC011 genome.

Genome assembly polishing greatly reduced the number of problematic gene models

To correct potential assembly errors in the RSC011 genome, we leveraged existing whole genome sequencing data from over 160 mutant lines that were derived from the RSC011 strain [14] and searched for substitutions and indels that were found in more than 100 of these mutant lines. This resulted in 1883 substitutions and 42,138 indels that likely represented assembly errors. We generated a polished version of the RSC011 genome assembly using the software mutsim [26]. Generating gene annotations for the polished genome assembly using the same computational procedures as described above ('PPCAC2 vanilla', Table1) reduced the numbers of problematic gene models (gene models with either more than three 3'UTR or 5'UTR exons) from 4113 to 2819. This reduction in the number of problematic gene models was also observed when running the original annotation procedure as described previously [14,15] on the polished genome ('PPCAC1 polished', Table 1). In addition, both sets of annotations showed a pronounced increase in the completeness value (Table 1), suggesting that even genome assemblies that were generated using HiFi reads may still contain substantial numbers of assembly errors. These errors will affect over one

thousand gene models and additional genome assembly polishing can thus also improve the quality of gene annotations.

Community curation corrects more than two thousand problematic gene models

In order to correct the remaining problematic gene models, we employed a community annotation effort where the list of 2819 problematic gene models were shared as an online spreadsheet among four community curators who inspected these cases in the genome browser and selected alternative gene models from various computationally derived models (RNA-seq gene models, Iso-seq gene models, RNA-seq derived ORFs only, gene models with truncated UTRs). This approach is different from platforms such as web apollo [27], which allow very flexible modifications of individual gene models. Only allowing curators to choose among predefined gene models, requires less training and makes this approach more scalable to larger numbers of candidate sets. In hindsight, many cases will remain uncorrected as the predefined gene models often do not allow a satisfiable solution. For those cases, the curators could note this as an additional comment. In most cases, the curators had to frequently decide whether a locus encoded two genes or one. Two or more genes can be merged due to overlapping transcribed loci (Fig 2A,B) or due to incorrectly predicted gene boundaries in homology derived models. During the community curation, we used clearly separated transcriptomic alignments between two neighboring gene models or opposite strand orientation with additional conservation in *C. elegans* (BLASTP e-value < 0.001) as strong indicators supporting the presence of two genes. Furthermore overlapping ORFs, RNA-seq coverage differences, and conservation in *C. elegans* were used as weaker evidence to support either the presence of one or two genes at a locus. After manual inspection and correction of all 2819 problematic gene models, the number of genes with more than three UTR exons could be reduced to 697. This confirms our previous experience

that community-based curations are an effective means to reduce the number of problematic gene models [16,17].

Genome-wide screens identify roughly 24% of gene models as problematic

During the community curations, we occasionally observed erroneous annotations of neighboring genes (e.g. artificially fused genes not resulting in multiple UTR exons, predicted antisense ORFs, homology derived gene models with neither transcriptomic support nor conservation in *C. elegans*). To screen and correct additional gene annotation errors, we scanned the complete RSC011 assembly in the genome browser at 20 kb resolution. This also allowed us to recheck and eventually modify curations from the first round. For the whole genome, this resulted in a total of 7528 corrections (S1 Table). Automated classification of these changes yielded 3562 removals, 2928 single gene replacements, 835 splits, and 204 added genes (Fig 3A). Note that some of those cases actually represent gene fusions (Fig 3B), but in the shared spreadsheet, such fusions were recorded as two independent corrections: one replacement and one removal. Overall, these results suggest that roughly 24% of the 31,726 automatically annotated gene models have obvious problems that could be easily detected by visual inspection in the genome browser.

Antisense coding potential frequently causes misannotated ORFs

The largest class of corrections corresponds to removed gene models. We subdivided this class based on exonic overlaps with gene models in our final gene annotation ('PPCAC2 cream', Table 1). Notably, the largest group consists of removed genes that overlap with protein coding exons on the opposite strand (Fig 3C,D). In many of those cases, one of the overlapping gene models shows conservation with *C. elegans*, suggesting that the antisense gene model is a result of misassigning the ORF to the wrong strand. In many cases, sense and antisense models share the same exon-intron structure suggesting that coding

sequences frequently have antisense coding potential, which can lead to incorrectly annotated ORFs. The second most abundant group of removed genes consists mostly of gene models that do not have homologs in *C. elegans* and show very little expression evidence in our transcriptomic data sets (Fig 3E). Many of those poorly supported gene models were already captured in the candidate set with multiple UTR exons as they are unlikely functional and intraspecies variation frequently introduces premature stops during the liftover of gene models from the *P. pacificus* reference strain PS312. Note that removing gene models based on the absence of transcriptomic inherently comes with the caveat that we can never be absolutely sure that a locus does not encode a gene. Eventually, such models will be reactivated if future studies provide more robust evidence that they are largely correct.

Among the removed gene models with same strand overlaps, we found many partial ORFs or isoforms that are part of other better supported gene models. However, such cases are far less abundant than the spurious antisense gene models. We encountered the problem of spurious antisense gene models already previously and therefore incorporated the removal of species-specific orphan genes with antisense overlaps in the annotation process for different *P. pacificus* strain genomes [17,28,29], but it appears that antisense coding potential is not only generating species-specific orphan genes, but can also create gene models with apparent protein conservation across species. This problem could in future be alleviated by incorporating homology information in the ORF prediction or by only using strand-specific RNA-seq data.

Heuristics favoring longer genes cause propagation of gene fusions errors

One of the most abundant gene annotation problems in the genome of the *P. pacificus* reference strain PS312 consisted in artificial gene fusions that likely resulted from failure of

gene prediction programs in identifying the exact gene boundaries [16]. While we have corrected thousands of those instances in our latest set of *P. pacificus* PS312 gene annotations (El Paco gene annotation 3) [16,17], it cannot be excluded that this set of gene annotation still harbors some artificially fused gene models [30]. Using this gene set as homology data to annotate the genome assemblies of other *P. pacificus* strains and other *Pristionchus* species may lead to propagation of these gene annotation errors, especially, when combined with a heuristic to choose the longest ORF per locus as representative gene model [15]. This likely explains the presence of 835 corrections that were classified as splits (Fig 3A). However, we need to emphasize here that the use of the El Paco annotations as homology data is still needed to annotate genes without any expression evidence (Fig 4A) or genes where transcriptomic data is not sufficient to generate a complete gene model (Fig 4B). Thus, future annotation pipelines need to be optimized in how they combine transcriptomic and protein homology evidence. Alternatively, an even better reference data set might prevent further error propagation issues.

The community curated RSC011 gene annotations exhibit the highest level of completeness

The primary focus of the previous RSC011 gene annotation was to identify protein-coding genes. Given that many assembled transcripts might be incomplete, ORFs were sometimes extended over the starting methionine as long as no inframe stop codon were encountered [14,15]. For this reason, many protein sequences did not start with methionine. In addition, generally no UTR regions were annotated. With the additional transcriptomic data generated for this study, we incorporated many more complete gene models, which should facilitate a better annotation of the starting methionine and the 3'UTR. To systematically compare the final community curated gene annotation with different automatically generated gene annotations, we calculated the number of genes with annotated 3'UTRs, the number of

problematic gene models, the number of protein products that start with a methionine, and the completeness value as estimated by the benchmarking of universally single-copy orthologs (BUSCO) approach (Table 1) [31]. This showed that polishing the genome assembly led to a pronounced reduction in the number of problematic gene models and a substantial increase in the BUSCO completeness. In comparison, the effect of community curations on the BUSCO completeness was rather minor. Furthermore, incorporation of additional transcriptomic data and restricting the ORF prediction to complete ORFs drastically increased the number of gene models with a starting methionine and a 3'UTR. At the same time, this additional transcriptomic data increases the chance that an assembled transcript will contain a retained intron. Also, prioritizing ORFs with a starting methionine and an annotated 3' UTR can sometimes lead to the selection of genes with shorter ORFs. These two problems largely explain the slight decrease in BUSCO completeness between 'PPCAC1 polished' and 'PPCAC2 vanilla' (Table 1). Thus, despite a reduction in gene count by more than two thousand, the community curated RSC011 gene annotations shows the highest level of completeness.

Discussion

In this study, we present an improved version of the genome for the *P. pacificus* strain RSC011. The improvements were achieved through multiple steps, such as genome assembly polishing, incorporation of additional transcriptomic resources, and manual curation. The fact that even after the first two steps of improvement, manual inspection identified around 24% of gene models as problematic, clearly highlights the need for manual curation. The resulting improved gene annotations will be an essential resource for future genomic and experimental research on transgenerational epigenetic inheritance [14]. In addition, this work represents a case study summarizing problems in gene annotation and might be useful for developers and users of gene annotation pipelines [32–38]. As such our

study highlights several problems that may impact gene annotation. First, genome assembly polishing is an important first step to improve gene annotation quality. Second, problems in transcriptome assembly may arise due to incompletely spliced reads, polycistronic transcripts, and overlapping genes. Such problems could be partially mitigated by the use of RNA-seq read coverage and homology data to separate such fusions at the transcript level. Third, antisense coding potential may cause ORFs to be misassigned to the wrong strand. This can be alleviated by the use of homology data during ORF prediction or strand-specific RNA-seq data. In addition, the ORF prediction should incorporate the possibility that a transcript could encode multiple ORFs. Fourth, it is not totally clear how to best integrate homology and transcriptomic data. While homology data can cause error propagation problems it may be helpful to fully annotate genes with no or only incomplete transcriptomic evidence. Finally, in the context of antisense transcripts, we saw a lot of transcripts where exons are located in introns of genes on the opposite strand, but encountered very little evidence for antisense overlap between exonic regions. This is consistent with previous analysis of strand-specific RNA-seq data in *P. pacificus* [39] and suggests that at least for *Pristionchus* species it should be legitimate to prohibit antisense exonic overlaps during the gene annotation process.

Notably, many if not all of our findings are not new. The difficulty of gene annotation and the problem of error propagation has been acknowledged previously [40]. Genome assembly polishing has been routinely applied to improve the quality of newly assembled genomes [41,42]. Also, the presence of polycistronic transcripts among Iso-seq reads [43] and spurious antisense gene models was known before [17]. Thus, it could have been possible to anticipate each of these problems in advance without the need to screen a whole genome for annotation artifacts and manually correct thousands of gene models. Confucius once said: “By three methods we may learn wisdom: First, by reflection, which is noblest; Second, by imitation, which is easiest; and third by experience, which is the bitterest.”

However, we consider as a major outcome of this study that we bring together all these different findings in a single study and highlight their impact on gene annotation. We hope that this knowledge will be helpful for future efforts in annotating genomes across the tree of life.

Methods

Worm culturing and transcriptome sequencing

In order to obtain stage-enriched transcriptomes for the *P. pacificus* strain RSC011, we prepared initial cultures by putting four J4 worms onto each of five 6cm plates with 300µl of *E. coli* OP50. After nine days the plates were full of eggs. We washed one of those plates with M9 to obtain a transcriptome representing mostly eggs. The other plates were grown for an additional day. Here two plates were washed with M9 for the J2 stage. The remaining two plates were washed using M9 into 15ml Falcon tubes and let stand for 5 min. Supernatant containing mostly J2 worms were distributed to fresh 6cm plates with 300µl OP50. After 24h we collected developmental stage J3, after 48h J4 and after 60h young Adult. The pellets of each developmental stage were frozen at -80°C until further use. RNA was extracted using the Zymo Direct-zol RNA Miniprep Kit. RNA was sequenced with Novogene. For the Iso-seq experiment worm pellets were prepared from mixed-stage cultures and RNA was extracted with the Zymo Direct-zol RNA Miniprep Kit. We then quantified the total RNA and quality-controlled it using the Bioanalyzer® RNA Pico Kit. All processed samples had a RIN number >9.0. We prepared Iso-Seq libraries using the following protocol: "Procedure & Checklist - Preparing Iso-Seq® libraries using SMRTbell® prep kit 3.0 (PN 102-396-000 REV02 APR2022)". We used 500ng of total RNA as input amount and performed duplicates of each sample to obtain enough cDNA. Briefly: we synthesize cDNA according to the protocol above and amplified it with 12 PCR cycles. We pooled together the duplicates of the same

sample. We indexed individual libraries using SMRTbell barcoded adaptors during the adapter ligation step of the library prep protocol. After the indexed libraries were quality-controlled using the Bioanalyzer® High Sensitivity DNA kit, we pooled the libraries by combining an equal mass of each barcoded library. We sequenced all the libraries on a Sequel® II system, using the Sequel II Sequencing Plate 2.0 and the Sequel II binding kit 3.1, which is recommended for libraries with fragments shorter than 3 kb.

Transcriptome analysis

From the raw PacBio reads, consensus HiFi reads were generated with the ccs software (version: 6.4.0 with `–min-rq 0.99` option) and demultiplexed by lima (version: 2.7.1 with `–split-bam-named –same –dump-removed` options). HiFi reads were then aligned against the RSC011 genome assembly by the software GMAP (version: 2024-02-22 with `-max-intron-length-middle=10000` option) and then assembled into transcripts by stringtie (version: v2.2.1) [22,23]. To annotate ORFs we modified the `ppcac_generate_annotations.pl` script of the PPCAC software to only consider ORFs with a starting Methionine. Raw RNA-seq reads from multiple developmental stages of *P. pacificus* strain RSC011 were assembled using the Trinity software (version: 2.2.0 with `–normalize_reads` option) [24]. Subsequently, the assembled transcripts were aligned against the polished RSC011 genome by GMAP. The resulting alignment file was taken as input to stringtie and was processed in the same way as the Iso-seq data. For visualization purposes, raw RNA-seq reads were also aligned against the polished RSC011 assembly by the software STAR (version: 2.7.10b with) and .bam files were created by samtools (version 0.1.18) [44,45].

Genome assembly polishing

In order to reduce the number of problematic gene models, we employed whole genome resequencing data from more than 160 mutant lines in the RSC011 to screen for

substitutions and indels that were called in more than 100 mutant lines [14]. This identified 1883 substitutions and 42,138 indels that were corrected in a new polished version of the RSC011 genome assembly (version 2) using the software mutsim [26].

Gene annotation

During the course of this study, we generated three alternative versions of gene annotations for the new RSC011 genome assembly. In order to assess the effect of genome assembly polishing on gene annotation, we reran the original PPCAC gene annotation pipeline on the polished assembly using the same input data sets and parameters as described previously [14,15]. Specifically, these annotations were generated from transcriptomic evidence (ORFs in Trinity assembled transcripts) and homology data from the *P. pacificus* PS312 El Paco gene annotations (version 3) [17]. One representative gene model per 100-bp window was chosen by selecting the gene model with longest ORF while all other intersection gene models were then discarded [15]. This version is labeled as PPCAC1 polished (Table 1). Next, we integrated the newly generated transcriptome data by providing them as additional input data into the PPCAC pipeline and prioritizing these gene models by adding a value of 100 to their ORF length. This version was labeled as PPCAC2 vanilla (Table 1) and formed the initial version for community curation. The final community curated version was labeled as PPCAC2 cream (Table 1).

Community curation

For initial community curation, we defined problematic gene models as genes with more than three exons that were either annotated as 3'UTR or 5'UTR. After an initial assessment of hundreds of candidates by an experienced curator, three other community curators were then recruited from within the laboratory. These curators were then trained in a dedicated seminar where typical errors, evidence types, and solutions were presented and discussed.

The list of problematic gene models genes was then shared as an online spreadsheet across all community curators who could manually inspect the problematic locus in the genome browser (jbrowse on pristonchus.org) [25]. Different tracks in the genome browser included the alignment files for Iso-seq and RNA-seq data, as well as alternative gene models that were either created by stringtie or by exonerate. In addition, we labeled gene models based on the best BLASTP hit (e-value < 0.001) in *C. elegans* to visualize ORFs that are highly conserved. Using these data, the curators eventually proposed alternative gene models for a given candidate genes. Note that the curators were explicitly asked to skip problematic cases where different evidence types (RNA-seq alignments, gene orientation, homology information) did not point towards a clear solution. After the curation of the gene models with long UTRs, we implemented all suggested changes in an intermediate annotation and then scanned the whole genome at 20 kb resolution for any obvious annotation problems and proposed additional corrections. This also served to detect and correct any inconsistencies in the first round of community curation (e.g. addition of overlapping redundant gene models). After both rounds of curation, we compiled the final set of gene annotation and assigned new gene identifiers to the new gene models (S2 Table). To facilitate comparisons between the previously published gene annotation (PPCAC1, Table 1) [14] and the current one (PPCAC2 cream, Table 1), we generated a mapping between gene models using an approach to identify syntenic orthologs between genomes [28].

Table 1. Comparison of different gene annotations.

Gene annotation set	Number of gene models	Number of problematic models	BUSCO (C[S,D]/F/M) %	with starting Methionine	with annotated 3'UTR
PPCAC1	30,074	2138	81.5[79.0,2.5]/1.9/16.6	14,016	3156

PPCAC1 polished	31,701	1114	87.4[84.5/2.9]/1.2/11.4	14,231	2019
PPCAC2 vanilla	31,726	2819	86.9[84.1/2.9]/1.6/11.5	22,921	16,049
PPCAC2 cream	29,497	697	88.6[85.6/3.0]/1.2/10.2	22,096	16,265

The table summarizes features of different gene annotations for the *P. pacificus* strains RSC011. The gene set 'PPCAC1' denotes the previously published gene annotation [14]. The gene set 'PPCAC1 polished' represents the reannotation of the polished genome assembly using the same protocol and data sets as in Quiobe et al. [14]. 'PPCAC2 vanilla' denotes a fully automated gene annotation that incorporates newly generated RNA-seq and Iso-seq data. 'PPCAC2 cream' denotes the final manually curated gene annotation. Problematic gene models were defined as gene models with three or more 3'UTR or 5'UTR exons. Completeness values were calculated using BUSCO (version 5) against the nematode odb10 data set. Note that the number of missing BUSCO genes is an overestimate as it has been demonstrated that extensive sequence divergence can result in a failure to detect orthologs [15].

Supporting information

S1 Table. Community curations.

The table summarizes the updates to the PPCAC2 vanilla gene set by the community curators together with their classification.

S2 Table. Gene Identifier conversion table.

The table shows the mapping between the new gene identifier in the PPCAC2 cream gene set and the original sequence identifier.

S3 Table. Gene Identifier mapping across versions.

The table shows a mapping between gene identifiers of the PPCAC2 cream annotation and the previous annotation [14]. This mapping was generated by a method to identify syntenic orthologs between genomes [28].

Data access

All transcriptomic data sets that were created for this study, have been submitted to the European Nucleotide Archive under the study accession PRJEB101809. The *P. pacificus* RSC011 genome assembly and gene annotations are also available on pristionchus.org.

Acknowledgements

The authors thank the whole Sommer laboratory for helpful discussions. The authors would also like to thank Alejandro Sanchez-Flores and one anonymous reviewer for helpful comments on the manuscript.

Author contributions

Conceptualization: Christian Rödelberger.

Data Curation: Christian Rödelberger.

Formal Analysis: Christian Rödelberger.

Funding Acquisition: Ralf J. Sommer.

Methodology: Christian Rödelberger.

Investigation: Christian Rödelberger, Neha Agyal, Shiela Pearl Quiobe, Hao Wu, Waltraud Röseler, Dafne Ibarra-Morales.

Project Administration: Christian Rödelberger, Ralf J. Sommer.

Resources: Ralf J. Sommer, Christian Rödelsperger.

Software: Christian Rödelsperger.

Supervision: Christian Rödelsperger, Ralf J. Sommer.

Validation: Christian Rödelsperger.

Visualization: Christian Rödelsperger.

Writing – Original Draft Preparation: Christian Rödelsperger.

Writing – Review & Editing: Ralf J. Sommer, Christian Rödelsperger.

References

1. Reich S, Loschko T, Jung J, Nestel S, Sommer RJ, Werner MS. Developmental transcriptomics in *Pristionchus pacificus* reveals the environmental responsiveness of a plasticity gene-regulatory network. *Genome Res.* 2025;35: 1560–1573.
2. Projecto-Garcia J, Biddle JF, Ragsdale EJ. Decoding the architecture and origins of mechanisms for developmental polyphenism. *Curr Opin Genet Dev.* 2017;47: 1–8.
3. Athanasouli M, Loschko T, Rödelsperger C. Interspecies systems biology links bacterial metabolic pathways to nematode gene expression, chemotaxis behavior, and survival. *Genome Res.* 2025. doi:10.1101/gr.280848.125
4. Lo W-S, Sommer RJ, Han Z. Microbiota succession influences nematode physiology in a beetle microcosm ecosystem. *Nat Commun.* 2024;15: 5137.
5. Nakayama K, Hiraga H, Manabe A, Chihara T, Okumura M. cGMP-dependent pathway and a GPCR kinase are required for photoreponse in the nematode *Pristionchus pacificus*. *PLoS Genet.* 2024;20: e1011320.
6. Hiramatsu F, Lightfoot JW. Kin-recognition and predation shape collective behaviors in the cannibalistic nematode *Pristionchus pacificus*. *PLoS Genet.* 2023;19: e1011056.
7. Ragsdale EJ, Müller MR, Rödelsperger C, Sommer RJ. A developmental switch coupled to the evolution of plasticity acts through a sulfatase. *Cell.* 2013;155: 922–933.
8. Bento G, Ogawa A, Sommer RJ. Co-option of the hormone-signalling module dafachronic acid-DAF-12 in nematode evolution. *Nature.* 2010;466: 494–497.
9. Sommer RJ. Phenotypic Plasticity: From Theory and Genetics to Current and Future Challenges. *Genetics.* 2020;215: 1–13.
10. Rödelsperger C, Neher RA, Weller AM, Eberhardt G, Witte H, Mayer WE, et al.

- 492 Characterization of genetic diversity in the nematode *Pristionchus pacificus* from
493 population-scale resequencing data. *Genetics*. 2014;196: 1153–1165.
- 494 11. McGaughan A, Rödelisperger C, Grimm DG, Meyer JM, Moreno E, Morgan K, et al.
495 Genomic profiles of diversification and genotype-phenotype association in island
496 nematode lineages. *Mol Biol Evol*. 2016;33: 2257–2272.
- 497 12. Zauner H, Mayer WE, Herrmann M, Weller A, Erwig M, Sommer RJ. Distinct patterns of
498 genetic variation in *Pristionchus pacificus* and *Caenorhabditis elegans*, two partially
499 selfing nematodes with cosmopolitan distribution. *Mol Ecol*. 2007;16: 1267–1280.
- 500 13. Dardiry M, Eberhard G, Witte H, Rödelisperger C, Lightfoot JW, Sommer RJ. Divergent
501 combinations of cis-regulatory elements control the evolution of phenotypic plasticity.
502 *PLoS Biol*. 2023;21: e3002270.
- 503 14. Quiobe SP, Kalirad A, Röseler W, Witte H, Wang Y, Rödelisperger C, et al.
504 EBAX-1/ZSWIM8 destabilizes miRNAs, resulting in transgenerational inheritance of a
505 predatory trait. *Sci Adv*. 2025;11: eadu0875.
- 506 15. Rödelisperger C. The community-curated *Pristionchus pacificus* genome facilitates
507 automated gene annotation improvement in related nematodes. *BMC Genomics*.
508 2021;22: 216.
- 509 16. Rödelisperger C, Athanasouli M, Lenuzzi M, Theska T, Sun S, Dardiry M, et al.
510 Crowdsourcing and the feasibility of manual gene annotation: A pilot study in the
511 nematode *Pristionchus pacificus*. *Sci Rep*. 2019;9: 18789.
- 512 17. Athanasouli M, Witte H, Weiler C, Loschko T, Eberhardt G, Sommer RJ, et al.
513 Comparative genomics and community curation further improve gene annotations in the
514 nematode *Pristionchus pacificus*. *BMC Genomics*. 2020;21: 708.
- 515 18. Moya ND, Stevens L, Miller IR, Sokol CE, Galindo JL, Bardas AD, et al. Novel and
516 improved *Caenorhabditis briggsae* gene models generated by community curation.
517 *BMC Genomics*. 2023;24: 486.
- 518 19. Doyle SR, Tracey A, Laing R, Holroyd N, Bartley D, Bazant W, et al. Genomic and
519 transcriptomic variation defines the chromosome-scale assembly of *Haemonchus*
520 *contortus*, a model gastrointestinal worm. *Commun Biol*. 2020;3: 656.
- 521 20. Bryant AS, Akimori D, Stoltzfus JDC, Hallem EA. A standard workflow for community-
522 driven manual curation of genome annotations. *Philos Trans R Soc Lond B Biol Sci*.
523 2024;379: 20220443.
- 524 21. Stevens L, Martínez-Ugalde I, King E, Wagah M, Absolon D, Bancroft R, et al. Ancient
525 diversity in host-parasite interaction genes in a model parasitic nematode. *Nat Commun*.
526 2023;14: 7776.
- 527 22. Wu TD, Watanabe CK. GMAP: a genomic mapping and alignment program for mRNA
528 and EST sequences. *Bioinformatics*. 2005;21: 1859–1875.
- 529 23. Pertea M, Pertea GM, Antonescu CM, Chang T-C, Mendell JT, Salzberg SL. StringTie
530 enables improved reconstruction of a transcriptome from RNA-seq reads. *Nat*
531 *Biotechnol*. 2015;33: 290–295.

- 532 24. Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, et al. Full-length
533 transcriptome assembly from RNA-Seq data without a reference genome. *Nat*
534 *Biotechnol.* 2011;29: 644–652.
- 535 25. Buels R, Yao E, Diesh CM, Hayes RD, Munoz-Torres M, Helt G, et al. JBrowse: a
536 dynamic web platform for genome visualization and analysis. *Genome Biol.* 2016;17:
537 66.
- 538 26. Krawitz P, Rödelsperger C, Jäger M, Jostins L, Bauer S, Robinson PN. Microindel
539 detection in short-read sequence data. *Bioinformatics.* 2010;26: 722–729.
- 540 27. Lee E, Helt GA, Reese JT, Munoz-Torres MC, Childers CP, Buels RM, et al. Web
541 Apollo: a web-based genomic annotation editing platform. *Genome Biol.* 2013;14: R93.
- 542 28. Prabh N, Rödelsperger C. Multiple *Pristionchus pacificus* genomes reveal distinct
543 evolutionary dynamics between de novo candidates and duplicated genes. *Genome*
544 *Res.* 2022;32: 1315–1327.
- 545 29. Röseler W, Sommer RJ, Rödelsperger C. Nematode genome announcement: A
546 chromosome-scale genome assembly for the reference mapping strain PS1843. *J*
547 *Nematol.* 2024;56: 20240063.
- 548 30. Ceron-Noriega A, Almeida MV, Levin M, Butter F. Nematode gene annotation by
549 machine-learning-assisted proteotranscriptomics enables proteome-wide evolutionary
550 analysis. *Genome Res.* 2023;33: 112–128.
- 551 31. Tegenfeldt F, Kuznetsov D, Manni M, Berkeley M, Zdobnov EM, Kriventseva EV.
552 OrthoDB and BUSCO update: annotation of orthologs with wider sampling of genomes.
553 *Nucleic Acids Res.* 2025;53: D516–D522.
- 554 32. Haas BJ, Salzberg SL, Zhu W, Pertea M, Allen JE, Orvis J, et al. Automated eukaryotic
555 gene structure annotation using EVIDENCEModeler and the Program to Assemble
556 Spliced Alignments. *Genome Biol.* 2008;9: R7.
- 557 33. Swain MT, Tsai IJ, Assefa SA, Newbold C, Berriman M, Otto TD. A post-assembly
558 genome-improvement toolkit (PAGIT) to obtain annotated genomes from contigs. *Nat*
559 *Protoc.* 2012;7: 1260–1284.
- 560 34. Holt C, Yandell M. MAKER2: an annotation pipeline and genome-database
561 management tool for second-generation genome projects. *BMC Bioinformatics.*
562 2011;12: 491.
- 563 35. Gabriel L, Brůna T, Hoff KJ, Ebel M, Lomsadze A, Borodovsky M, et al. BRAKER3: Fully
564 automated genome annotation using RNA-seq and protein evidence with GeneMark-
565 ETP, AUGUSTUS, and TSEBRA. *Genome Res.* 2024;34: 769–777.
- 566 36. Kirkland TN, Beyhan S, Stajich JE. Evaluation of different gene prediction tools in
567 *Coccidioides immitis*. *J Fungi (Basel).* 2023;9. doi:10.3390/jof9111094
- 568 37. Chao K-H, Heinz JM, Hoh C, Mao A, Shumate A, Pertea M, et al. Combining DNA and
569 protein alignments to improve genome annotation with LiftOn. *Genome Res.* 2025;35:
570 311–325.
- 571 38. Varabyou A, Erdogdu B, Salzberg SL, Pertea M. Investigating open reading frames in

- known and novel transcripts using ORFanage. Nat Comput Sci. 2023;3: 700–708.
39. Rödelberger C, Menden K, Serobyen V, Witte H, Baskaran P. First insights into the nature and evolution of antisense transcription in nematodes. BMC Evol Biol. 2016;16: 165.
40. Salzberg SL. Next-generation genome annotation: we still struggle to get it right. Genome Biol. 2019;20: 92.
41. Walker BJ, Abeel T, Shea T, Priest M, Abouelliel A, Sakthikumar S, et al. Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement. PLoS One. 2014;9: e112963.
42. Kim C, Kim J, Kim S, Cook DE, Evans KS, Andersen EC, et al. Long-read sequencing reveals intra-species tolerance of substantial structural variations and new subtelomere formation in. Genome Res. 2019;29: 1023–1035.
43. Gordon SP, Tseng E, Salamov A, Zhang J, Meng X, Zhao Z, et al. Widespread polycistronic transcripts in fungi revealed by single-molecule mRNA sequencing. PLoS One. 2015;10: e0132628.
44. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics. 2013;29: 15–21.
45. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The sequence alignment/map format and SAMtools. Bioinformatics. 2009;25: 2078–2079.

Figure legends

Figure 1 Gene annotation workflow. The flow diagram shows the simplified workflow for annotating the *P. pacificus* RSC011 genome using the PPCAC pipeline [14,15]. For this study, we extended this workflow in order to integrate newly generated Iso-seq and RNA-seq data.

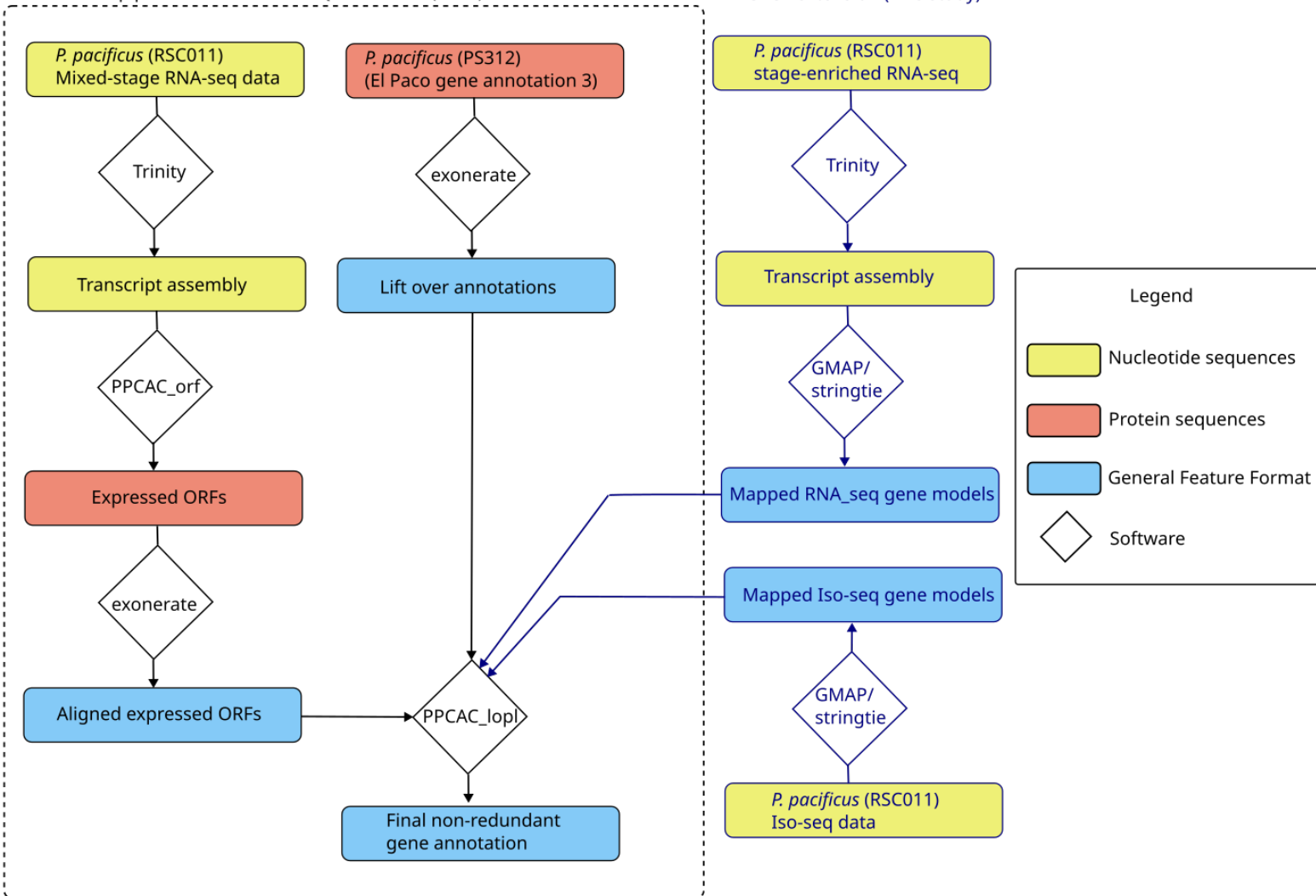
Figure 2 Retained introns, overlapping genes, polycistronic reads, and assembly

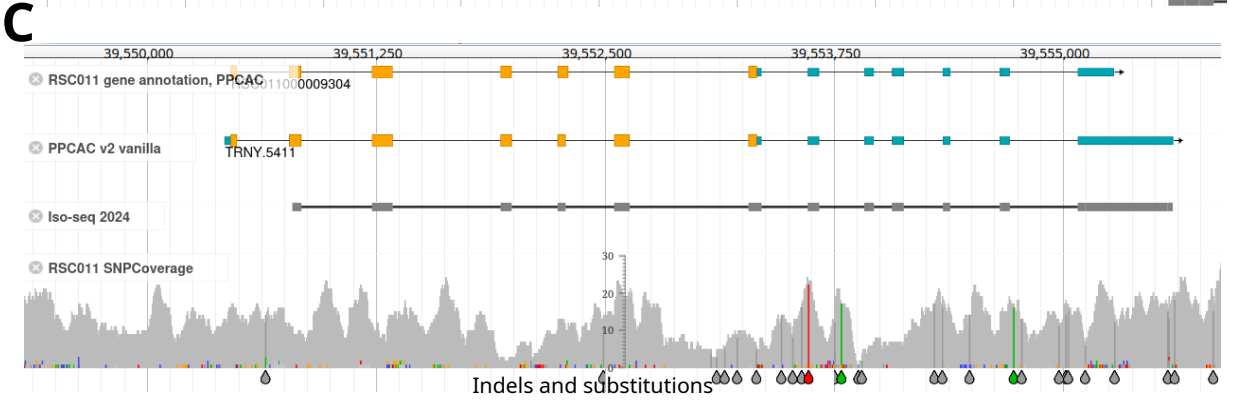
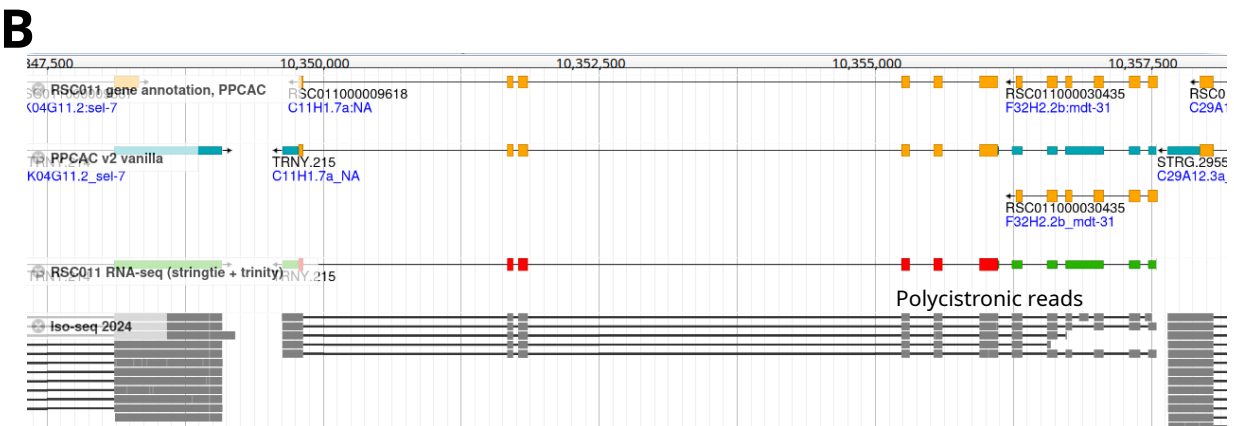
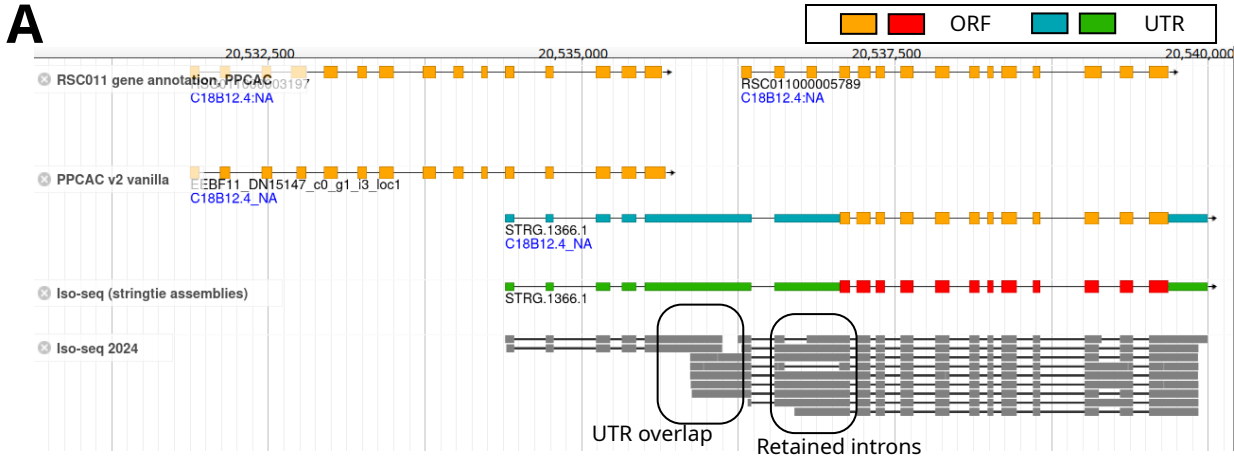
errors cause gene models with unusually long UTRs. A) The genome browser screenshot shows a roughly 10 kb region in the RSC011 genome assembly with the original annotation as topmost track [14]. BLASTP searches of the two paralogous genes against *C. elegans* support that these are two separate genes since both copies are homologous to the

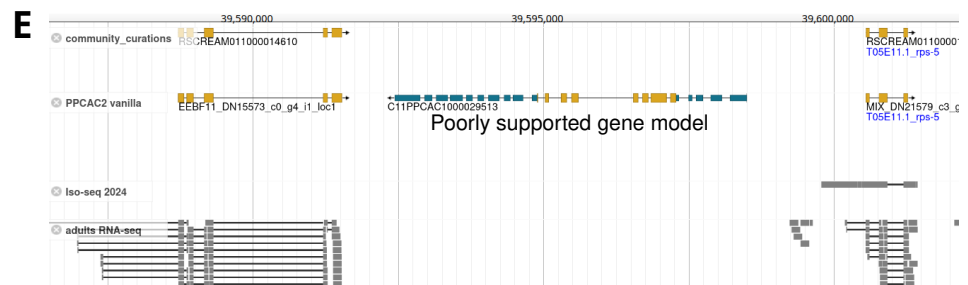
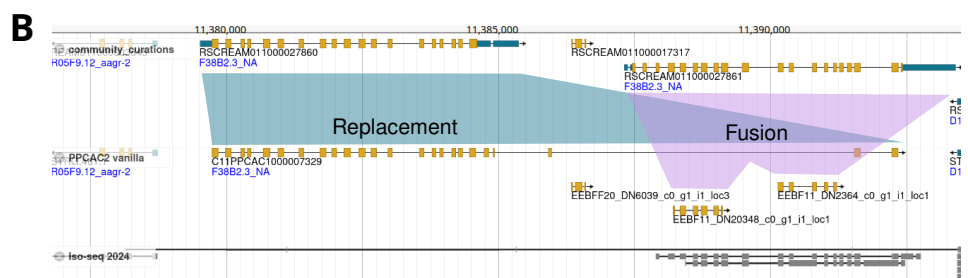
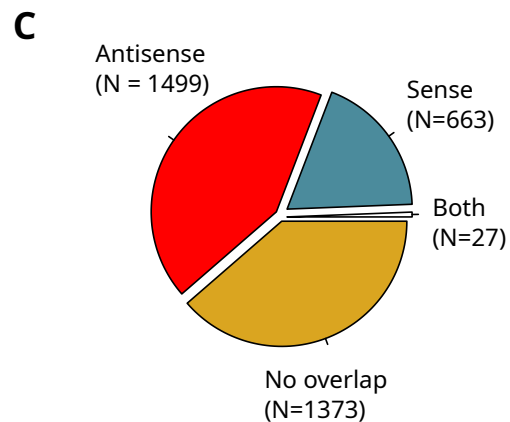
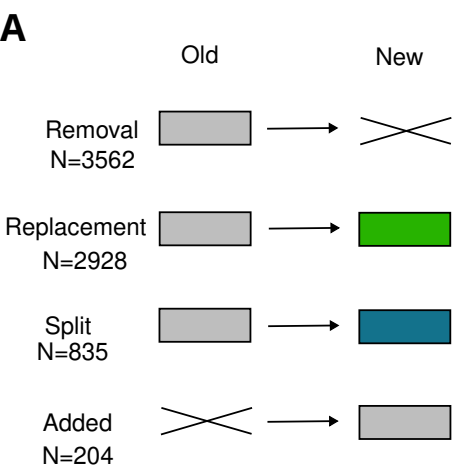
same part of the *C. elegans* gene *C18B12.4*. The new annotation (PPCAC v2), the raw Iso-seq alignments together with the assembled transcripts are shown in separate tracks. While both copies are at least partially covered by Iso-seq reads, both genes appear to overlap in the UTR sequences. Together with frequently retained introns, this leads to a transcript assembly with unusually long 5' UTR sequence (STRG.1366.1). **B)** This genomic region encodes two genes that are homologous to two different *C. elegans* genes. However, some polycistronic Iso-seq reads span both genic loci. **C)** This genome browser screenshot shows a gene model with a long UTR sequence. The additional RSC011 SNPCoverage track summarizes whole genome resequencing data at this locus and reveals SNPs and indels that likely introduce frameshifts and early stop in the ORF.

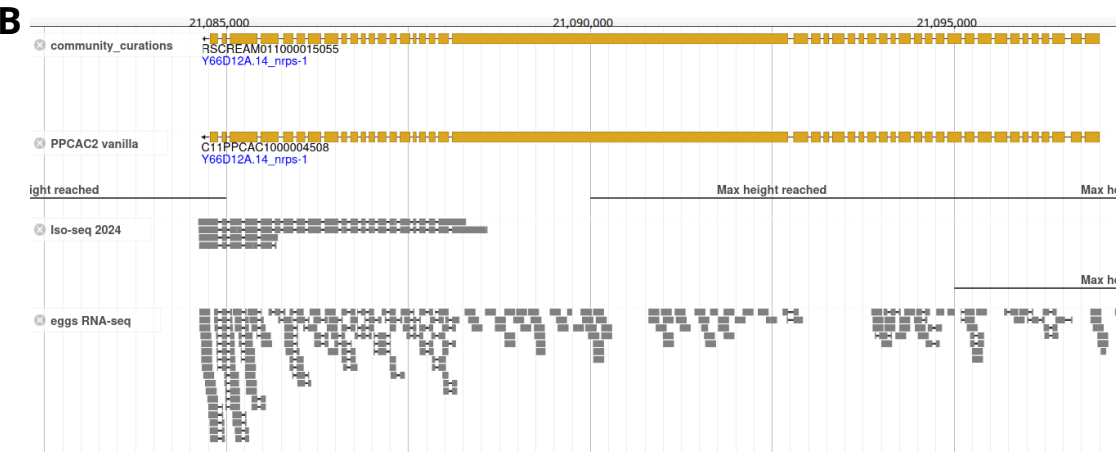
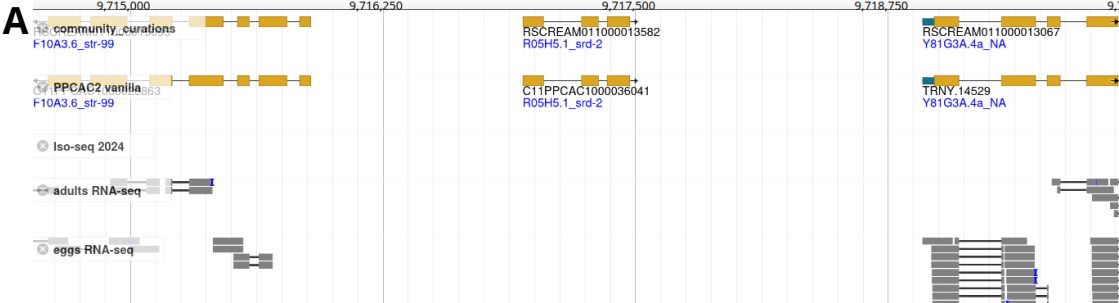
Figure 3 Roughly 24% of gene models are problematic. A) Genome-wide screening for obvious gene annotation artifacts led to over 7500 proposed corrections. Those corrections were classified into replacements, splits, removals and additions. **B)** At this locus, C11PPCAC1000027860 was chosen initially as the gene model with the longest ORF. Subsequently, only smaller gene models were added in order to prevent overlaps. However, transcriptomic data supports two genes at this locus. This could be corrected by one replacement and one fusion. **C)** Removed genes were further subgrouped based on overlaps with the final community curated gene annotations. The largest group consisted of antisense gene models and poorly supported gene models without any overlaps. **D)** This genome browser screenshot shows an example of an antisense gene model that has very similar gene structure with a gene on the opposite strand that encodes a protein with a homolog in *C. elegans*. **E)** This region shows a poorly supported gene model that was removed after community curation.

631 **Figure 4 Homology data and gene prediction program help to annotate genes without**
632 **complete transcriptomic support. A)** This genomic region shows an example of a gene
633 that has no transcriptomic support in our data, but has a homolog in *C. elegans*. **B)** This
634 genomic locus shows a gene with nearly complete homology in *C. elegans*, but without full
635 transcriptomic support.









**Bacterial diet and genetic background influence mutation rate in
*Pristionchus pacificus***

Shiela Pearl Quiobe*, Yinan Wang*, Penghieng Theam*, Waltraud Röseler, Hanh
Witte, Christian Rödelberger, Ralf J. Sommer

(published as Wang *et al.*)

G3 Genes|Genomes|Genetics: jkag038, (2026). DOI: 10.1093/g3journal/jkag038

Bacterial diet influences mutation rate in *Pristionchus pacificus*

Yinan Wang,[†] Penghieng Theam ,[†] Shiela Pearl Quiobe,[†] Waltraud Röseler, Hanh Witte, Christian Rödelsperger ,
Ralf J. Sommer *

Department for Integrative Evolutionary Biology, Max Planck Institute for Biology Tübingen, Max-Planck Ring 9, Tübingen 72076, Baden-Württemberg, Germany

*Corresponding author: Department for Integrative Evolutionary Biology, Max Planck Institute for Biology Tübingen, Max-Planck Ring 9, Tübingen 72076, Baden-Württemberg, Germany.

Email: ralf.sommer@tuebingen.mpg.de

[†]Contributed equally.

Mutation is a major force of evolution and its accumulation is suggested to be influenced by environmental and genetic factors in both unicellular and multicellular species. While ample of evidence showed an effect of temperature on mutation rate, the influence of diet is less well characterized, especially in multicellular organisms. Here, we present mutation accumulation (MA) rate differences for the same nematode species comparing a variety of bacterial diets. MA rates were estimated from whole-genome sequencing data of MA lines of different natural isolates of the free-living nematode *Pristionchus pacificus* on various bacterial diets isolated from *Pristionchus*-associated environments. Average single-nucleotide mutation rates varied between 1.69×10^{-9} and 2.23×10^{-9} nucleotide site⁻¹ × generation⁻¹, whereas the average insertion rates varied between 1.53×10^{-10} and 2.90×10^{-10} nucleotide site⁻¹ × generation⁻¹ and the average deletion accumulation rates varied between 3.01×10^{-10} nucleotide site⁻¹ × generation⁻¹ and 4.51×10^{-10} nucleotide site⁻¹ × generation⁻¹. We observed around a 1.4-fold mutation rate difference among groups on bacterial diets. Despite mutation-rate differences, the mutation spectra are largely unchanged. These results suggest that bacterial diet influences MA rate without drastically changing other mutational features.

Keywords: *Pristionchus pacificus*; *Caenorhabditis elegans*; mutation accumulation; mutation rate; nematodes; dietary influence

Introduction

Mutations are one of the major evolutionary forces and they are influenced by environmental and genetic factors in both unicellular and multicellular organisms (Matsuba et al. 2013; Lynch et al. 2023). Mutation accumulation (MA) experiments represent a powerful approach to study the influence of various factors on mutation rate and mutation spectra with many case studies in various organisms (Denver et al. 2012; Matsuba et al. 2013; Weller et al. 2014; Ness et al. 2015; Liu and Zhang 2019; Waldvogel and Pfenninger 2021; Lynch et al. 2023). For example, genetic factors were shown to influence mutation rate up to seven-fold in the green alga *Chlamydomonas reinhardtii* (Ness et al. 2015).

Among environmental factors potentially influencing MA, temperature has been studied in multiple animal species (Matsuba et al. 2013; Waldvogel and Pfenninger 2021). The influence of high temperature is suggested to be achieved through the increased level of stress on the organism in the nematode model organism *Caenorhabditis elegans* (Matsuba et al. 2013). In contrast to temperature, the influence of dietary conditions on MA is less studied, in particular in animals. Previous studies in *Drosophila melanogaster* suggest that poor nutritional conditions can contribute to the accumulation of deleterious mutations (Agrawal

and Wang 2008). However, it is currently unclear whether diet has an influence on MA in nematodes.

In multicellular organisms, MA experiments are performed by propagating organisms for a certain number of generations, with only one or two individuals picked for establishing the next generation (Denver et al. 2012; Matsuba et al. 2013; Weller et al. 2014; Waldvogel and Pfenninger 2021). Experiments are often performed in the laboratory under highly controlled environmental conditions and with ample resources to reduce competition (Denver et al. 2012; Matsuba et al. 2013; Weller et al. 2014; Waldvogel and Pfenninger 2021). Usually, MA experiments are performed in species with a short generation time and simple maintenance. Meeting those criteria, MA experiments can be performed in the nematode *Pristionchus pacificus*, a free-living species with a generation time of around four days. This species has recently been established as a model organism for studying developmental plasticity of two alternative mouth forms, potential predation on other nematodes and self-recognition largely by using the laboratory strain *P. pacificus* PS312 (Sommer et al. 1996; Lightfoot et al. 2019; Schroeder 2021; Sommer 2025). As *P. pacificus* is often found in association with scarab beetles, a multitude of wild isolates is available for this species (McCaughan et al. 2016). In recent times, several of these wild isolates were started to be used in natural variation studies (Dardiry et al. 2023).

To investigate how diverse dietary sources contribute to MA, we used *P. pacificus* strains of different genetic backgrounds and exposed worms of one of these strains to distinct bacterial diets. In this study, we performed MA experiments of the *P. pacificus* wild isolate RSC019 on different bacteria isolated from *Pristionchus*-associated environment. Specifically, MA data were newly generated for *P. pacificus* RSC019 on 3 bacterial diets, *E. coli* OP50, *Hafnia alvei* LRB17 (*H. alvei* LRB17), and *Agrobacterium tumefaciens* L27 (*A. tumefaciens* L27). *H. alvei* LRB17 is also known to reduce predation and life span compared to *E. coli* OP50 in *P. pacificus* PS312, while *A. tumefaciens* L27 increased the predation but did not show any influence on life span (Akduman et al. 2018, 2020). We also obtained MA data of *P. pacificus* RSC011, a wild isolate that was subject to a recent long-term environmental induction experiment, which used a similar experimental setup as MA experiments (Quiobe et al. 2025). In this experiment, *P. pacificus* RSC011 were raised on an alternative diet *Novosphingobium* sp. L76 (referred to as “*N. sp. L76*” for the rest of the manuscript) for 100 generations. *N. sp. L76* is known to have multiple influences on the laboratory strain *P. pacificus* PS312 including increased predatory behavior and reduced adult lifespan (Akduman et al. 2018, 2020). *N. sp. L76* can also increase brood size, developmental speed and the probability of producing the predatory mouth form in *P. pacificus* RSC011 (Dardiry et al. 2023; Quiobe et al. 2025; Quiobe and Sommer 2025). Comparing MA properties in those newly generated data together with the MA data generated previously in *P. pacificus* PS312 on *E. coli* OP50 (Weller et al. 2014), we compared MA properties in *P. pacificus* from three different genetic backgrounds and raised on four bacterial diets. In total, we observed a 1.4-fold change in accumulation rate of all three types under different dietary conditions. However, between the 2 different genetic backgrounds we observed no difference in single-nucleotide variation (SNV) accumulation rate, a two-fold change in insertions accumulation and 1.4-fold in deletion accumulation.

Materials and methods

Generation of MA lines

All *P. pacificus* and bacterial strains were from the Sommer lab collection (McGaughan et al. 2016; Akduman et al. 2018). Except for the standard *E. coli* OP50, bacterial strains used in this study were isolated from *Pristionchus*-associated environments (Akduman et al. 2018). Note that the original taxonomic assignments of L27 and LRB17 in Akduman et al. (2018) were revised to *A. tumefaciens* L27 and *H. alvei* LRB17 after whole genome sequencing (Akduman et al. 2018; Athanasouli et al. 2025).

To generate MA lines of *P. pacificus* RSC019, J4 progenies of a single hermaphrodite of RSC019 were grown on the standard lab food source *E. coli* OP50 following a protocol similar to Molnar et al. (2011). For *P. pacificus* RSC019 MA lines propagated on non-*E. coli* OP50 diets, the F1 worms were moved as J4 progenies of the same single hermaphrodite to the new diets. To remove remnants of *E. coli* OP50 cells F1 worms were transferred to new plates five times. In each transfer, worms were let to feed on the new bacteria for at least 15 min. From F2 to F100, all MA lines were raised at 20 °C and only one hermaphroditic J4-stage individual was transferred every generation in each line. Two previous generations of each line were kept and used to rescue when necessary (due to contamination of plates or the reduction of brood size). In *P. pacificus* RSC019, 20 lines each were grown on *E. coli* OP50, *A. tumefaciens* L27, and *H. alvei* LRB17. All lines were frozen in liquid nitrogen every 10 generations. At the start of the experiments, the parental line was also frozen.

The MA experiment in RSC011 was done as described previously (Quiobe et al. 2025). In short, to initiate the experiment, 110 clonal lines were established by isolating individual J4 larvae onto *N. sp. L76* and 10 lines onto *E. coli* OP50 from the complete brood of a single hermaphrodite. Each line was transferred every 4 days by a single J4 animal with fresh *N. sp. L76* or *E. coli* OP50, resulting in a continuous propagation for 1 year (101 generations). Populations were cryopreserved in liquid nitrogen every 10 generations (up to F100). For this study, we randomly selected 20 lines on *N. sp. L76* and eight lines (out of the 10 original lines) on *E. coli* OP50 after 100 generations.

Whole-genomic sequencing (WGS) library preparation

To gain enough material for WGS for the frozen ancestral line and MA lines of *P. pacificus* RSC019, five hermaphroditic J4 individuals were picked from each line transferred to five 6-cm nematode growth medium (NGM) plates. Progenies were collected for DNA extraction after the bacteria were depleted. DNA extraction and genomic sequencing were done at the same time for all the lines of *P. pacificus* RSC019 including the ancestral line to avoid potential batch effects. WGS library preparation was done using the Monarch Genomic DNA Purification Kit. The manufacturer's protocol was followed with some adjustments for nematode tissues. Briefly, each sample was lysed with 250 µl of tissue lysis buffer and 15 µl of proteinase K at 56 °C and kept agitated at 400 rpm for 1–1.5 h. It was then centrifuged for 3 min at maximum speed and supernatant was transferred to a new Eppendorf tube. Five microliters of RNase was added to the lysate that was then lightly and thoroughly mixed and kept agitated at 400 rpm for 10 min at 56 °C. Afterward, 400 µl of gDNA binding buffer was added to the sample. After mixing thoroughly, the sample was loaded onto the gDNA purification column. This was followed by a 3-min centrifugation to bind to the purification column at 100×g. Then, the same was centrifuged for 1 min more at maximum speed to remove the supernatant. The washing step was repeated twice with 500 µl of gDNA wash buffer and 1-min centrifugation at maximum each time. Finally, 32 µl of pre-heated elution buffer at 60 °C was added to the sample, which was allowed to stand for 1 min at room temperature. The elution was done by centrifuging the sample at maximum speed. WGS libraries of *P. pacificus* RSC011 MA lines were also generated with Monarch® Genomic DNA Purification Kit and in a separate single batch, following the manufacturer's instructions. All sequencing of lines of *P. pacificus* RSC019 and RSC011 were conducted by Novogene. Raw WGS data for *P. pacificus* PS312 on *E. coli* OP50 and *P. pacificus* RSC011 on *N. sp. L76* were obtained from a previous publication (Weller et al. 2014; Quiobe et al. 2025).

Generation of a reference genome for RSC019

To reduce the influence of the divergence of genetic background on mutation detection, all resequencing data were mapped to the PacBio genomes of the corresponding genetic backgrounds. Specifically, the El Paco genome was used for *P. pacificus* PS312, and the PacBio genome generated previously was used for RSC011 (Rödelsperger et al. 2017; Quiobe et al. 2025). To generate a genome assembly of the *P. pacificus* RSC019, we followed previously established protocols (Röseler et al. 2024). In short, sequencing of a PacBio library yielded 15 Gb HiFi reads that were assembled into a raw assembly by the software Canu (version 1.4) (Korean et al. 2017). This assembly was scaffolded by the software RagTag (version 2.1.0), which used the chromosome-scale *P. pacificus* assembly for the strain PS312 (version El Paco) as reference (Rödelsperger et al. 2017; Alonge et al. 2022). Evidence-based gene

annotations were generated by the software PPCAC based on strain-specific RNA-seq data and the community curated *P. pacificus* gene annotations (version El Paco annotations 3) (Athanasoulis et al. 2020; Rödelberger 2021). The transcriptomic data for the RSC019 strain were generated as described previously (Sun et al. 2021). The final assembly spans 152.6Mb with an N50 value of 23.3Mb. The resulting 27,588 gene models showed a BUSCO completeness of 94.9% (version 3 with the nematode odb9 data set) (Simão et al. 2015).

Raw data analysis

Mutation detection was performed following the GATK pipeline (version 4.2.5.0) for germline short variant discovery (SNPs + indels) (Poplin et al. 2018). Specifically, alignment of raw sequencing data to the corresponding reference genome was done with bwa mem (version 0.7.17) (Li 2013). HaplotypeCaller, GenomicsDBImport, and GenotypeGVCFs in the GATK pipeline were used separately for variant calling for each sample, further consolidate GVCFs, and the final VCFs (Poplin et al. 2018).

Mutation sharing detection

To detect if there were oversharing among lines in the same group, we compared the number of shared alternative homozygotes loci among pairs of line in the three genetic background separately. To have a fair comparison among pairs and remove the influence of potential background heterozygosity, we only counted loci with all lines covered by more than five reads less than 30% alternative homozygotes. Results are shown in [Supplementary Figs. 1–3](#).

Mutation detection

Line-specific mutations following similar criteria to those previously described, including SNPs and indels, were identified as accumulated mutations (Weller et al. 2014). Specifically, accumulated mutations were defined if one meets the following criteria: (i) only one type of homozygotes genotype exists with more than one line on the focal site; (ii) the alternative homozygotes only occurred in one line with no less than five reads in total supporting the genotype (DP in the vcf file); (iii) no alternative heterozygotes were inferred in any other line in the same group; (iv) at least 10 lines of the group had the reference homozygotes with support from more than five good quality reads; (v) genotype quality of the accumulated homozygotes was larger than 20 (GQ in the vcf file); and (vi) no other linking accumulated SNVs or indels in the same line. To reduce the influence of group size on mutation detection, lines were grouped into *P. pacificus* PS312 on *E. coli* OP50, RSC011 on *N. sp.* L76, RSC019 on *E. coli* OP50, RSC019 on *H. alvei* LRB17, and RSC019 on *A. tumefaciens* L27, allowing each group having animals within 19 to 22 lines. Potential cross contamination occurred among lines would reduce the number of detected accumulated mutations. Therefore, we removed PT34 in RSC019 on *A. tumefaciens* L27, PT7 in RSC019 on *E. coli* OP50, MAN_44 in RSC011 on *N. sp.* L76, and all eight lines in the whole group of RSC011 on *E. coli* OP50. Mutations were filtered in each group separately.

To compare the MA rate between RSC011 on the two bacterial diets, we separately grouped the eight lines from RSC011 on *E. coli* OP50 with 19 lines of RSC011 on *N. sp.* L76 to avoid the influence the potential cross contamination among lines. The estimated mutations of the 19 lines of RSC011 on *N. sp.* L76 showed no difference indicating comparable estimated mutation rates among analysis. The estimated SNV accumulation rates were higher and deletion accumulation rate were lower in RSC011 on *E. coli* OP50 ([Supplementary Fig. 4](#), [Supplementary Table 5](#)).

Calculation of mutation rate

To reduce the influence of sequencing depth on MA rate calculation, the MA rate in each line was calculated as the total number of mutations divided by the generations and length of genomic regions with more than half lines in the group having more than five reads mapped to the loci. The influences of mutations were annotated with the bcftools (version 1.15) csq function (Danecek and McCarthy 2017). Mutations were defined as coding ones if their annotation contained one of the following consequences: missense, synonymous, or stop_gained.

Statistical test

A Wilcoxon rank sum test was applied to test if MA rates were different among groups with wilcox.test function in R, an F-test was applied to test if variants of MA rates were different with var.test function in R, and a fisher's exact test was applied for testing if the mutation spectra were different among groups with fisher.test function in R.

Estimation of false positive and false negative mutation rates

To estimate the false-positive (FP) and false-negative (FN) rate for mutation detection, we employed both experimental and computational methods. Experimentally, we tested the detection of 16 randomly picked accumulated mutations from PT9 and PT10 in the group of *P. pacificus* RSC019 on *E. coli* OP50 with Sanger sequencing. Two of those mutations were in a distance of 2 bp, so the sequenced region was largely overlapping and covered both mutations. In total, 15 regions on the genome from the two lines were tested. Information about the primers and mutation detection in those two lines can be found in [Supplementary Table 7](#). Sanger sequencing results were transformed to fasta format using the Bio.SeqIO package in python with the first and last 50 bp sequence with potentially low quality being removed. The generated sequences in fasta format were then used as query to blast to the reference genome of *P. pacificus* RSC019 for testing the mutation detection. Both FP and FN rates in those two lines could be estimated from Sanger sequencing of those regions in both lines. The only case with a discrepancy between the Sanger sequencing result and GATK reported mutations was found in the tested region with two mutations within a 2 bp distance (mutation set number 12 in [Supplementary Table 7](#)).

To estimate FP rates computationally, we used the WGS data of the ancestral line of *P. pacificus* RSC019 (PT1) on *E. coli* OP50. The FP rate of the ancestral line was calculated as the proportion of alternative homozygotes that were only detected in the ancestral line among all (3 out of 5,101 alternative homozygotes in the region with the potential to call mutations in the group of *P. pacificus* RSC019 on *E. coli* OP50).

To estimate FN rates computationally, we introduced random mutations into the reference genome of all three *P. pacificus* genetic backgrounds. We calculated the FN rate of insertions and false recall rates of deletions as the proportion of inserted mutations that were not detected after applying the same mutation detection pipeline among all inserted ones in the region with enough coverage for each sample. We randomly inserted SNVs, insertions and deletions to the chromosomes of all three reference genomes, and rerun the GATK pipeline with the edited reference genome and WGS of *P. pacificus* PS312, RSC011, and RSC019 on *E. coli*. To simplify mutation detection, we only inserted mutations in the region where there was no mutation detected within 200 bp in the flanking region. Inserted SNVs were considered as detected if

Table 1. Statistics of the number of mutations and properties of accumulated mutations in each mutation accumulation groups.

Strain	Bacteria	SNV	Insertion	Deletion	ts/tv	Coding/Noncoding	Genic/Intergenic	ATBias	uSNV ($\times 10^{-9}$)	uInsertion ($\times 10^{-10}$)	uDeletion ($\times 10^{-10}$)
PS312	<i>E. coli</i> OP50	944	243	253	0.79	0.43	2.59	5.60	2.35 ± 1.14	5.95 ± 0.92	6.18 ± 5.73
RSC011	<i>N. sp.</i> L76	484	66	123	1.03	0.34	2.72	3.30	1.88 ± 0.65	2.57 ± 2.43	4.78 ± 4.42
RSC019	<i>A. tumefaciens</i> L27	431	56	77	1.30	0.41	1.99	4.66	1.69 ± 0.55	2.19 ± 2.09	3.01 ± 3.01
RSC019	<i>E. coli</i> OP50	569	74	115	1.21	0.41	2.16	5.24	2.23 ± 0.70	2.90 ± 2.70	4.51 ± 3.66
RSC019	<i>H. alvei</i> LRB17	466	41	89	0.96	0.39	1.93	5.24	1.74 ± 0.69	1.53 ± 1.87	3.32 ± 2.86

Note: The unit of mutation rates are per-site, per-generation. 95% confidence of mutation rates are shown in the table.

the alternative homozygotes with the same changes were detected in the same loci as they were inserted. Inserted indels were considered to be detected if alternative homozygotes of mutations as the same type were detected within 5 bp upstream, since assigning indels uniquely is not always possible.

Results

Mutation accumulation experiments in different *P. pacificus* isolates

Diet is an important environmental factor for all animals. In this study, we investigated MA in *P. pacificus* under different bacterial diets and genetic backgrounds. Specifically, MA data were generated for *P. pacificus* RSC019 on three bacterial diets and for RSC011 on two bacterial diets, propagating worms for 100 generations via single-worm descend under laboratory conditions. The *P. pacificus* RSC019 data is described here for the first time, whereas the *P. pacificus* RSC011 lines on *E. coli* OP50 and *N. sp.* L76 were previously generated in a long-term environmental induction experiment in the context of developmental plasticity. The whole-genome sequencing data for the RSC011 lines on *N. sp.* L76 had been characterized previously (Quiobe et al. 2025), whereas the corresponding data on *E. coli* OP50 is described here for the first time. In total, 20 lines of *P. pacificus* RSC011 on *N. sp.* L76, 8 on *E. coli* OP50, and 60 lines of RSC019 separately on *E. coli* OP50, *H. alvei* LRB17, and *A. tumefaciens* L27 and the ancestral line of RSC019 were sequenced. Those data were combined with previously published MA data of the standard laboratory strain *P. pacificus* PS312 on *E. coli* OP50 propagated for 142 generations (Weller et al. 2014). To reduce the influence of difference in genetic backgrounds on mutation detection, raw sequencing data were mapped to the reference genome for the corresponding *P. pacificus* strains. Published reference genomes were used for *P. pacificus* PS312 and RSC011 (Rödelsperger et al. 2017; Quiobe et al. 2025), while the PacBio genome assembly for RSC019 was newly generated for this study using previously established protocols (Röseler et al. 2024). Detection of mutations followed the GATK pipeline (Poplin et al. 2018). While analyzing the data, we observed pairs of samples with higher number of shared mutations, indicating potential sample swap/cross contamination during the experiment (Supplementary Figs. 1–3). Therefore, one RSC019 sample on *E. coli* OP50, one on *A. tumefaciens* L27, one RSC011 sample on *N. sp.*, and eight in RSC011 on *E. coli* OP50 were removed from the main analyses of our study. SNVs and indels which were only detected in one line in each group were identified as accumulated mutations and used for the following analysis. In total, 2,894 SNPs and 1,138 indels were detected for 99 samples in total (Supplementary Tables 1 and 2).

Mutation rates are largely consistent between two genetic backgrounds

To study the influence of genetic background on the MA, we compared the accumulation rates of SNVs and indels of groups of

P. pacificus PS312 and RSC019 on *E. coli* OP50. For *P. pacificus* strain PS312, the average MA rate of SNVs is $2.35 \pm 1.14 \times 10^{-9}$ nucleotide site⁻¹ $\times$ generation⁻¹ with an average insertion rate of $5.95 \pm 5.92 \times 10^{-10}$ nucleotide site⁻¹ $\times$ generation⁻¹ and a deletion rate of $6.18 \pm 5.73 \times 10^{-10}$ nucleotide site⁻¹ $\times$ generation⁻¹ (Table 1). For RSC019, the MA rate of SNVs is $2.23 \pm 0.70 \times 10^{-9}$ nucleotide site⁻¹ $\times$ generation⁻¹ with an insertions rate of $2.90 \pm 2.70 \times 10^{-10}$ nucleotide site⁻¹ $\times$ generation⁻¹ and a deletion rate of $4.51 \pm 3.66 \times 10^{-10}$ nucleotide site⁻¹ $\times$ generation⁻¹ (Table 1). Thus, we observed differences in MA rates for insertions and deletions but not for SNVs between these two groups (Fig. 1a, Wilcoxon rank sum tests, Supplementary Table 4). The variance of mutation rates of *P. pacificus* PS312 raised on *E. coli* OP50 is higher than RSC019 on the same bacteria for insertions as well as SNVs but not deletions (Supplementary Table 4, two-sided F-test).

We further tested differences in other properties of those three types of mutations. We found a significant difference in mutation spectrum between *P. pacificus* PS312 and RSC019 (*P*-value = 0.015, Fisher's exact test, Fig. 2, Supplementary Table 4). The ratios of transitions and transversions were also significantly different between *P. pacificus* PS312 and RSC019 (*P*-value = 5×10^{-5} , Fisher's exact test, Supplementary Table 4), whereas the distribution of mutations across coding and noncoding regions and genic and intergenic regions were not (*P*-value > 0.05, Fisher's exact test, Supplementary Table 4). Length distribution of deletions but not insertions in *P. pacificus* PS312 was different from that in *P. pacificus* RSC019 (*P*-value < 0.05 with Fisher's exact test, 5.1 bp for PS312 and 8.1 bp for RSC019, Supplementary Tables 3 and 4). These results show that the indel properties also had subtle changes.

Mutation accumulation rate changed with bacterial diet

To study the effect of bacterial diet on the mutation rate, we compared the MA rates of *P. pacificus* RSC019 on different bacteria (average MA rates on *E. coli* OP50: SNV: $2.23 \pm 0.70 \times 10^{-9}$ nucleotide site⁻¹ $\times$ generation⁻¹, insertion: $2.90 \pm 2.70 \times 10^{-10}$ nucleotide site⁻¹ $\times$ generation⁻¹, deletion: $4.51 \pm 3.66 \times 10^{-10}$ nucleotide site⁻¹ $\times$ generation⁻¹, on *A. tumefaciens* L27: SNV: $1.69 \pm 0.55 \times 10^{-9}$ nucleotide site⁻¹ $\times$ generation⁻¹, insertion: $2.19 \pm 2.09 \times 10^{-10}$ nucleotide site⁻¹ $\times$ generation⁻¹, deletion: $3.01 \pm 3.01 \times 10^{-10}$ nucleotide site⁻¹ $\times$ generation⁻¹, and on *H. alvei* LRB17: SNV: $1.74 \pm 0.70 \times 10^{-9}$ nucleotide site⁻¹ $\times$ generation⁻¹, insertion: $1.53 \pm 1.87 \times 10^{-10}$ nucleotide site⁻¹ $\times$ generation⁻¹, deletion: $3.32 \pm 2.86 \times 10^{-10}$ nucleotide site⁻¹ $\times$ generation⁻¹, Table 1). In *P. pacificus* RSC019, the SNV accumulation rate of worms grown on *E. coli* OP50 was detected to be higher than those on the other two bacterial diets (Fig. 1a, Table 1, *P*-value < 0.05 compared to RSC019 on *A. tumefaciens* L27 and *H. alvei* LRB17, Wilcoxon rank sum test, Supplementary Table 4). The insertion accumulation rates of *P. pacificus* RSC019 on *E. coli* OP50 showed significantly higher accumulation rates of insertions compared to those on *H. alvei* LRB17, and higher deletion

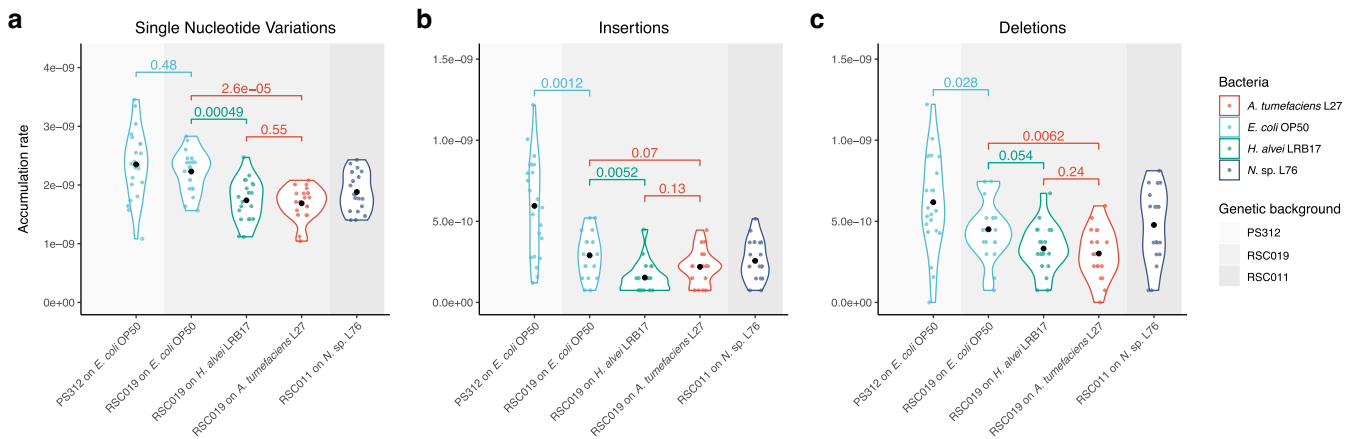


Fig. 1. Summary of mutation accumulation rates across different conditions. Accumulation rates of a) single-nucleotide variations (SNVs), b) insertions, and c) deletions. The color in the background indicates the genetic background of each group, and the color indicates the bacterial diet. Unit of y-axis is nucleotide site⁻¹ × generation⁻¹. P-values are shown for each pair of comparisons, and statistical tests were done with the Wilcoxon's rank sum test.

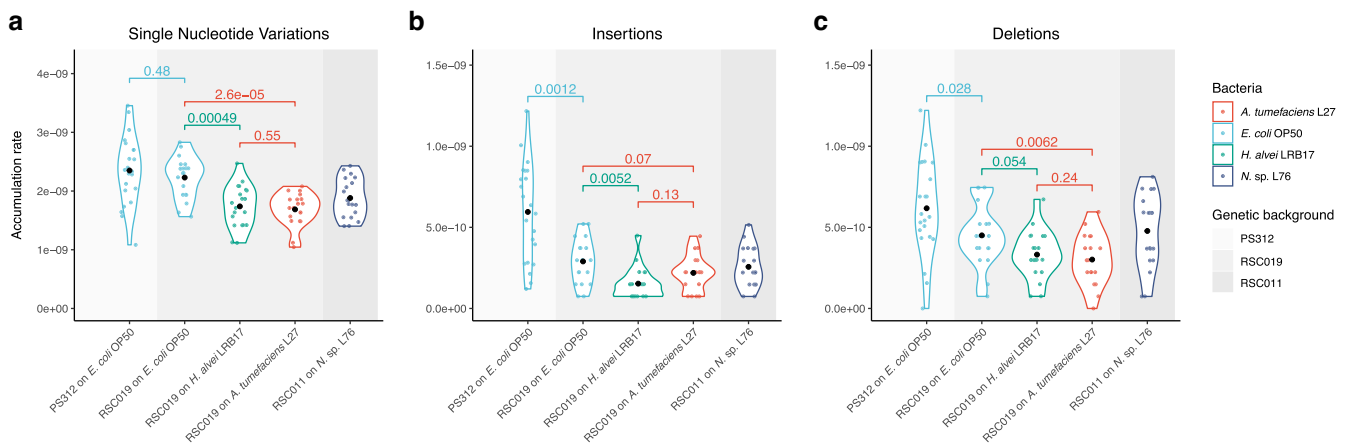


Fig. 2. Summary of the mutation spectra across different conditions. a) Spectra of SNV and length distribution of b) insertion and c) deletions of each group. Color in the background indicates the genetic background, and color corresponds to the types of mutations.

accumulation rates compared to *A. tumefaciens* L27 (Fig. 1, b and c, Table 1). The length distributions of insertions and deletions were detected to be different between *P. pacificus* RSC019 on *E. coli* OP50 and those on *H. alvei* LRB17 (*P*-value < 0.05, Fisher's exact test, 2.8 and 8.1 bp for *E. coli* OP50 group and 4.4 and 10.8 bp for *H. alvei* LRB17 group, Supplementary Table 4), but not between the other two groups. The difference in mutation rate between diets is also supported by a separate comparison of our RSC011 data on *N. sp.* L76 and *E. coli* OP50 (Supplementary Fig. 4, Supplementary Table 5). Thus, while the SNV mutation rates are largely consistent across genetic backgrounds, we observe an up to 1.3-fold increase in mutation rates between bacterial diets.

Estimation of false positive and false negative mutation detection with GATK

Finally, we wanted to validate the quality of our mutation detection by performing false-positive (FP) and false-negative (FN) analyses. For estimating the FP and FN rates, we employed both experimental and computational strategies. Experimentally, we estimated FP and FN rates within two lines (PT9 and PT10) in the group of *P. pacificus* RSC019 on *E. coli* OP50 as described in more details in Materials and Methods. We investigated mutation detection in both lines in 15 regions covering six SNVs, six insertions

and four deletions Sanger sequencing (Supplementary Table 6). All mutations were detected as originally reported except for two closely located indels where the discrepancy in results could be attributed to the ambiguity in indel annotation. Therefore, we did not consider that specific case as FN. Given that this experimental validation did not identify any FP or FN, we would estimate the associated FP and FN rates to be close to 0%.

In addition, we computationally estimated the FP and FN rates in all three *P. pacificus* genetic backgrounds. We randomly inserted around 400 SNVs, around 3,000 insertions and deletions into the reference genome of those three genetic backgrounds and tested the detection of this set of mutations with those edited reference genome in around 20 lines from each genetic background (Supplementary Table 7). The FN rates were defined as the ratio of simulated mutations that could not be detected in the regions covered by sufficient reads in each line. We observed FN rates varied within 0–5.8% for SNVs, and false recall rates of 3.9–17.2% for insertions and 2.7–9.1% for deletions among lines (Supplementary Table 7), and estimated lower number of FN rates for insertions and deletions. To estimate the FP rates, we incorporated WGS data of the ancestral line of RSC019 (PT1), the only sequenced ancestral line among all three groups. The FP rate of the ancestral line was 0.06% and was calculated as the proportion of alternative

homozygotes that were only detected in the ancestral line among all when comparing to the group of *P. pacificus* RSC019 (Supplementary Table 7). Thus, the experimental validation and computational evaluation points toward low error rates of our mutation detection procedure.

Discussion

In this study, we compared SNV and indel accumulation rates of *P. pacificus* nematodes between genetic backgrounds and various bacterial diets. The average SNV accumulation rates observed in this study ($1.08\text{--}3.34 \times 10^{-9}$ nucleotide site $^{-1} \times$ generation $^{-1}$) span a similar range with previously reported rates in two free-living nematodes of the genus *Caenorhabditis* ($0.78\text{--}2.14 \times 10^{-9}$ nucleotide site $^{-1} \times$ generation $^{-1}$) (Denver et al. 2012). When further split SNVs based on their mutation and sequence context, we observed similar trends of an excess of mutations between T and A under the sequence context of TTA or TAA between *P. pacificus* groups and *C. elegans* N2 (Supplementary Fig. 5, Konrad et al. 2019). Thus, our results suggest that the magnitude of mutation rates and fold changes appears to be conserved across the two nematode genera *Caenorhabditis* and *Pristionchus*, which were recently estimated to have diverged roughly 300 MYA (Qing et al. 2025). This indicates that mutation rates and spectra can overall be stable during nematode evolution, and we therefore speculate that these values can serve as a first estimate of mutation rate even for more distantly related free-living nematodes.

While we could not detect significant differences in the average SNV accumulation rates between genetic backgrounds, we observed significant differences in the mean and variance of indel frequencies between PS312 and RSC019. However, we would like to point out that our data sets for PS312 and RSC019 on *E. coli* are not completely comparable, as both experiments have not been carried out for the same number of generations (142 generations in the case of PS312 vs 100 generations for RSC019) and previous comparisons of first-order and second-order MA lines have identified significant differences in indel frequencies between both regimes of MA lines pointing toward the evolvability of the mutational process (Saxena et al. 2019). Thus, we cannot completely rule out an effect of such a process on the mean and variance of the observed results.

Previous studies have indicated that organisms under stressful conditions such as high temperature or poor diet quality resulting in lower fecundity or body mass can experience increased mutation rate (Agrawal and Wang 2008; Sharp and Agrawal 2012; Matsuba et al. 2013; Waldvogel and Pfenninger 2021). In our study, we showed, to the best of our knowledge for the first time, that diet can influence the MA in nematodes. One potential explanation for the difference in MA rate is the physiological stress caused by diet. Among all tested bacteria, *N. sp.* L76 and *H. alvei* LRB17 could potentially cause stress responses in *P. pacificus* PS312, since they induced reduced adult survival compared to the other tested bacteria (Akduman et al. 2018). In contrast, *P. pacificus* RSC019 on *H. alvei* LRB17 had a slightly lower mutation rate compared to that on *E. coli* OP50. Thus, the biological processes that affect mutation rates across different microbiota remain to be elucidated.

Data availability

The Pacbio genome assembly of *P. pacificus* RSC019 and WGS data for *P. pacificus* RSC011 on *E. coli* OP50 and all *P. pacificus* RSC019 are newly generated for this study and available on European Nucleotide Archive under the study accession PRJEB94012. The RSC019 genome assembly and gene annotations are available at

<http://www.pristionchus.org>. All strains used in this study are available upon request from the corresponding author.

Supplemental material available at G3 online.

Acknowledgments

We thank all members of the Sommer lab for fruitful discussions throughout these studies. We would also like to thank the *Caenorhabditis* Genetics Center for providing us with a strain of *E. coli* OP50.

Funding

This work was fully funded by the Max-Planck Society.

Conflicts of interest

None declared.

Literature cited

- Agrawal AF, Wang AD. 2008. Increased transmission of mutations by low-condition females: evidence for condition-dependent DNA repair. *PLoS Biol.* 6:e30. <https://doi.org/10.1371/journal.pbio.0060030>.
- Akduman N et al. 2020. Bacterial vitamin B12 production enhances nematode predatory behavior. *ISME J.* 14:1494–1507. <https://doi.org/10.1038/s41396-020-0626-2>.
- Akduman N, Rödelberger C, Sommer RJ. 2018. Culture-based analysis of *Pristionchus*-associated microbiota from beetles and figs for studying nematode-bacterial interactions. *PLoS One.* 13:e0198018. <https://doi.org/10.1371/journal.pone.0198018>.
- Alonge M, et al. 2022. Automated assembly scaffolding using RagTag elevates a new tomato system for high-throughput genome editing. *Genome Biol.* 23:258. <https://doi.org/10.1186/s13059-022-02823-7>.
- Athanasouli M et al. 2020. Comparative genomics and community curation further improve gene annotations in the nematode *Pristionchus pacificus*. *BMC Genomics.* 21:708. <https://doi.org/10.1186/s12864-020-07100-0>.
- Athanasouli M, Loschko T, Rödelberger C. 2025. Interspecies systems biology links bacterial metabolic pathways to nematode gene expression, chemotaxis behavior, and survival. *Genome Res.* 35:2363–2374. <https://doi.org/10.1101/gr.280848.125>.
- Danecek P, McCarthy SA. 2017. BCFtools/csq: haplotype-aware variant consequences. *Bioinformatics.* 33:2037–2039. <https://doi.org/10.1093/bioinformatics/btx100>.
- Dardiry M, Piskobulu V, Kalirad A, Sommer RJ. 2023. Experimental and theoretical support for costs of plasticity and phenotype in a nematode cannibalistic trait. *Evol Lett.* 7:48–57. <https://doi.org/10.1093/evlett/qrac001>.
- Denver DR et al. 2012. Variation in base-substitution mutation in experimental and natural lineages of *Caenorhabditis* Nematodes. *Genome Biol Evol.* 4:513–522. <https://doi.org/10.1093/gbe/evs028>.
- Konrad A, Brady MJ, Berghthorsson U, Katju V. 2019. Mutational landscape of spontaneous base substitutions and small indels in experimental *Caenorhabditis elegans* populations of differing size. *Genetics.* 212:837–854. <https://doi.org/10.1534/genetics.119.302054>.
- Koren S, et al. 2017. Canu: scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. *Genome Res.* 27:722–736. <https://doi.org/10.1101/gr.215087.116>.

- Li H. 2013. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM [preprint]. arXiv 3997. [accessed 2025 Jul 15]. <http://arxiv.org/abs/1303.3997>. <https://doi.org/10.48550/arXiv.1303.3997>
- Lightfoot JW et al. 2019. Small peptide-mediated self-recognition prevents cannibalism in predatory nematodes. *Science*. 364:86–89. <https://doi.org/10.1126/science.aav9856>.
- Liu H, Zhang J. 2019. Yeast spontaneous mutation rate and spectrum vary with environment. *Curr Biol*. 29:1584–1591.e3. <https://doi.org/10.1016/j.cub.2019.03.054>.
- Lynch M et al. 2023. The divergence of mutation rates and spectra across the Tree of Life. *EMBO Rep*. 24:e57561. <https://doi.org/10.15252/embr.202357561>.
- Matsuba C, Ostrow DG, Salomon MP, Tolani A, Baer CF. 2013. Temperature, stress and spontaneous mutation in *Caenorhabditis briggsae* and *Caenorhabditis elegans*. *Biol Lett*. 9: 20120334. <https://doi.org/10.1098/rsbl.2012.0334>.
- McGaughan A et al. 2016. Genomic profiles of diversification and enotype-phenotype association in island nematode lineages. *Mol Biol Evol*. 33:2257–2272. <https://doi.org/10.1093/molbev/msw093>.
- Molnar RI, Bartelmes G, Dinkelacker I, Witte H, Sommer RJ. 2011. Mutation rates and intraspecific divergence of the mitochondrial genome of *Pristionchus pacificus*. *Mol Biol Evol*. 28:2317–2326. <https://doi.org/10.1093/molbev/msr057>.
- Ness RW et al. 2015. Extensive de novo mutation rate variation between individuals and across the genome of *Chlamydomonas reinhardtii*. *Genome Res*. 25:1739–1749. <https://doi.org/10.1101/gr.191494.115>.
- Poplin R et al. 2018. Scaling accurate genetic variant discovery to tens of thousands of samples [preprint]. bioRxiv 201178. [accessed 2024 Apr 3]. <https://www.biorxiv.org/content/10.1101/201178v3>. <https://doi.org/10.1101/201178>
- Qing X et al. 2025. Phylogenomic insights into the evolution and origin of *Nematoda*. *Syst Biol*. 74:349–358. <https://doi.org/10.1093/sysbio/syae073>.
- Quiobe SP et al. 2025. EBAX-1/ZSWIM8 destabilizes miRNAs, resulting in transgenerational inheritance of a predatory trait. *Sci Adv*. 11:eadu0875. <https://doi.org/10.1126/sciadv.adu0875>.
- Quiobe SP, Sommer RJ. 2025. Adaptive consequences of transgenerational inheritance of a predatory mouth-form trait in nematodes [preprint]. bioRxiv 680509. [accessed 2025 Nov 2]. <https://www.biorxiv.org/content/10.1101/2025.10.05.680509v1>. <https://doi.org/10.1101/2025.10.05.680509>
- Rödelsperger C et al. 2017. Single-molecule sequencing reveals the chromosome-scale genomic architecture of the nematode model organism *Pristionchus pacificus*. *Cell Rep*. 21:834–844. <https://doi.org/10.1016/j.celrep.2017.09.077>.
- Rödelsperger C. 2021. The community-curated *Pristionchus pacificus* genome facilitates automated gene annotation improvement in related nematodes. *BMC Genomics*. 22:216. <https://doi.org/10.1186/s12864-021-07529-x>.
- Röseler W, Sommer RJ, Rödelsperger C. 2024. Nematode genome announcement: a chromosome-scale genome assembly for the *Pristionchus pacificus* reference mapping strain PS1843. *J Nematol*. 56:20240063. <https://doi.org/10.2478/jofnem-2024-0063>.
- Saxena AS, Salomon MP, Matsuba C, Yeh S-D, Baer CF. 2019. Evolution of the mutational process under relaxed selection in *Caenorhabditis elegans*. *Mol Biol Evol*. 36:239–251. <https://doi.org/10.1093/molbev/msy213>.
- Schroeder NE. 2021. Introduction to *Pristionchus pacificus* anatomy. *J Nematol*. 53:e2021–e2091. <https://doi.org/10.21307/jofnem-2021-091>.
- Sharp NP, Agrawal AF. 2012. Evidence for elevated mutation rates in low-quality genotypes. *Proc Natl Acad Sci U S A*. 109:6142–6146. <https://doi.org/10.1073/pnas.1118918109>.
- Simão FA, Waterhouse RM, Ioannidis P, Kriventseva EV, Zdobnov EM. 2015. BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics*. 31: 3210–3212. <https://doi.org/10.1093/bioinformatics/btv35>.
- Sommer RJ. 2025. *Pristionchus*—beetle associations: towards a new natural history. *J Invertebr Pathol*. 209:108243. <https://doi.org/10.1016/j.jip.2024.108243>.
- Sommer RJ, Carta L, Kim S-Y, Sternberg PW. 1996. Morphological, genetic and molecular description of *Pristionchus pacificus* sp. n. (Nematoda: Neodiplogastridae). *Fundament Appl Nematol*. 19:511–521.
- Sun S, Rödelsperger C, Sommer RJ. 2021. Single worm transcriptomics identifies a developmental core network of oscillating genes with deep conservation across nematodes. *Genome Res*. 31:1590–1601. <https://doi.org/10.1101/gr.275303.121>.
- Waldvogel A-M, Pfenninger M. 2021. Temperature dependence of spontaneous mutation rates. *Genome Res*. 31:1582–1589. <https://doi.org/10.1101/gr.275168.120>.
- Weller AM, Rödelsperger C, Eberhardt G, Molnar RI, Sommer RJ. 2014. Opposing forces of A/T-biased mutations and G/C-biased gene conversions shape the genome of the nematode *Pristionchus pacificus*. *Genetics*. 196:1145–1152. <https://doi.org/10.1534/genetics.113.159863>.

Editor: E. Haag

Supplementary Material

Supplementary Figure 1: Number of shared alternative homozygotes in pairs in PS312 on *E. coli* OP50. Results indicate little mutation sharing among lines.

Supplementary Figure 2: Number of shared alternative homozygotes in pairs in RSC019. Results indicate large proportion of mutation sharing between PT33 and PT34, and mild mutation sharing between PT6 and PT7.

Supplementary Figure 3: Number of shared alternative homozygotes in pairs in RSC011. Results indicate mild mutation sharing between MAN_35 and MAN_44, and large proportion of mutation sharing among MAE lines (RSC011 on *E. coli* OP50).

Supplementary Figure 4: Mutation rate comparison between RSC011 on *E. coli* OP50 and *N. sp.* L76. Each line of RSC011 on *E. coli* OP50 were group with the group on *N. sp.* L76 separately for accumulated mutation detection and mutation rates calculation. Mutation rates of RSC011 on *N. sp.* L76 were also estimated in these eight groups and shown.

Supplementary Figure 5: Mutational spectra across 96 SNV types in *P. pacificus* and *C. elegans* N2. SNVs are divided into 96 types based on the mutation and the nucleotide 1 bp up and down stream of it. a-f) mutation spectra of *P. pacificus* groups and g) mutation spectra of *C. elegans* N2.

Supplementary Table 1: Summary information of all mutations.

Supplementary Table 2: Summary information of all lines.

Supplementary Table 3: Full list of statistics of mutations in all five groups.

Supplementary Table 4: *p*-values of comparisons of mutation properties among groups.

Supplementary Table 5: Mutation rates estimated for RSC011 on *E. coli* OP50 and on *N. sp.* L76.

Supplementary Table 6: Primers used for sanger sequencing.

Supplementary Table 7: Computational estimation of FN rates.

The logic of transgenerational inheritance of cannibalism

Shiela Pearl Quiobe, Ata Kalirad, Ralf J. Sommer

Under Review in *iScience*

The logic of transgenerational inheritance of cannibalism

Shiela Pearl Quiobe ¹, Ata Kalirad ¹, Ralf J. Sommer^{1,*}

**1 Department for Integrative Evolutionary Biology, Max Planck Institute for
Biology Tübingen, 72076 Tübingen, Germany**

*** ralf.sommer@tuebingen.mpg.de**

ORCID: 0000-0002-6261-2657 (SPQ)

ORCID: 0000-0002-9500-3903 (AK)

ORCID: 0000-0003-1503-7749 (RJS)

Abstract

Epigenetic inheritance of environmentally-induced phenotypes across generations has been observed across taxa. However, the dynamics of non-genetic inheritance as a consequence of environmental variability remains poorly understood. Here, we take advantage of the transgenerational epigenetic inheritance (TEI) of an environmentally-induced cannibalistic trait in the nematode *Pristionchus pacificus*. Using alternative environmental induction (AIE) and superinduction experiments, we illustrate how environmental variability regimes influence TEI and uncover the dosage-dependent nature of the trigger molecule – vitamin B12 – on the onset of TEI. Our study provides a unique example of the dynamics of TEI of an ecologically consequential trait as a consequence of environmental variation

Keywords: Transgenerational epigenetic inheritance, dynamics of non-genetic inheritance, cannibalism, developmental plasticity, nematode

Introduction

Epigenetic inheritance has been reported in a plethora of organisms, ranging from inheritance of odor sensitivity in mice and longevity in *Caenorhabditis elegans* to famine-induced epigenetic changes in humans (1–3). Despite these examples, the importance of epigenetic inheritance in evolution remains disputed (4–6). Importantly, the inheritance of epigenetic changes across three generations or more following the exposure to an environmental cue – referred to as transgenerational epigenetic inheritance (TEI) (7, 8) – is thought to provide evolutionary benefits in fluctuating environment. However, there are few experimental studies of the dynamics of TEI (e.g., (9)) and, to our knowledge, there have been no case studies which investigated the pattern of TEI in response to environmental variability for an ecologically-relevant phenotype.

The nematode *Pristionchus pacificus* provides a perfect model to study TEI (10). This worm exhibits developmental plasticity of its mouth parts with two alternative forms in response to environmental variation (11). Specifically, *P. pacificus* individuals adopt a *stenostomatous* (St) morph with a single tooth and a narrow buccal cavity, or alternatively, a *eurystomatous* (Eu) morph with two teeth and a broad cavity (Figure 1A). This developmental decision is irreversible and affects the feeding capabilities of adult worms. St individuals are strict bacterial feeders, whereas Eu animals are omnivores that can also kill other nematodes (12, 13). As *P. pacificus* is a self-fertilizing hermaphrodite with many of the same appealing characteristics as *Caenorhabditis elegans* (short generation time, easy husbandry and isogenic propagation), the regulatory mechanisms controlling mouth-form plasticity have been investigated by forward and reverse genetics. By now, a detailed gene regulatory network has been identified (14–18). Importantly, the mouth-form decision in *P. pacificus* is in parts stochastic in nature, and is deeply influenced by a slew of environmental factors, most notably diet (19, 20). Together, this mouth form polyphenism results in intraguild life-history predation that could greatly shape the ecological dynamics of communities of *P. pacificus* and its relatives in the wild (13, 21).

In nature, *P. pacificus* is found in association with beetles of the family

Scarabaeidae (22). Specifically, dauer larvae of *P. pacificus* and other nematodes reside on adult beetles (for review see (23)). Following the natural death of a beetle in the soil, dauer larvae develop into adults, forming a short-lived community sustained by bacteria and other microbes that decompose the insect carcass. Subsequently, new dauer larvae dispers into the environment in search for new beetle hosts. In the context of the beetle life cycle, mouth-form polyphenism in *P. pacificus* with its associated predation, is expected to greatly affect the dynamics of the transient ecological competition that unfolds on the decaying carcass. However, despite multiple recent studies on the ecological significance of phenotypic plasticity in *P. pacificus* in promoting and / or hindering co-existence (20, 24, 25), little is known about the role of epigenetic processes and potential TEI in the wild, particularly in the context of the decaying beetle carcasses.

Recently, we demonstrated that dietary induction of the predatory mouth form can be transgenerationally inherited for multiple generations (26). Using a wild isolate of *P. pacificus* from a high altitude location on La Réunion Island, RSC011, a long-term environmental induction experiment has been performed. When grown on the standard laboratory food source *E. coli* OP50, a *P. pacificus* PSC011 adult has around 30% chance of developing the Eu mouth form (20). In contrast, exposure of these worms to *Novosphingobium* L76 – a bacterium derived from *Pristionchus*’s natural environment on La Réunion (19) – increases the probability of developing the Eu mouth form to 100% (26) (Figure 1B). Specifically, this dietary induction of the predatory mouth form is immediate (in the first generation of exposure), systemic (found in all the lines exposed to the inducing diet), and complete (all the progeny of an exposed mother show the Eu morph). Strikingly, dietary reversal from *Novosphingobium* L76 to *E. coli* OP50 will result in the TEI of the predatory mouth form (26) (Figure 1B). This transgenerational memory was observed for a minimum of four generations, but required an original exposure to *Novosphingobium* L76 for at least five generations. This pattern of TEI is phenomenologically different from the majority of examples of TEI observed in *C. elegans* (26). Subsequently, we have shown that bacterial-derived vitamin B12 – through its role in bringing about vitellogenin provisioning – is necessary and sufficient to induce TEI of predatory mouth form in *P. pacificus* (27).

It is important to note that these laboratory studies do not mimic the conditions in nature. In the ecological context of decaying scarab beetles, spatial and temporal variability with respect to diversity of bacteria in the growing microbial biomass on the carcass is expected. To explore how such variability in the exposure to the inducing agent might affect the TEI of the predatory morph in *P. pacificus*, we designed multiple alternative induction experiments in which *P. pacificus* are alternatively exposed to *Novosphingobium* L76 or *E. coli* OP50 for different numbers of generations.

In this manuscript, firstly we develop simple theoretical models that indicate the potential patterns of TEI in *P. pacificus* as a function of environmental variability. Secondly, we introduce a two-pronged experimental approach to understand which theoretical model better explains the TEI of mouth-form polyphenism. Our approach relies on two experimental designs: (a) alternative induction experiment, which enables a systematic exploration of how TEI behaves as a function of environmental variability and (b) superinduction experiment, designed to assess the behavior of TEI with respect to the intensity of the environmental cue. Taken together, our results shed light on the dynamics of TEI as a function of environmental variability, and they link the observed changes to a theoretical framework, thus adding to a growing body of mechanistic understanding of the TEI of the predatory morph in *P. pacificus*.

Methods

Maintenance of nematodes

The ancestral *P. pacificus* RSC011 isolate was cryopreserved within the first 10 generations after its initial collection from Coteau Kerveguen, La Réunion. Worms were reared at 20°C under standard conditions on Nematode Growth Medium (NGM) plates seeded with either *E. coli* OP50 or *Novosphingobium* L76. Bacterial strains were cultured overnight in Lysogeny Broth (LB) with kanamycin (50µg/ml) when required. Specifically, OP50 was grown overnight at 37°C in LB without shaking while *Novosphingobium* L76 was grown at 30°C at 180 rpm. NGM plates were seeded with

300 μ l of overnight cultures and incubated for 2 days prior to use.

93

Dietary induction and food reversals

94

Worms were propagated on *Novosphingobium* for 5 and 10 generations, respectively. To transfer from nematodes from *Novosphingobium* L76 to *E. coli* OP50, worms were first transferred from *Novosphingobium* L76 to NGM plates supplemented with (50 μ g/ml) of kanamycin with a kanamycin resistant *E. coli* strain as food for one generation, before being reintroduced to standard *E. coli* OP50-seeded NGM plates for subsequent generations.

95

96

97

98

99

100

Mouth-form phenotyping

101

Mouth-form phenotyping was performed by observing the nematode buccal cavity using a Zeiss Discovery V.20 stereo microscope ($\times 150$ magnification) based on mouth-form identities as described before (14). On each plate, 20 young-adult animals were assayed.

102

103

104

Dietary supplementation

105

Methylcobalamin (Vitamin B12 CAS No. 13422-55-4) – from Sigma – was dissolved in water to prepare the stock solution shortly before each experiment. solutions were mixed with NGM agar at the required concentration just before pouring to 6 cm plates. Plates were allowed to dry at room temperature for two days and then spotted with *Novosphingobium* L76.

106

107

108

109

110

Statistical analysis

111

The probability of assuming the predatory mouth form by the adult nematodes was calculated using the following hierarchical bayesian model (Figure S1A):

112

113

$$\begin{aligned}
y_i &\sim \text{Binomial}(N, \theta_i) \\
\theta_i &= \text{logit}^{-1}(\eta_i) \\
\eta_i &= \mu + z_i \sigma \\
z_i &\sim \text{Normal}(0, 1) \\
\mu &\sim \text{Normal}(0, 2) \\
\sigma &\sim \text{HalfNormal}(2)
\end{aligned} \tag{1}$$

Using the inferred parameters, the predictive probability for the development of the predatory mouth form is calculated:

$$\begin{aligned}
\tilde{z} &\sim \text{Normal}(0, 1) \\
\tilde{\theta} &= \text{logit}^{-1}(\mu + \tilde{z}\sigma) \quad .
\end{aligned} \tag{2}$$

The 95% highest density interval (HDI) for $\tilde{\theta}$ is used to visualize the inferred probability of developing the Eu mouth form.

The effect of TEI is quantified using the risk difference (RD) between generation i and the first generation following reversal as baseline:

$$RD = \tilde{\theta}_i - \tilde{\theta}_{\text{baseline}} \quad . \tag{3}$$

The model was fitted to the laboratory measurements using PyMC (28) using Python 3.12 with Numpy 2.3.0 (29). We ensured that the estimated values were stable using common convergence diagnostics (30) (Figure S1B).

The TEI models 123

Systems of ODEs, representing different models of TEI dynamics, were numerically integrated in Python 3.12 using SciPy 1.15.3 (31). 125

Results

126

The expected dynamics of TEI

127

There have been several theoretical attempts to incorporate epigenetic inheritance in an evolutionary framework (5, 32). However, such models are primarily concerned with the potential role of epigenetic inheritance as an additional source of correlation between parents and offspring and its consequence on the response of the population to selection. In contrast, we are interested in the dynamics of TEI itself, as a function of the environment. A suitable model in this context was previously introduced (9):

128

129

130

131

132

133

$$\frac{dz}{dt} = xy + \gamma \frac{z^n}{z^n + k^n} - \alpha z \quad , \quad (4)$$

where z is the state of a gene which has been silenced by trigger x , which is present only at t_0 and decays with rate α . This model is based on three rules observed with respect to the TEI of gene silencing induced by double-stranded RNA in *Caenorhabditis elegans*: (i) correlation between offspring response and parental state, (ii) stochastic inheritance state (y) in parents that are exposed to the trigger, and (iii) transgenerational momentum, i.e., the likelihood of TEI increases the longer it persists.

134

135

136

137

138

139

140

The TEI of mouth-form state in *P. pacificus* seems to deviate from rules (ii) and (iii): the response to the environmental trigger (*Novosphingobium* L76) was systemic, resulting in all the lines assuming predatory mouth form, and little variation was observed in TEI amongst lines that were exposed to trigger from 5 to 95 generations (26). For an individual i of strain j , its mouth-form state X_i , in environment E_1 can be described as $X_i \sim \text{Binom}(P_{j|E_1})$, and when exposed to E_2 , $X_i \sim \text{Binom}(P_{j|E_2})$, where P_j is the probability of developing either of the two mouth forms in a given environment. However, TEI implies $P_{j|E_2}$ should be affected by the environment experienced by the parent. We propose a model of TEI in *P. pacificus* (26), represented by a causal network:

141

142

143

144

145

146

147

148

149

150

$$\text{Trigger} \longrightarrow X \text{---}\vdash Y \text{---}\vdash Z \quad , \quad (5)$$

where an unknown trigger induces the activity of enzyme X (EBAX-1 ubiquitin

151

ligase), which suppresses the microRNA Y (miR-2235a/miR-35). In the absence of the trigger or following the degradation of enzyme X , Y degrades a microRNA target (Z), which prevents the TEI of the predatory mouth-form in *P. pacificus*. Although more mechanistic details of this process – at least for the moment – remain elusive, this causal model can enable us to construct a phenomenological model. A simple circuit could be specified as

$$\begin{aligned}\frac{dx}{dt} &= \alpha_X u(t) - \beta_X x, \\ \frac{dy}{dt} &= \frac{\alpha_Y}{1 + \left(\frac{x}{K_{XY}}\right)^{n_{XY}}} - \beta_Y y, \\ \frac{dR}{dt} &= \frac{1 - R}{\tau_R} - k_R u(t) R, \\ \frac{dz}{dt} &= \alpha_Z u(t) R - \beta_Z z,\end{aligned}\tag{6}$$

where $u(t)$ is the trigger at time t , α indicates maximum production, and β reflects degradation. An additional component R is added, simply to enable an explicit inclusion of history in the system. We construct two variations of the above model to investigate three possible TEI regulatory circuits: (i) The constant TEI circuit, which repeatedly exhibits the same TEI pattern regardless of its history of past exposures to the trigger, (ii) the acclimating TEI circuit, which exhibits a weaker TEI pattern given a history of intermittent exposure to the trigger, and (iii) the sensitizing TEI circuit, which exhibits stronger patterns of TEI if it was exposed to the trigger in the past (Figure 1C, Table S1).

These models illustrate three general patterns of TEI that one could observe in *P. pacificus* (Figure 1D). Neither of the three models can *a priori* be ruled out, and all could be justified in an ecological and/or evolutionary context. Although, the details of these models are not a direct reflection of data, they provide us with toy models in order to guide our experimental design.

A black-box approach to relate theory to reality

To relate these theoretical expectation to reality, we designed two types of experimental approaches: an alternative induction experiment and a superinduction experiment. The reasoning behind these designs are to treat the gene regulatory circuitry responsible for the induction of the predatory Eu mouth form in *P. pacificus* as a black box (33): since the inner workings of the molecular machinery are not fully understood, by manipulating the input (environmental cue) and observing the output (the pattern of TEI) we aim to reveal the dynamics of this system.

The design of the alternative induction experiment

At the start of the experiment, a single self-fertilizing hermaphrodite of *P. pacificus* RSC011 is transferred from the standard *E. coli* OP50 (non-inducing environment) to *Novosphingobium* L76 (inducing environment). In each generation, a single healthy J4 juvenile progeny was moved to a new plate. The single-worm bottleneck means that the population genetic changes are driven by drift; following the same procedure as described in (26). After propagation on *Novosphingobium* L76 for n generations, the next generation would be propagated on *E. coli* OP50, with the following generation being transferred back to *Novosphingobium* L76. The alternative exposure to *Novosphingobium* L76 determines the number of generations during which the worms are exposed to the trigger, before the interruption of the exposure for a single generation (Figure 2A). We use alternative induction experiments (AIEs) with $n = 1, 2, 3, 4, 5$ to illustrate the dynamics of TEI in *P. pacificus* when exposed to changing environments.

The design of the superinduction experiment

In contrast to the AIE, in which the duration of the induction is manipulated, in a superinduction experiment, we manipulate the intensity of the environmental cue. Previously, we have shown that vitamin B12 is both necessary and sufficient to induced TEI of the predatory mouth form in *P. pacificus* (27). To increase the intensity of the inducing agent, we supplement *Novosphingobium* L76 with 1500nM vitamin B12. We hypothesized that the manipulation of the concentration of trigger in the superinduction experiment supplement the AIE design to reveal the dynamics of TEI in *P. pacificus*.

The TEI of cannibalism in *P. pacificus* follows a mixed model.

In AIE with $n = 1$, i.e., a single generation of exposure, a sensitizing TEI would result in an increasing probability of developing the predatory mouth form in the non-inducing environment. However, in such an AIE, no clear trend is observed in 18 generations (Figure 2B). This lack of pattern becomes evident when comparing the predictive risk difference (RD) for the probability of developing the predatory mouth form between generations grown on the non-inducing diet (Figure S2). Similarly, no such trend was observed in $n = 2$ (Figure 2C, S3A). These results hint at a history-independent TEI that only switches on following a minimum of 5 generations of exposure to the inducing environment, as previously observed in (26). The AIE with $n = 3$ and $n = 4$, both were consistent with this model (Figure 2D,E). However, for $n = 3$, comparison between generation 4 and 12, $0.076 \leq 95\% \text{HDI}(\text{RD}) \leq 0.34$, suggesting an effect on the response on non-inducing diet (Figure S3B). In contrast, AIE with $n = 5$ – which corresponds to the minimum number of generations required for TEI of predatory mouth form in *P. pacificus* – exhibited a pattern consistent with a sensitizing TEI: a second exposure to the inducing environment for five generations increased the probability of assuming the predatory mouth form on the non-inducing environment ($0.17 \leq 95\% \text{HDI}(\text{RD}) \leq 0.43$ when comparing generation 12 with 6) (Figure 2F, S3D) (RD for all experiments in 2B – F are found in Figures S2 and S3).

Trigger concentration modulates TEI of cannibalism in *P. pacificus*.

We have previously proposed that the key trigger of the TEI of mouth-form polyphenism in *P. pacificus* is vitamin B12, which is produced by *Novosphingobium* L76 (27). Consequently, provided that the uptake of the trigger by the worm is not the limiting factor, supplementing the inducing diet (*Novosphingobium* L76) with additional B12 would increase the concentration of the trigger that the worm encounters. Increase in the concentration of the trigger can result in the early onset of TEI and/or change the duration of TEI. To test our hypothesis, we designed the superinduction experiment.

In the superinduction experiment, we exposed *P. pacificus* RSC011 worms to either

Novosphingobium L76 (inducing condition) or *Novosphingobium* L76 plus 1500nM vitamin B12 (superinducing condition) and then transferred them back to the non-inducing environment (*E. coli* OP50). In the absence of any information in regard to the effect of B12 concentration on TEI, we explored a range of induction and superinduction periods ranging from one to five generations (Figure 3). We have previously reported that a minimum of five generation of exposure to *Novosphingobium* L76 is required to induce TEI in *P. pacificus* RSC011 (26).

A single generation of exposure under inducing or superinducing conditions has no credible effect on the predictive probability of developing the predatory mouth form in the non-inducing environment (Figure 3A, B, S4A, and S4B). Exposure for two generations to the superinducing condition elevates the predictive probability of developing the predatory mouth form in the first generation in the non-inducing environment (For inducing, $0.45 \leq 95\%HDI(\tilde{\theta}_{R1}) \leq 0.72$; for superinducing, $0.62 \leq 95\%HDI(\tilde{\theta}_{R1}) \leq 0.84$) (Figure 3C,D,S4C, and S4B). The RD for the superinducing condition suggest an effect when comparing the first generation on non-inducing diet (R1) and the third generation (R3) ($-0.38 \leq 95\%HDI(RD_{R3 \text{ vs } R1}) \leq 0.0$) (Figure S4D).

The effect of superinduction in early onset of TEI becomes evident when comparing experiments in which induction or superinduction was carried out for three generations (Figure 3E and F). Whereas $\tilde{\theta}_{Ri}$ shows a minor effect following induction, in the superinduction case, $0.69 \leq 95\%HDI(\tilde{\theta}_{R1}) \leq 0.92$, which remains high for the second generation in the non-inducing environment ($0.66 \leq 95\%HDI(\tilde{\theta}_{R2}) \leq 0.88$), before going to levels comparable to the inducing case (Figure S4E and S4F).

Induction and superinduction for four generations results in a striking pattern: the probability of developing the predatory mouth form on the non-inducing diet immediately after superinduction is very high ($0.63 \leq 95\%HDI(\tilde{\theta}_{R1}) \leq 1$) compared with the inducing case ($0.45 \leq 95\%HDI(\tilde{\theta}_{R1}) \leq 0.7$) (Figure 3G, H, S5A, and S5B). However, the second generation in the non-inducing environment following superinduction shows a decline in the probability of developing the predatory mouth form

($0.55 \leq 95\% \text{HDI}(\tilde{\theta}_{R2}) \leq 0.77$), which is followed by an increase in the following generation ($0.66 \leq 95\% \text{HDI}(\tilde{\theta}_{R3}) \leq 0.9$) (Figure S5B). It remains to be seen, to what extent such effects on TEI can be attributed to the broader effects of vitamin B12 on the nematode.

Induction and superinduction for five generations clearly demonstrates the effect of the concentration of the trigger molecule on the duration of the TEI of mouth-form polyphenism (Figure 3I and J). Consistent with our previous findings (26), five generation of exposure to the inducing conditions results in an elevated probability of developing the predatory mouth form immediately following induction ($0.87 \leq 95\% \text{HDI}(\tilde{\theta}_{R1}) \leq 0.1$). This response gradually declines, finally reaching $0.29 \leq 95\% \text{HDI}(\tilde{\theta}_{R4}) \leq 0.74$ in our experiment (Figure 3I and S5C). However, the superinduction slows the rate of decline of the TEI, resulting in $0.49 \leq 95\% \text{HDI}(\tilde{\theta}_{R4}) \leq 0.75$ after four generations on the non-inducing environment and remains relatively high in the subsequent generation ($0.4 \leq 95\% \text{HDI}(\tilde{\theta}_{R5}) \leq 0.7$) (Figure 3J and S5D).

Discussion

In this study we investigated the logic and dynamics of TEI of a predatory trait. The nematode *P. pacificus* is well suited to investigate TEI in response to environmental variability because it exhibits one of a few cases of TEI with an ecologically-relevant phenotype. Building on simple theoretical models indicating the potential patterns of TEI of the predatory mouth form in *P. pacificus*, we performed two sets of experiments. Alternative induction experiments revealed that TEI of the predatory morph is largely history-independent. Superinduction experiments on the other hand indicated that an earlier onset of TEI is possible, suggesting that the nematode itself is not the limiting factor of the initial TEI. These findings help to further increase our understanding of this complex memory phenotype and add information necessary to better understand the relevance of this phenomenon in nature.

Alternative induction experiments followed a mixed model with AIE with $n < 5$ are

history-independent, whereas AIE with $n = 5$ exhibited a sensitized TEI (Figure 2).
The pattern in AIE with $n = 5$ is puzzling: why didn't the interruption in this AIE
reset the effect in non-inducing environment, but it was enough in AIEs with $n < 5$ to
disrupt the memory? One possible strategy to resolve this puzzle is to conduct AIEs on
a longer temporal scale to see if enough trigger accumulates over longer periods.
However, that solution complicates the issue since random mutations can fix in AIEs,
given its similarity to a mutation-accumulation experiment. Previously (26), we
documented such mutations during the long-term experimental induction in that study.
We reasoned that the pattern observed in AIEs, with clear sensitization in AIE with
 $n = 5$, can be explained by two alternative hypotheses: (i) there is a limit to the
amount of trigger absorbed by a worm per generation and five generations are needed to
accumulate enough trigger across generations to switch on the TEI, or (ii) the
concentration of the trigger in *Novosphingobium* L76 is not enough, and if presented
with higher amount of trigger, *P. pacificus* would express TEI after shorter exposure to
the inducing environment.

Our superinduction experiments strongly favor the second model as vitamin B12
supplementation on top of a *Novosphingobium* diet will result in higher transmission of
the predatory mouth form (Figure 3). It has to be noted that the exact concentrations
of vitamin B12 in individual worms cannot be determined as reliable vitamin B12
sensors are still not available (34, 35). While the concentrations of vitamin B12 in these
supplementation experiments followed those of previous studies on surplus killing (19)
and transgenerational memory (26), *Pristionchus* vitamin B12 applications have to use
higher doses than those used in *C. elegans* (1000 or 1500 nM versus 63 nM).
Interestingly, previous studies showed that the induction of the predatory mouth form
requires much less vitamin B12, i.e. supplementation of around 1nM is sufficient,
whereas only very high, in parts, supraphysiological concentrations can cause the TEI
after vitamin B12 supplementation.

Transgenerational plasticity (TGP) is a broad term that describes non-genetic
inheritance when the effect of the environment in one generation affects a subsequent
generation (36). Such a broad concept is adequate when discussing our results,

especially the results of the superinduction experiments, where TEI, with its strict
criterion concerning the number of affected generations, does not reflect the intricacies
of the observed dynamics. Additionally, our results illustrate the insights gained from
our experimental designs reveals the intricacies beneath these terms once one delves into
the detailed behavior of an exemplar of TGP. Specifically, our results indicate how the
induction of developmental plasticity in *P. pacificus* and the patterns of TGP are not
one and the same. Rather, while the induction of the predatory mouth form is systemic
and immediate, the onset of TGP is both influenced by the number of generations
during which the worms are exposed to the environmental trigger and the concentration
of that trigger, best described as combination of the constant and sensitizing TEI
models (Figure 1D).

It is assumed that the evolution of TEI is directly dependent on environmental
variability, where the evolution of developmental plasticity itself is possible when
withing generation environmental variability is low and between generation variability
exists and TEI requires longer periods of stability (37). Our exploration of TEI of a
cannibalistic trait in *P. pacificus* provides a unique example to understand how
epigenetic inheritance unfolds in a trait that directly affects competition via predation
in an organism that lives in a complex yet tractable ecological settings (Figure 4).
Luther Burbanks’ use of “ever-moving life forces” in his description of the role of
environment on heredity (38) is an apt description of the attempt to better understand
the dynamics of non-genetic inheritance, a necessity if we are to characterize the role of
non-genetic inheritance in ecology and evolution.

Data availability

Code and data are available on the Github repository associated with this manuscript
(<https://github.com/Kalirad/NematodeSuperinduction>)

Authors’ contributions

352

SPQ, AK, and RJS designed the experiments. SPQ and AK conducted the experiments.

353

SPQ collected the data. AK analyzed and visualized the results. Mathematical

354

modeling was done by AK. SPQ, AK, and RJS wrote the manuscript.

355

Conflict of interest declaration

356

We declare we have no competing interests.

357

Funding

358

This work was funded by the Max Planck Society.

359

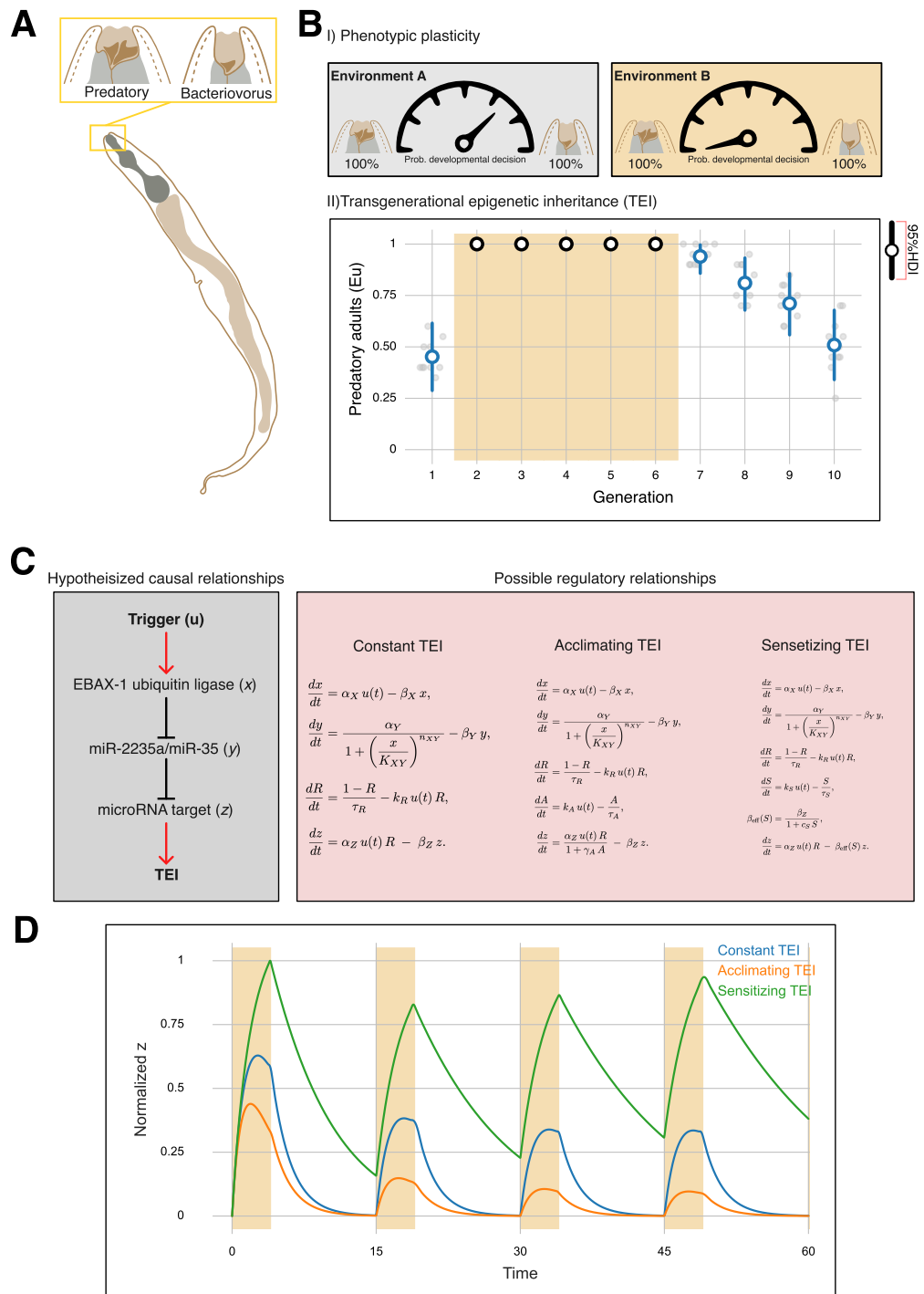


Figure 1

Figure 1 caption

(A) The nematode *Pristionchus pacificus* develops either of two mouth forms: a wide eurystomatous (Eu) form which enables predation of other nematodes in addition to bacterivory, and a narrow non-predatory stenostomatous (St) form. The irreversible decision to express a mouth form is taken during the development from juvenile to adult stage. **(B)** The mouth-form polyphenism in *P. pacificus* is characterized by change in the probability of the developmental switch between the predatory and non-predatory mouth forms depending on the environmental conditions (I), and transgenerational epigenetic inheritance (TEI), where an exposure for at least five generations to an inducing environment results in epigenetic inheritance for three generations in the non-inducing environment (II). **(C)** We have previously suggested a hypothetical causal model which connect the environmental trigger to TEI (26). However, different regulatory models can be constructed based on our causal model. A constant TEI model describes a case where TEI is independent of environmental variability. An acclimating TEI describes a scenario in which repeated exposure to the environmental trigger dampens the TEI. Finally, in a sensitizing TEI model, the TEI amplifies with more periodic exposure to the inducing environment. **(D)** The dynamics of the three models of TEI with periodic exposure to the environmental trigger.

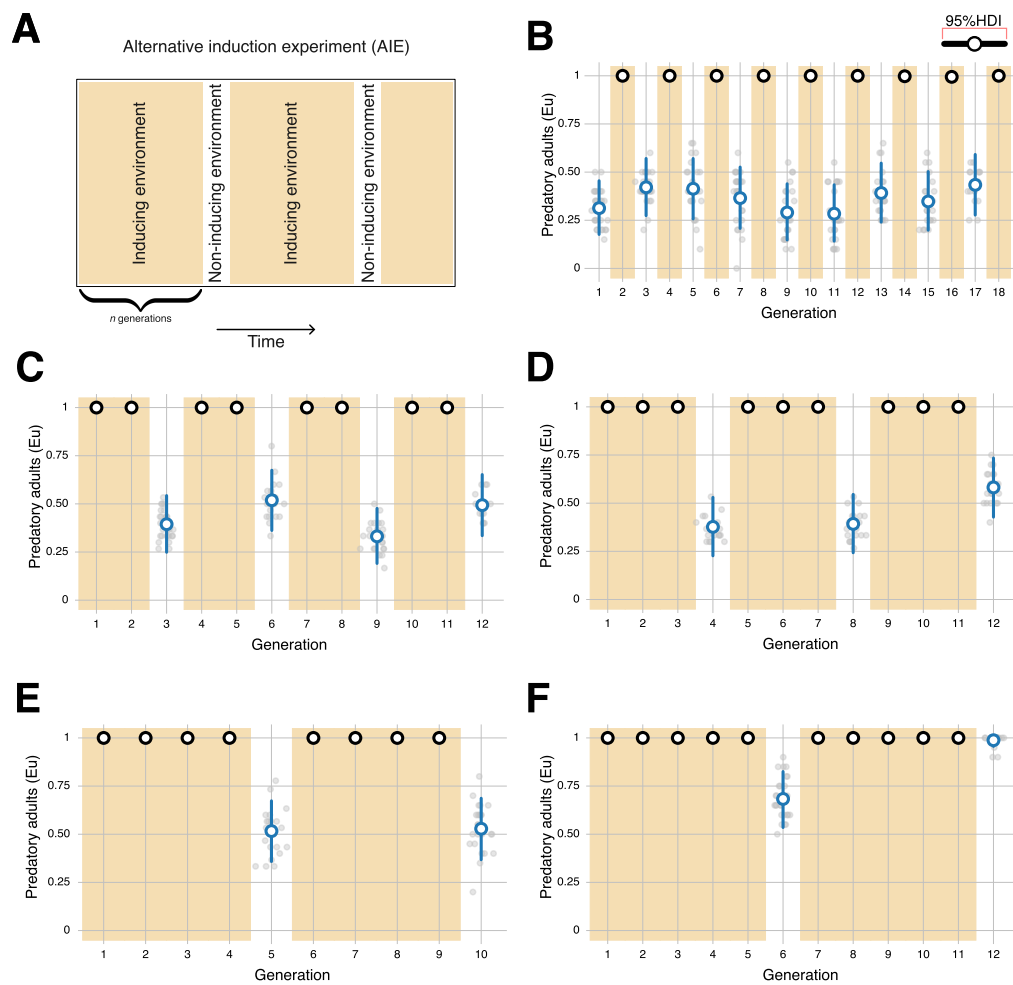


Figure 2

Figure 2 caption

(A) In the alternative induction experiment (AIE), nematodes are grown for n generations to the inducing environment (*Novosphingobium* L76), followed by a single generation of growth on the non-inducing environment (*Escherichia coli* OP50). This pattern is repeated during the experiment, to infer the dynamics of the TEI of mouth-form state as a function of environmental variability. A single hermaphrodite is used to establish new population in each generation, ensuring that the observed patterns are not due to selection. **(B)** AIE with $n = 1$ result in no accumulating of TEI over the course of the experiment, suggesting that in this scenario the TEI does not sensitize and instead resets by the removal of the environmental trigger. In AIE with $n = 2$ **(C)**, $n = 3$ **(D)**, and $n = 4$ **(E)**, the same constant response in TEI, independent of the duration of exposure to the environmental trigger, is observed. However, with $n = 5$ **(F)**, a sensitizing dynamics is observed, as the TEI is elevated after the second phase of exposure. In **(B) – (F)**, vertical lines indicate the 95% highest density interval (HDI) inferred using a bayesian model fitted to observed number of predatory adults out of 20 adults assayed per plate (see Methods). For each experiment in **(B) – (F)**, > 6000 nematodes were phenotyped.

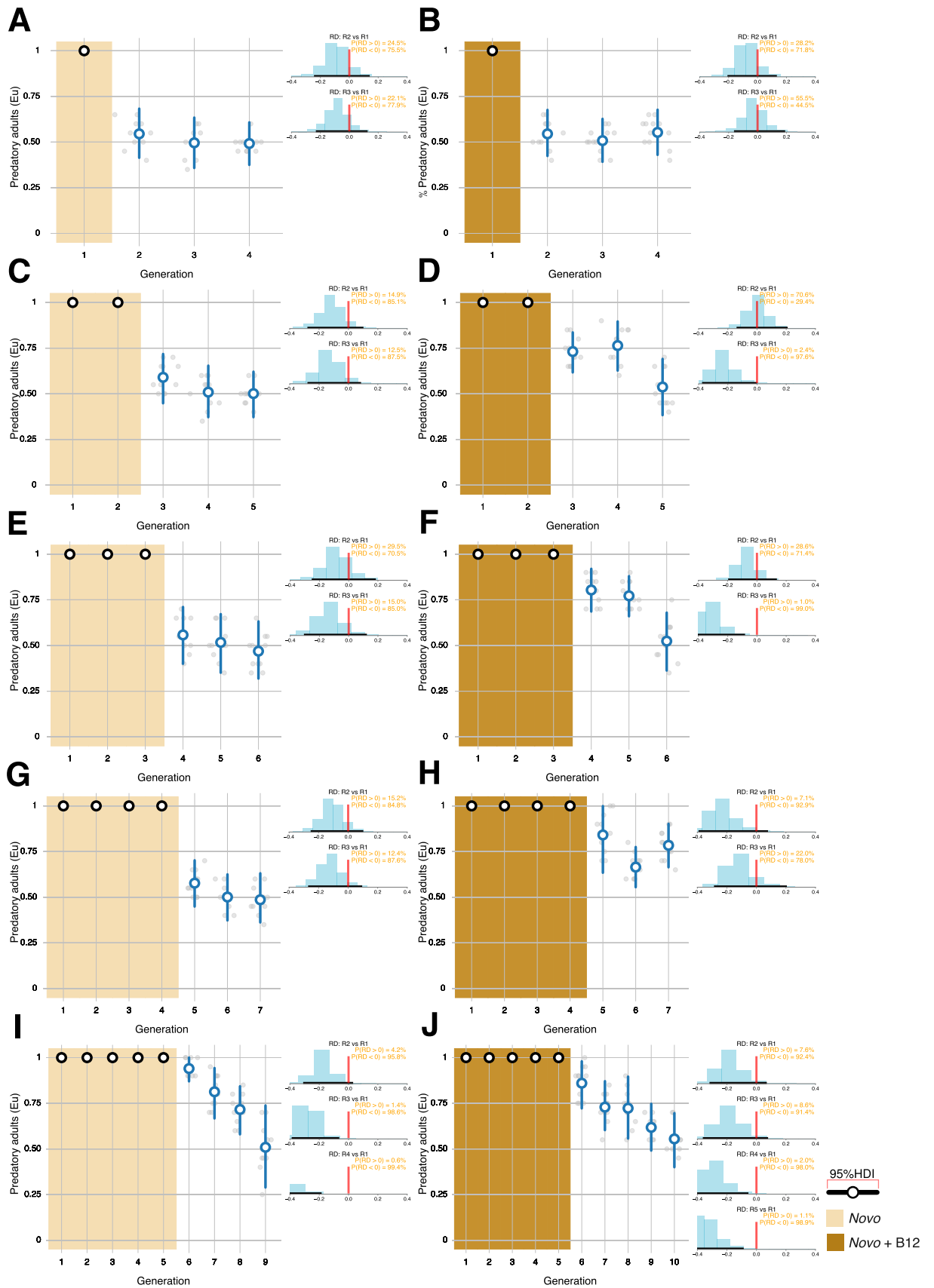


Figure 3

Figure 3 caption

The pattern of TEI in induction (**A**, **C**, **E**, **G**, and **I**) or superinduction experiments (**B**, **D**, **F**, **H**, and **J**). In each experiment, worms were propagated on *Novosphingobium* L76 (induction) or *Novosphingobium* L76 plus 1500nM vitamin B12 (superinduction) for n generations before being transferred back to the non-inducing environment (*E. coli* OP50). To illustrate the pattern of TEI in each experiment, we measured RD by comparing the posterior predictive for $\tilde{\theta}$ after m generations in the non-inducing environment (R_m) relative to that of the first generation grown on the non-inducing environment following induction or superinduction (R_1). The posterior predictive for RD for each experiment is shown as histograms, with the red line indicating zero –i.e., no difference– and the horizontal bar indicating the 95% HDI for RD for a given comparison. The orange text indicates the percentage of the distribution for RD that is lesser or greater than zero. For each experiment, between 1120 to 2280 nematodes were phenotyped.

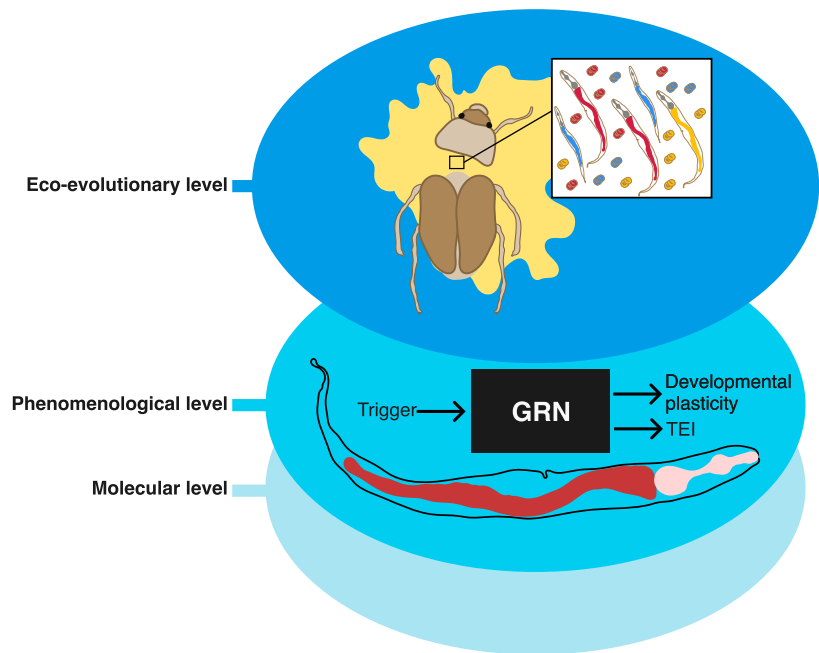


Figure 4

Figure 4 caption

Mouth-form polyphenism in *P. pacificus* provides a unique case where the molecular underpinnings of this case of developmental plasticity is tractable – given the wide array of tools available to study this phenomenon in a nematode. We have outlined an approach where hypothesized phenomenological models enable understanding the dynamics of TEI in *P. pacificus* using two experimental designs. Crucially, the observed dynamics is related to competition on a decaying beetle, suggesting a framework suitable to place developmental plasticity and TEI to its ecological context.

References

1. Brian G Dias and Kerry J Ressler. Parental olfactory experience influences behavior and neural structure in subsequent generations. *Nature Neuroscience*, 17(1):89–96, 2014. doi: 10.1038/nn.3594. URL <https://doi.org/10.1038/nn.3594>.
2. Qinghao Zhang, Weiwei Dang, and Meng C Wang. Lysosomes signal through the epigenome to regulate longevity across generations. *Science*, 389(6767):1353–1360, Sep 2025. ISSN 1095-9203 (Electronic); 0036-8075 (Linking). doi: 10.1126/science.adn8754.
3. Bastiaan T Heijmans, Elmar W Tobi, Aryeh D Stein, Hein Putter, Gerard J Blauw, Ezra S Susser, P Eline Slagboom, and L H Lumey. Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proc Natl Acad Sci U S A*, 105(44):17046–17049, Nov 2008. ISSN 1091-6490 (Electronic); 0027-8424 (Print); 0027-8424 (Linking). doi: 10.1073/pnas.0806560105.
4. Thomas E. Dickins and Qazi Rahman. The extended evolutionary synthesis and the role of soft inheritance in evolution. *Proceedings of the Royal Society B: Biological Sciences*, 279(1740):2913–2921, 2012. doi: 10.1098/rspb.2012.0273. URL <https://royalsocietypublishing.org/doi/abs/10.1098/rspb.2012.0273>.
5. Pim Edelaar, Jun Otsuka, and Victor J. Luque. A generalised approach to the study and understanding of adaptive evolution. *Biological Reviews*, 98(1):352–375, 2023. doi: <https://doi.org/10.1111/brv.12910>. URL <https://onlinelibrary.wiley.com/doi/abs/10.1111/brv.12910>.
6. Irene Adrian-Kalchhauser, Sonia E Sultan, Lisa NS Shama, Helen Spence-Jones, Stefano Tiso, Claudia Isabelle Keller Valsecchi, and Franz J Weissing. Understanding ‘non-genetic’ inheritance: insights from molecular-evolutionary crosstalk. *Trends in ecology & evolution*, 35(12):1078–1089, 2020.
7. Russell Bonduriansky and Troy Day. Nongenetic inheritance and its evolutionary implications. *Annual Review of Ecology, Evolution, and Systematics*, 40(Volume

-
- 40, 2009):103–125, 2009. ISSN 1545-2069. doi:
<https://doi.org/10.1146/annurev.ecolsys.39.110707.173441>. URL
<https://www.annualreviews.org/content/journals/10.1146/annurev.ecolsys.39.110707.173441>.
8. Adam Klosin and Ben Lehner. Mechanisms, timescales and principles of trans-generational epigenetic inheritance in animals. *Curr Opin Genet Dev*, 36: 41–49, Feb 2016. ISSN 1879-0380 (Electronic); 0959-437X (Linking). doi: 10.1016/j.gde.2016.04.001.
 9. Leah Hour-Zeevi, Yael Korem Kohanim, Olga Antonova, and Oded Rechavi. Three rules explain transgenerational small rna inheritance in *c. elegans*. *Cell*, 182(5):1186–1197.e12, 2020. ISSN 0092-8674. doi: <https://doi.org/10.1016/j.cell.2020.07.022>. URL <https://www.sciencedirect.com/science/article/pii/S0092867420309302>.
 10. Ralf J Sommer. Phenotypic plasticity: from theory and genetics to current and future challenges. *Genetics*, 215(1):1–13, 2020.
 11. Gilberto Bento, Akira Ogawa, and Ralf J Sommer. Co-option of the hormone-signalling module dafachronic acid–daf-12 in nematode evolution. *Nature*, 466(7305):494–497, 2010.
 12. James W Lightfoot, Martin Wilecki, Christian Rödelisperger, Eduardo Moreno, Vladislav Susoy, Hanh Witte, and Ralf J Sommer. Small peptide-mediated self-recognition prevents cannibalism in predatory nematodes. *Science*, 364(6435): 86–89, 2019.
 13. James W Lightfoot, Mohannad Dardiry, Ata Kalirad, Stefano Giaimo, Gabi Eberhardt, Hanh Witte, Martin Wilecki, Christian Rödelisperger, Arne Traulsen, and Ralf J Sommer. Sex or cannibalism: Polyphenism and kin recognition control social action strategies in nematodes. *Science Advances*, 7(35):eabg8042, 2021.
 14. Erik J Ragsdale, Manuela R Müller, Christian Rödelisperger, and Ralf J Sommer. A developmental switch coupled to the evolution of plasticity acts through a sulfatase. *Cell*, 155(4):922–933, 2013.

-
15. Bogdan Sieriebriennikov, Neel Prabh, Mohannad Dardiry, Hanh Witte, Waltraud Röseler, Manuela R Kieninger, Christian Rödelsperger, and Ralf J Sommer. A developmental switch generating phenotypic plasticity is part of a conserved multi-gene locus. *Cell Reports*, 23(10):2835–2843, 2018.
 16. Bogdan Sieriebriennikov, Shuai Sun, James W Lightfoot, Hanh Witte, Eduardo Moreno, Christian Rödelsperger, and Ralf J Sommer. Conserved nuclear hormone receptors controlling a novel plastic trait target fast-evolving genes expressed in a single cell. *PLoS genetics*, 16(4):e1008687, 2020.
 17. Michael S Werner, Tobias Loschko, Thomas King, Shelley Reich, Tobias Theska, Mirita Franz-Wachtel, Boris Macek, and Ralf J Sommer. Histone 4 lysine 5/12 acetylation enables developmental plasticity of *Pristionchus* mouth form. *nature communications*, 14(1):2095, 2023.
 18. Eleni Katsougia, Samantha J Connors, and Erik J Ragsdale. A species-specific nuclear receptor was integrated into a developmental polyphenism pathway. *Genetics*, 231(1):iyaf140, 07 2025. ISSN 1943-2631. doi: 10.1093/genetics/iyaf140. URL <https://doi.org/10.1093/genetics/iyaf140>.
 19. Nermin Akduman, James W Lightfoot, Waltraud Röseler, Hanh Witte, Wen-Sui Lo, Christian Rödelsperger, and Ralf J Sommer. Bacterial vitamin b12 production enhances nematode predatory behavior. *The ISME journal*, 14(6): 1494–1507, 2020.
 20. Mohannad Dardiry, Veysi Piskobulu, Ata Kalirad, and Ralf J Sommer. Experimental and theoretical support for costs of plasticity and phenotype in a nematode cannibalistic trait. *Evolution letters*, 7(1):48–57, 2023.
 21. Tess Renahan and Ralf J Sommer. Nematode interactions on beetle hosts indicate a role of mouth-form plasticity in resource competition. *Frontiers in Ecology and Evolution*, 9:752695, 2021.
 22. Natsumi Kanzaki, Matthias Herrmann, Christian Weiler, Waltraud Röseler, Tobias Theska, Jürgen Berger, Christian Rödelsperger, and Ralf J Sommer. Nine new *Pristionchus* (Nematoda: Diplogastridae) species from China. *Zootaxa*, 4943(1):zootaxa-4943, 2021.

-
23. Ralf J. Sommer. Pristionchus – beetle associations: Towards a new natural history. *Journal of Invertebrate Pathology*, 209:108243, 2025. ISSN 0022-2011. doi: <https://doi.org/10.1016/j.jip.2024.108243>. URL <https://www.sciencedirect.com/science/article/pii/S0022201124001861>.
 24. Ata Kalirad and Ralf J Sommer. The role of plasticity and stochasticity in coexistence. *Ecology Letters*, 27(2):e14370, 2024.
 25. Ata Kalirad and Ralf J Sommer. Spatial and temporal heterogeneity alter the cost of plasticity in *Pristionchus pacificus*. *PLOS Computational Biology*, 20(1):e1011823, 2024.
 26. Shiela Pearl Quiobe, Ata Kalirad, Waltraud Röseler, Hanh Witte, Yinan Wang, Christian Rödelsperger, and Ralf J Sommer. Ebax-1/zswim8 destabilizes mirnas, resulting in transgenerational inheritance of a predatory trait. *Science Advances*, 11(11):eadu0875, 2025.
 27. Shiela Pearl Quiobe, Ata Kalirad, Raphaela Zurheide, Hanh Witte, Christian Rödelsperger, and Ralf J. Sommer. Diet-derived vitamin b12 induces transgenerational inheritance of nematode predation through elevated vitellogenin provisioning. *bioRxiv*, 2025. doi: 10.1101/2025.05.21.655301. URL <https://www.biorxiv.org/content/early/2025/05/26/2025.05.21.655301>.
 28. Oriol Abril-Pla, Virgile Andreani, Colin Carroll, Larry Dong, Christopher J. Fonnesbeck, Maxim Kochurov, Ravin Kumar, Junpeng Lao, Christian C. Luhmann, Osvaldo A. Martin, Michael Osthege, Ricardo Vieira, Thomas Wiecki, and Robert Zinkov. PyMC: A modern and comprehensive probabilistic programming framework in Python. *PeerJ Computer Science*, 9(e1516), 2023. doi: 10.7717/peerj-cs.1516.
 29. Charles R. Harris, K. Jarrod Millman, Stéfan J. van der Walt, Ralf Gommers, Pauli Virtanen, David Cournapeau, Eric Wieser, Julian Taylor, Sebastian Berg, Nathaniel J. Smith, Robert Kern, Matti Picus, Stephan Hoyer, Marten H. van Kerkwijk, Matthew Brett, Allan Haldane, Jaime Fernández del Río, Mark Wiebe, Pearu Peterson, Pierre Gérard-Marchant, Kevin Sheppard, Tyler Reddy, Warren Weckesser, Hameer Abbasi, Christoph Gohlke, and Travis E. Oliphant. Array

-
- programming with NumPy. *Nature*, 585(7825):357–362, September 2020. doi: 10.1038/s41586-020-2649-2. URL <https://doi.org/10.1038/s41586-020-2649-2>.
30. John K Kruschke. Bayesian analysis reporting guidelines. *Nature human behaviour*, 5(10):1282–1291, 2021.
31. Pauli Virtanen, Ralf Gommers, Travis E. Oliphant, Matt Haberland, Tyler Reddy, David Cournapeau, Evgeni Burovski, Pearu Peterson, Warren Weckesser, Jonathan Bright, Stéfan J. van der Walt, Matthew Brett, Joshua Wilson, K. Jarrod Millman, Nikolay Mayorov, Andrew R. J. Nelson, Eric Jones, Robert Kern, Eric Larson, C J Carey, İlhan Polat, Yu Feng, Eric W. Moore, Jake VanderPlas, Denis Laxalde, Josef Perktold, Robert Cimrman, Ian Henriksen, E. A. Quintero, Charles R. Harris, Anne M. Archibald, Antônio H. Ribeiro, Fabian Pedregosa, Paul van Mulbregt, and SciPy 1.0 Contributors. SciPy 1.0: Fundamental Algorithms for Scientific Computing in Python. *Nature Methods*, 17:261–272, 2020. doi: 10.1038/s41592-019-0686-2.
32. Troy Day and Russell Bonduriansky. A unified approach to the evolutionary consequences of genetic and nongenetic inheritance. *The American Naturalist*, 178(2):E18–E36, 2011. doi: 10.1086/660911. URL <https://doi.org/10.1086/660911>. PMID: 21750377.
33. William Ross Ashby. *An introduction to cybernetics*. Wiley, New York, 1956.
34. Luciana Hannibal, Vegard Lysne, Anne-Lise Bjørke-Monsen, Sidney Behringer, Sarah C Grünert, Ute Spiekerkoetter, Donald W Jacobsen, and Henk J Blom. Biomarkers and algorithms for the diagnosis of vitamin b12 deficiency. *Front Mol Biosci*, 3:27, 2016. ISSN 2296-889X (Print); 2296-889X (Electronic); 2296-889X (Linking). doi: 10.3389/fmolb.2016.00027.
35. Seyed Mohammad Taghi Gharibzahedi and Zeynep Altintas. Optical bio/chemical sensors for vitamin b12 analysis in food and pharmaceuticals: state of the art, challenges, and future outlooks. *Beilstein Journal of Nanotechnology*, 16:2207–2244, 2025. ISSN 2190-4286. doi: 10.3762/bjnano.16.153. URL <https://doi.org/10.3762/bjnano.16.153>.

-
36. Caitlin McAndry, Alastair J. Wilson, Peter A. Cotton, and Manuela Truebano. Transgenerational plasticity research: Challenges and opportunities. *Functional Ecology*, 39(11):3083–3104, 2025. doi: <https://doi.org/10.1111/1365-2435.70139>. URL <https://besjournals.onlinelibrary.wiley.com/doi/abs/10.1111/1365-2435.70139>.
 37. Emilie C. Snell-Rood and Sean M. Ehlman. *Phenotypic Plasticity & Evolution: Causes, Consequences, Controversies*, chapter Ecology and Evolution of Plasticity. CRC Press, 1st edition, 2021.
 38. Luther Burbank. *The Training of the Human Plant*. The Century Co., New York, 1907. p. 68.

The logic of transgenerational inheritance of cannibalism

Table S1. Parameters used in three models of TEI (Figure 1C)

Component	Parameter	Value	Model(s)
<i>X Dynamics</i>	Production rate (α_X)	2.0	All
	Decay rate (β_X)	1.0	All
<i>Y Dynamics</i>	Production rate (α_Y)	1.5	All
	Decay rate (β_Y)	0.2	All
	Repression coefficient (K_{XY})	0.3	All
	Hill coefficient (n_{XY})	3.0	All
<i>Z Dynamics</i>	Base production rate (α_Z)	1.5	All
	Base decay rate (β_Z)	0.5	All
<i>Resource (R)</i>	Recovery timescale (τ_R)	25.0	All
	Depletion rate (k_R)	0.3	All
<i>Trigger</i>	Pulse period (T_{period})	15.0	All
	Pulse width	4.0	All
<i>Acclimation (A)</i>	Production rate (k_A)	0.2	Acclimating TEI
	Decay timescale (τ_A)	30.0	Acclimating TEI
	Repression strength (γ_A)	2.0	Acclimating TEI
<i>Sensitization (S)</i>	Production rate (k_S)	0.2	Sensitizing TEI
	Decay timescale (τ_S)	40.0	Sensitizing TEI
	Sensitization strength (c_S)	3.0	Sensitizing TEI

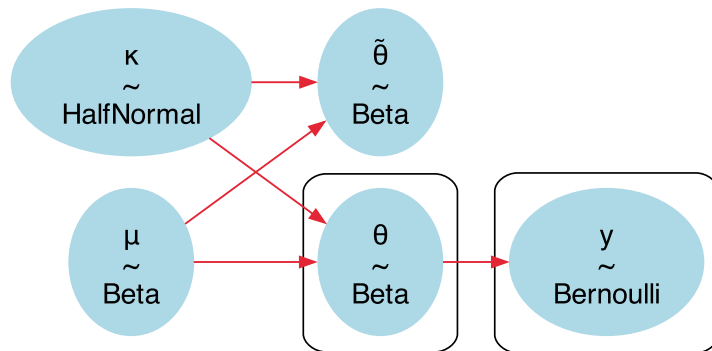
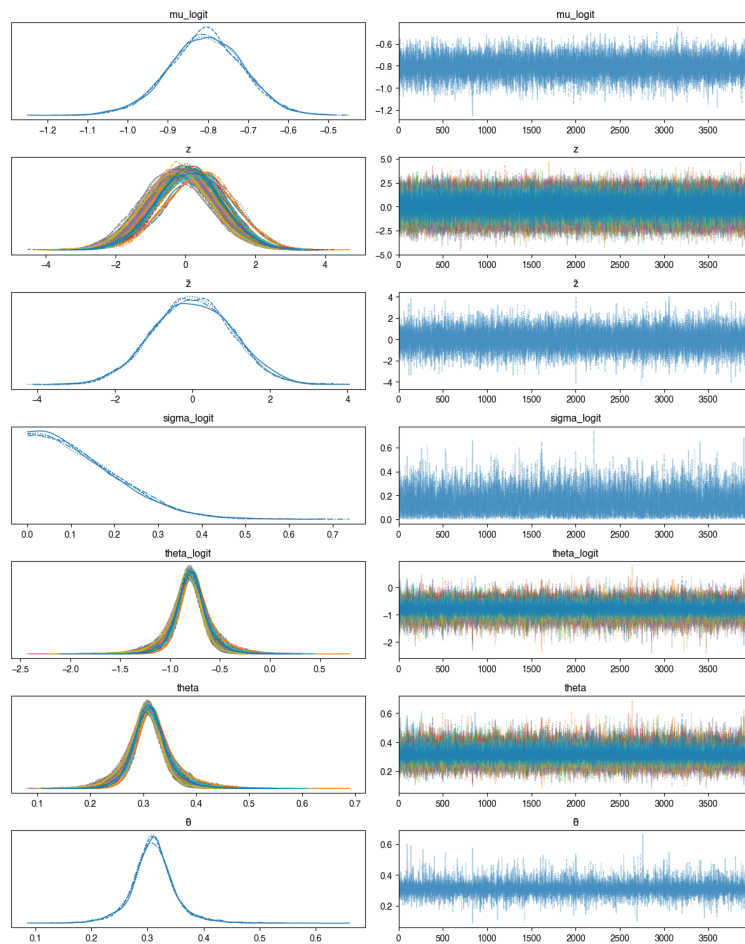
A**B****Figure S1**

Figure S1 caption

(A) The structure of the bayesian hierarchical model (equations (1) and (2)). The number of predatory adults per plate is used as the input, resulting in inference of θ_i for plate i . **(B)** An example of the typical output of the model, visualized using ArviZ 0.21., which represents the posterior density plots (left column) for chains, including for parameters that are estimated on the plate level, and the trace plots (right columns) indicating the convergence of the model.

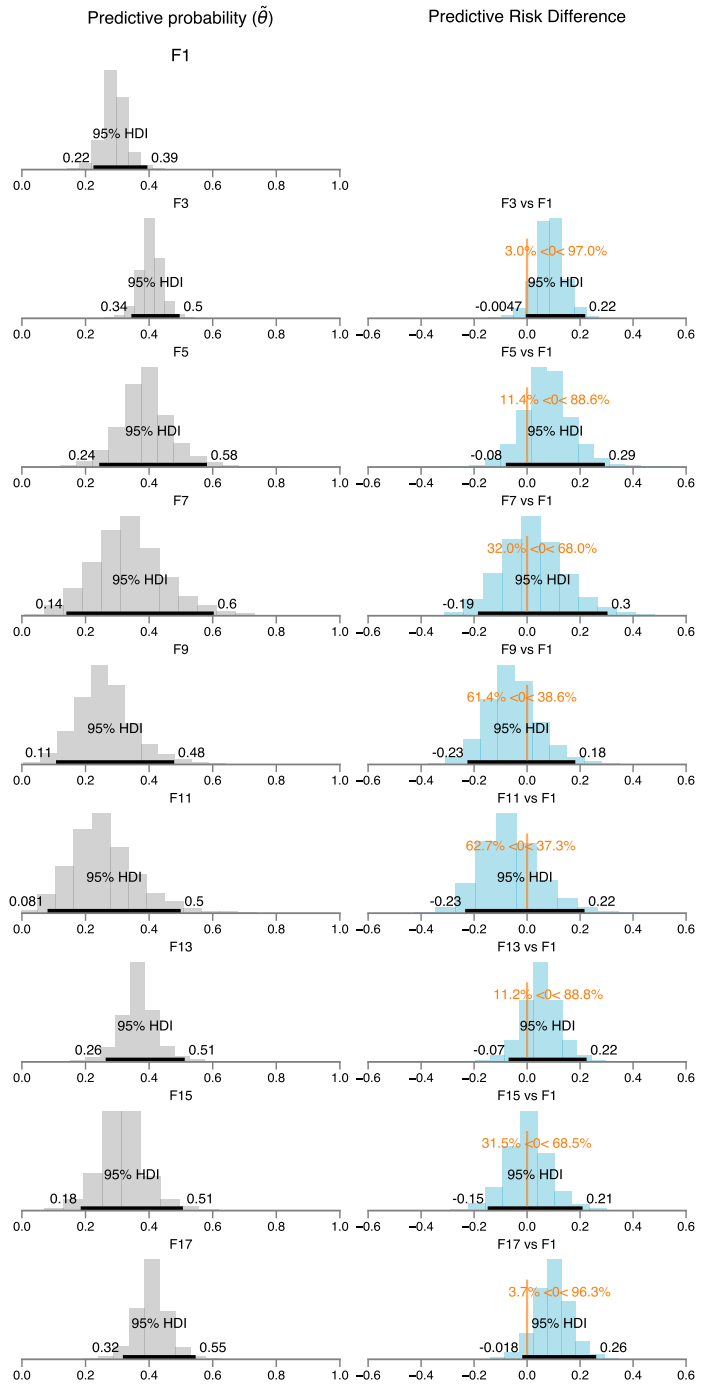


Figure S2

Figure S2 caption

The expanded analysis for the AIE experiment shown in Figure 2B. The left column visualizes the posterior predictive of $\tilde{\theta}$ for each generation Fi . The right column indicates the predictive RD, calculated by subtracting the inferred $\tilde{\theta}$ for each generation from that of the first generation (F1). The orange text indicates the percentage of the distribution for RD that is lesser or greater than zero. The black horizontal bar indicates the 95% HDI for each parameter.

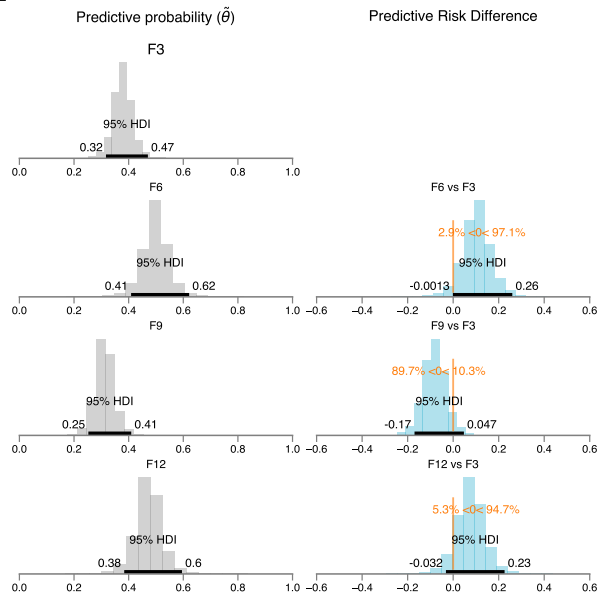
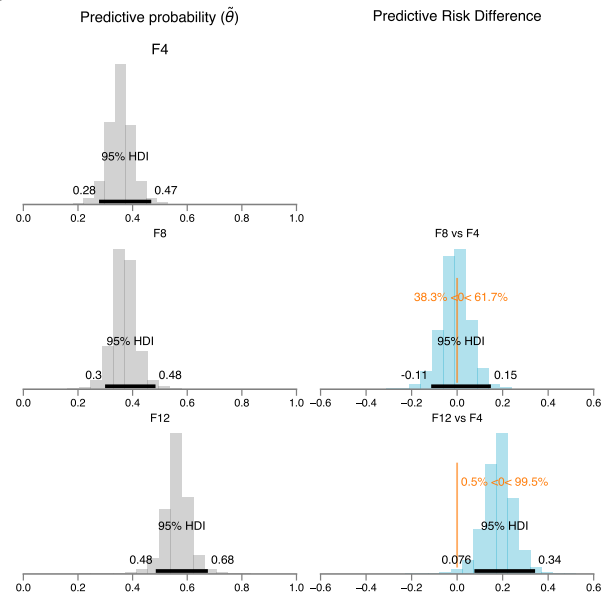
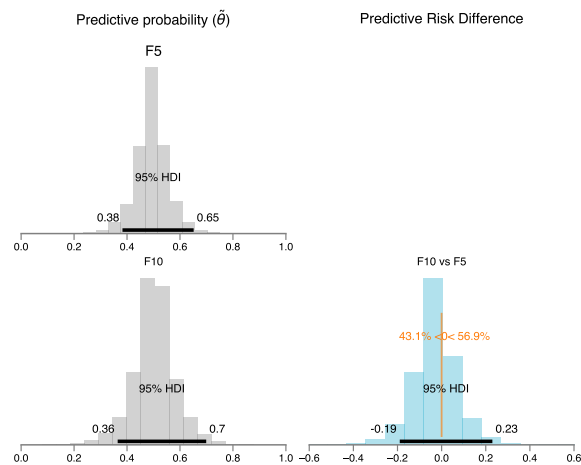
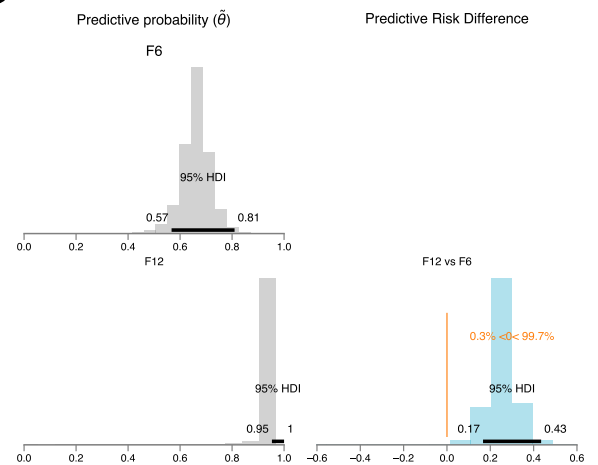
A**B****C****D****Figure S3**

Figure S3 caption

The expanded analysis for the AIE experiment shown in Figure 2C (**A**), Figure 2D (**B**), Figure 2D (**C**), and Figure 2E (**D**). In each subplot, the left column visualizes the posterior predictive of $\tilde{\theta}$ for each generation Fi . The right column indicates the predictive RD, calculated by subtracting the inferred $\tilde{\theta}$ for each generation from that of the first generation (F1). The orange text indicates the percentage of the distribution for RD that is lesser or greater than zero. The black horizontal bar indicates the 95% HDI for each parameter.

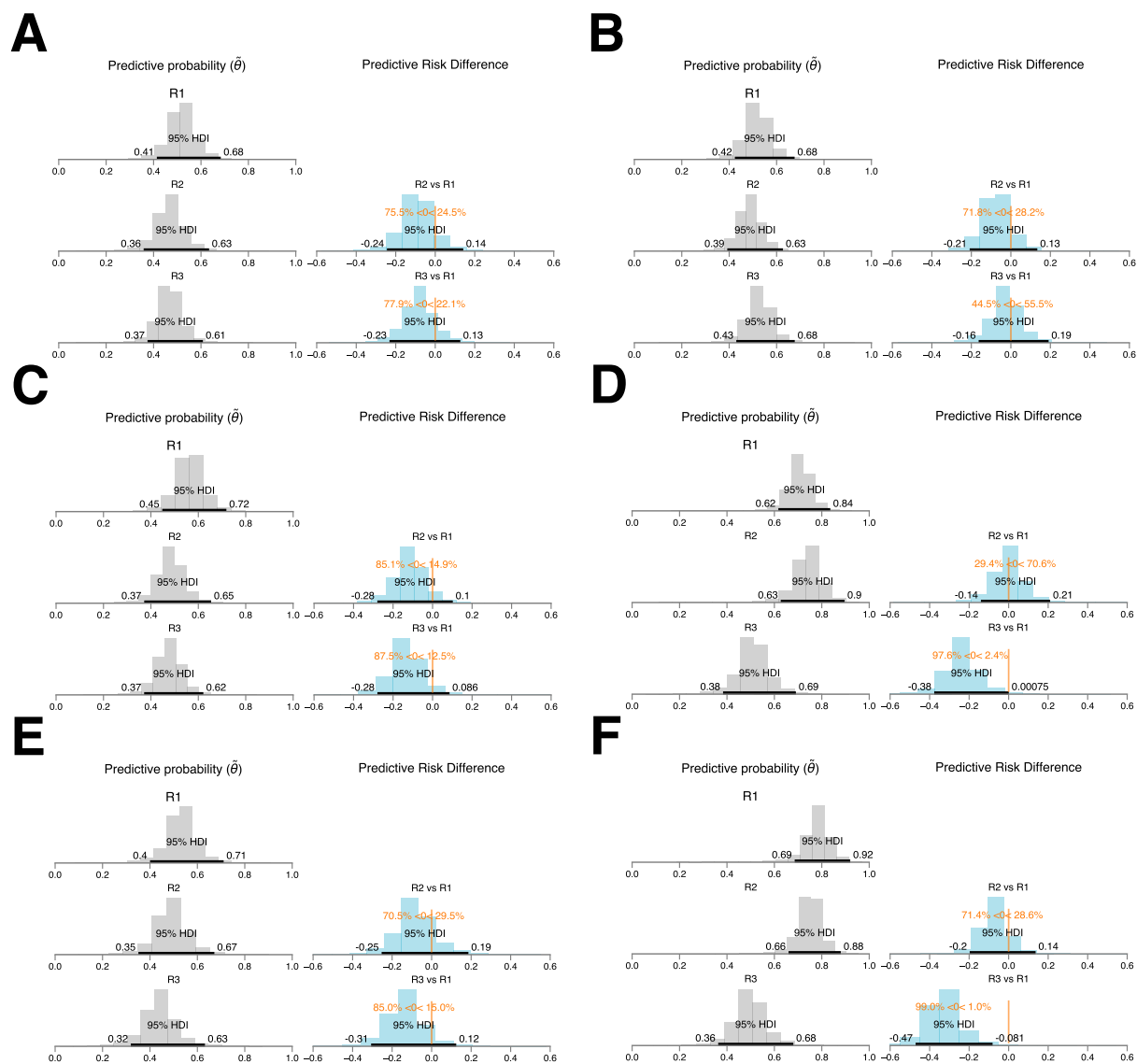


Figure S4

Figure S4 caption

The expanded analysis for the superinduction experiment shown in Figure 3A–F. In each subplot, the left column visualizes the posterior predictive of $\tilde{\theta}$ for each generation in the non-inducing environment Ri following j generation under induction (*Novosphingobium* L76) (**A**, **C**, **E**) or superinduction (*Novosphingobium* L76 +1500nM vitamin B12) (**B**, **D**, **F**). The right columns indicate the predictive RD, calculated by subtracting the inferred $\tilde{\theta}$ for generation i in the non-inducing environment from that of the first generation after transfer to the non-inducing environment (R1). The orange text indicates the percentage of the distribution for RD that is lesser or greater than zero. The black horizontal bar indicates the 95% HDI for each parameter.

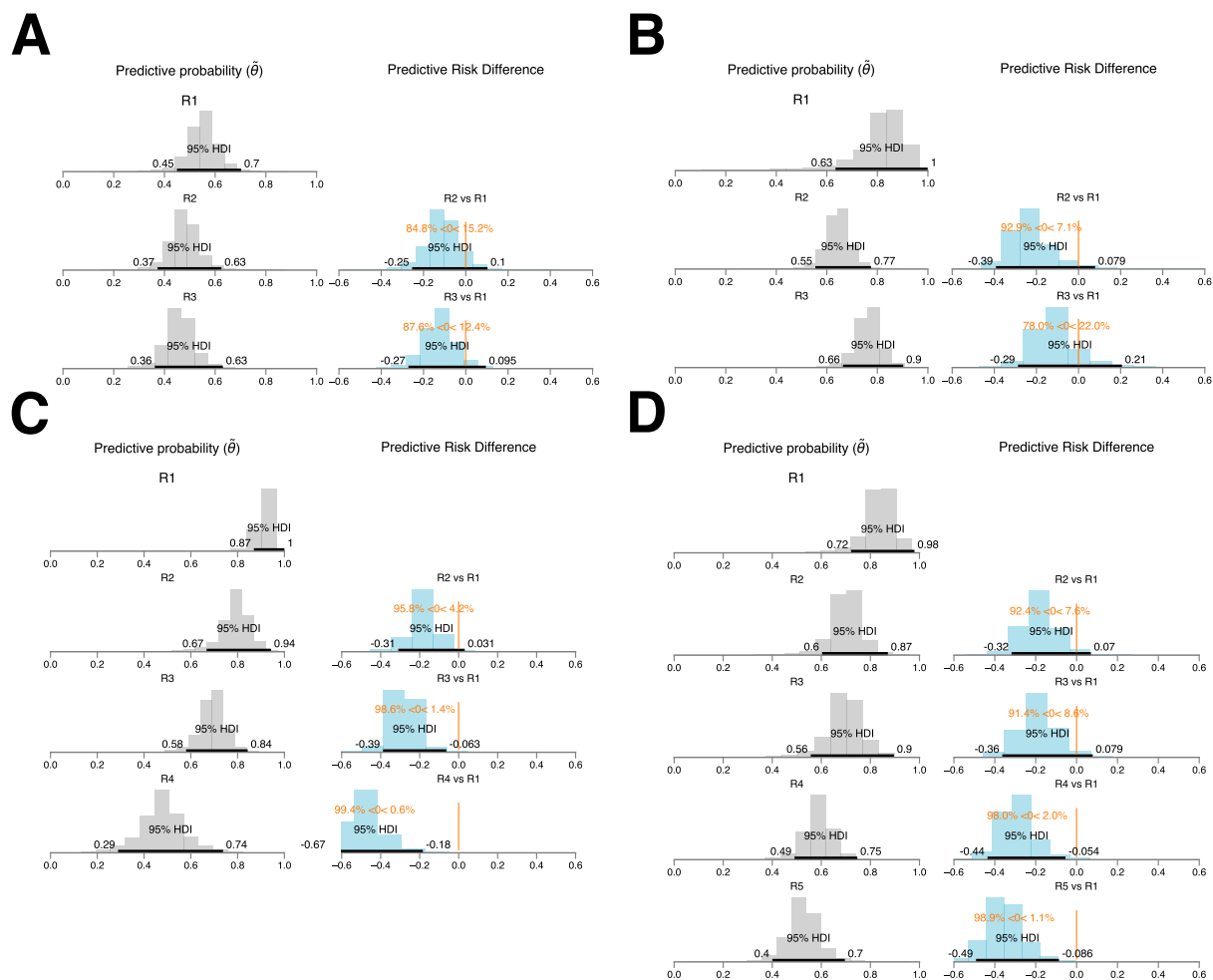


Figure S5

Figure S5 caption

The expanded analysis for the superinduction experiment shown in Figure 3G–J. In each subplot, the left column visualizes the posterior predictive of $\tilde{\theta}$ for each generation in the non-inducing environment Ri following j generation under induction (*Novosphingobium* L76) (**A** and **C**) or superinduction (*Novosphingobium* L76 +1500nM vitamin B12) (**B**, and **D**). The right columns indicate the predictive RD, calculated by subtracting the inferred $\tilde{\theta}$ for generation i in the non-inducing environment from that of the first generation after transfer to the non-inducing environment (R1). The orange text indicates the percentage of the distribution for RD that is lesser or greater than zero. The black horizontal bar indicates the 95% HDI for each parameter.

Darwinian games in a cannibalistic polyphenism and its transgenerational inheritance

Ata Kalirad, **Shiela Pearl Quiobe**, Ralf J. Sommer

Under Review in *Nature Communications*

Darwinian games in a cannibalistic polyphenism and its transgenerational inheritance

Ata Kalirad ¹, Shiela Pearl Quiobe ¹, Ralf J. Sommer^{1,*}

1 Department for Integrative Evolutionary Biology, Max Planck Institute for Biology Tübingen, 72076 Tübingen, Germany

*** ralf.sommer@tuebingen.mpg.de**

ORCID: 0000-0002-9500-3903 (AK)

ORCID: 0000-0002-6261-2657 (SPQ)

ORCID: 0000-0003-1503-7749 (RJS)

Abstract

The adaptive consequences of non-genetic inheritance in ecology and evolution remain disputed, a state of affair largely stemming from a paucity of tractable model organisms that would both reliably exhibit phenotypic plasticity in an ecologically-relevant trait and exhibit associated non-genetic inheritance. We combine experimental and game-theoretical approaches to address this question using *P. pacificus*, a nematode characterized by developmental plasticity in a cannibalistic trait that can be epigenetically inherited for multiple generations. We show that the epigenetic inheritance is accompanied by an elevated fecundity. Furthermore, we develop a theoretical model based on the Darwinian dynamics framework to explore the eco-evolutionary consequence of mouth-form polyphenism in *P. pacificus* when considering tradeoffs between plasticity and fecundity. Together, this integrative experimental and theoretical approach present a unique case study on how plasticity could be placed in the broader eco-evolutionary context.

Keywords: Developmental plasticity, adaptive value, life-time reproductive success, tradeoff, Darwinian dynamics

Introduction

Non-genetic inheritance of environmental effects across generations are increasingly recognized as widespread in nature. However, the evolutionary importance of such non-genetic inheritance, especially when it lasts three generations or more – i.e., transgenerational epigenetic inheritance (TEI) (1) – is contentious, since its adaptive value, often assumed to be intertwined with tradeoffs, is hard to measure (2–4). Theoretically, the evolution of TEI is linked to the degree of environmental variability, where relatively slower rate of change favors non-genetic inheritance across generations (5). However, despite the growing body of literature on TEI (1, 6–8) – in the absence of information on the fitness consequence and tradeoffs resulting from it – inference of the eco-evolutionary consequence of TEI remains elusive.

Pristionchus pacificus, an androdioecious hermaphroditic nematode, provides an ideal case study to explore the eco-evolutionary consequence of TEI. *P. pacificus* has been established as a plasticity model organism given its mouth-form polyphenism, with a life-cycle that in nature is defined by its association with Scarab beetles (9). Mouth-form polyphenism in *P. pacificus* is characterized by the effect of various environmental conditions – including bacterial diet – on the bias of the decision taken by the gene regulatory network that underpins the development of one of two possible mouth forms in the adults: the stenostomatous (St) morph with a narrow mouth and single dorsal tooth or the eury stomatous (Eu) form with a wide mouth and two teeth (10, 11) (Figure 1A). The predatory Eu adults can kill pre-adult stages of competing nematodes (12), resulting in life-history intraguild predation (13). The decision of this bi-stable switch is intrinsically stochastic, i.e., the development of either of the two mouth forms in genetically-identical individuals in a given environment can be best described as a binomial process with genotype-specific probabilities for a given condition (14, 15). The molecular details of the gene regulatory network responsible for the mouth-form polyphenism has been rigorously studied (16–18), including the

elucidation of the epigenetic mechanisms involved (19, 20).

We have previously shown the effect of diet on the induction of the predatory Eu mouth form in *P. pacificus* and its subsequent TEI in the absence of the environmental cue (21) (Figure 1B and 1C). The influence of bacterial diet on mouth-form polyphenism is mediated via the ubiquitin ligase EBAX-1/ZSWIM8 and the heat shock protein Hsp90 and the TEI following the change in diet is attributed to the destabilization of clustered microRNAs (21). Subsequently, we have shown that bacterial-derived vitamin B12 is necessary and sufficient to induce the TEI of the predatory mouth form (22).

Understanding the eco-evolutionary importance of TEI is a daunting task as multiple criteria must be met to enable addressing such question. For example, Baugh and Day (1) proposed that to understand the adaptive value for a given TEI, (i) it should be unequivocally shown that the effect is reliably inherited for multiple generations in the absence of the cue, (ii) its underlying mechanism should be explained, and (iii) its fitness consequence should be shown. The last criterion itself is challenging, since tradeoffs between plastic response and other traits are expected to exist, thus complicating a reliable assessment of the fitness consequence of TEI (6).

The characteristics of diet-induced mouth-form polyphenism in *P. pacificus* makes it well-suited to study the fitness consequence of TEI. Specifically, the diet-induced mouth-form polyphenism and the subsequent TEI of the predatory Eu mouth form are both understood at the molecular level, and a major component of fitness – i.e., life-time reproductive success (LRS) (23) – can be measured across conditions in *P. pacificus* given its short generation time (3–4 days at 20°C) and simple husbandry on monoxenic bacterial diet. Here, we experimentally illustrate the relationship between the induction of the predatory Eu mouth form and the resulting TEI with LRS. To illustrate the eco-evolutionary consequence of TEI in *P. pacificus*, we construct a modeling approach based on the Darwinian dynamics framework to study the interplay between environmental variability – an expected feature of the natural habitats in which *P. pacificus* resides (9) – and the competition between different strategies of TEI. This study presents a novel and integrative contribution to the literature on the adaptive

value of phenotypic plasticity and TEI.

Methods

Bacterial and nematode culture maintenance

The ancestral *P. pacificus* RSC011 isolate was cryopreserved within the first 10 generations after its initial collection from Coteau Kerveguen, La Réunion. Worms were reared at 20°C under standard conditions on Nematode Growth Medium (NGM) plates seeded with either *E. coli* OP50 or *Novosphingobium* L76. Bacterial strains were cultured overnight in Lysogeny Broth (LB) with kanamycin (50µg/ml) when required. Specifically, OP50 was grown overnight at 37°C in LB without shaking while *Novosphingobium* L76 was grown at 30°C at 180 rpm. NGM plates were seeded with 300µl of overnight cultures and incubated for 2 days prior to use.

Dietary induction and food reversals

Dietary induction on *Novosphingobium* and subsequent reversal experiments on *E. coli* OP50 were based on the long-term environmental induction (LTEI) protocol described previously (21). For this study, worms were propagated on *Novosphingobium* for 5 and 10 generations, respectively. For food reversals after the F5 and F10 generations, worms were first transferred from *Novosphingobium* L76 to NGM plates supplemented with (50µg/ml) of kanamycin with a kanamycin resistant *E. coli* strain as food for one generation, before being reintroduced to standard *E. coli* OP50-seeded NGM plates for subsequent generations. Reversal experiments were carried out for 3- 4 generations following the initial *Novosphingobium* exposure.

Assays for lifetime reproductive success

To obtain synchronized worm cultures, a bleaching process is widely used in protocols for assessing brood size. However, exposure to stressors such as bleaching, starvation, or extreme temperature fluctuation are known to directly alter the mouth-form phenotype. Therefore, to avoid effects that could directly or indirectly influence life-history phenotypes, no bleaching was performed. To assess lifetime reproductive success, J4

animals were individually transferred on separate plates with 75 μ l of bacterial lawn (Figure 2A). Upon reaching adulthood, individual worms were transferred daily to fresh plates for four days. Almost 90% of self-progeny are laid within the first 3 days. Worms were allowed to lay eggs on the day-4 plates for several additional days to provide an accurate estimate for the remaining $\sim 10\%$ of self-progeny. For LRS assays, 50 – 60 independent replicates (mothers) were measured for each generation.

Statistical analysis

To analyze LRS data, we implemented a Bayesian model using PyMC version 5.16.2 (24). For LRS for a given generation, we implemented a bayesian negative binomial regression. In this model, the total number of viable offspring of mother i (Y_i) is drawn from a negative binomial distribution (Figure S1):

$$Y_i \sim \text{NegativeBinomial}(\mu_i, \alpha) \quad . \quad (1)$$

To compare any given generation to the naive P0 generation with m other generations, we defined

$$\log(\mu_i) = \beta_0 + \sum_{k=1}^m \beta_k X_{i,k} \quad , \quad (2)$$

where $X_{i,k}$ is the binary indicator for mother i belonging to treatment group k . The following priors were used for this model:

$$\begin{aligned} \beta_0 &\sim \text{Normal}(5, 2) \\ \beta_k &\sim \text{Normal}(0, 2) \quad \text{for } k \in \{1, \dots, m\} \\ \alpha &\sim \text{Exponential}(1) \quad . \end{aligned} \quad (3)$$

To compare the change in LRS relative to P0, rate ratio (RR) for generation k was calculated:

$$\text{RR}_k = \exp(\beta_k) \quad . \quad (4)$$

The effect of the mouth form of a mother on its LRS was calculated using daily brood size. The brood size of mother i on day j is drawn from a negative binomial

distribution (Figure 2):

107

$$Y_{ij} \sim \text{NegativeBinomial}(\mu_{ij}, \alpha_j) \quad . \quad (5)$$

We defined the following link function

108

$$\log(\mu_{ij}) = \beta_{0,j} + \beta_{1,j}X_i \quad , \quad (6)$$

where X_i is the binary indicator, which reflects if the mother had a predatory

109

($X_i = 1$) or non-predatory ($X_i = 0$) mouth form. $\beta_{0,j}$ represents the expected log-brood

110

size for the St condition, and $\beta_{1,j}$ is the condition effect (the log-rate ratio). The

111

following priors were used in this model:

112

$$\beta_{0,j} \sim \text{Normal}(5, 2)$$

$$\beta_{1,j} \sim \text{Normal}(0, 2)$$

$$\alpha_j \sim \text{Exponential}(1) \quad .$$

The effect of predatory (Eu) mouth form on the brood size on day j was calculated

113

as

114

$$\text{RR}_j = \exp(\beta_{1,j}) \quad . \quad (7)$$

Posterior inference was performed using No-U-Turn Sampler (NUTS) by sampling 4

115

chains with 2000 tuning iterations followed by 4000 sampling iterations.

116

Eco-evolutionary model

117

In order to understand how the trade-off between fecundity and the induction of the

118

predatory mouth form would affect the eco-evolutionary dynamics, we developed an

119

approach based on the Darwinian dynamics framework (25). Each *P. pacificus* isolate i

120

in the community consists of three classes:

121

- J_i : juveniles,

122

- P_i : Adults with the Eu mouth form, 123
- N_i : Adults with St mouth form, with $A_i = P_i + N_i$. 124

For a community composed of s isolates the density of various classes in the 125
community is a vector: 126

$$\mathbf{X} = [x_{J_0}, x_{P_0}, x_{N_0} \mid \dots \mid x_{J_s}, x_{P_s}, x_{N_s}] \quad , \quad (8)$$

where x_{l_m} is the abundance of class l of isolate m . 127

We assume that nematodes consume resource y (bacterial food), which continuously 128
flows into the system, i.e., bacteria regrows: 129

$$\dot{y} = \rho(K - y) - \sum_{i=1}^s \left(c_{J,i} f(y) x_{Ji} + c_{A,i} f(y) x_{Ai} \right) \quad , \quad (9)$$

where ρ is the rate of resource regeneration, K is the maximum resource level, and 130
 $c_{l,m}$ is the consumption rate of stage l of isolate m . Resource accessibility, $f(y)$ is 131
modeled as a Monod function: 132

$$f(y) = \frac{y}{H + y} \quad , \quad (10)$$

where H is the half-saturation constant. 133

Each isolate i in the community possess a strategy u_i in a given environment. For a 134
community composed of s isolate, strategies found in the community can be represented 135
as a vector: 136

$$\mathbf{u} = [u_0 \mid \dots \mid u_s] \quad . \quad (11)$$

Strategy u_i of isolate i determines two key aspects of its phenotype: (i) the 137
proportion of predatory adults developed by individuals belonging to this isolate (p_i), 138
which is determined by 139

$$p(u_i) = \frac{1}{1 + \exp[-\sigma u_i]} \quad , \quad (12)$$

and (ii) the tradeoff between predatory mouth form and fecundity of this isolate (b_i), 140
represented by 141

$$b(u_i) = b_0 [1 - \kappa p_i(u_i)] \quad \text{where } 0 < \kappa < 1 \quad , \quad (13)$$

where κ determines the intensity of the tradeoff.

The mouth-form polyphenism results in life-history intraguild predation by predatory adults in *P. pacificus*. This aspect is modeled as type-II functional response:

$$\Phi_{ji}(x) = \frac{\alpha_{ji} x_{Pj} x_{Ji}}{1 + \sum_{m=1}^s h_{jm} \alpha_{jm} x_{Jm}} \quad , \quad (14)$$

where $\phi_{ji}(x)$ is the total killing rate of the predatory adults of isolate j on juveniles of isolate i , α_{ji} is the attack rate, and h_{jm} is the handling time.

For a given isolate i , a G-matrix is defined that describes the per capita growth rate of individuals belonging to this isolate:

$$\mathbf{G}_i(u_i, \mathbf{u}, \mathbf{x}) = \begin{pmatrix} -\lambda f(y) - \delta_J - \sum_{k=1}^s \Phi_{ki}(x) & b(u_i) f(y) & b(u_i) f(y) \\ p(u_i) \lambda f(y) & -\delta_A & 0 \\ (1 - p(u_i)) \lambda f(y) & 0 & -\delta_A \end{pmatrix} \quad . \quad (15)$$

Transgenerational inheritance

For environment e , there exist a target strategy $u_i^*(e)$ for species i . In the absence of TEI, following induction, reversal to the non-inducing environment would immediately result in the assumption of $u_i^*(e)$ by species i . Assuming TEI, the strategy would slowly revert to the target strategy:

$$\dot{u}_i = \frac{u_i^*(e) - u_i}{\tau_m} \quad m \in \{\text{down, up}\} \quad , \quad (16)$$

where τ_m determines the pace at which the predatory trait is induced in the new environment (τ_{up}) and the duration of TEI of the induced trait (τ_{down}).

Pairwise invasion assay

The fitness of a mutant strategy that invades a resident strategy can be formulated as the Jacobian matrix $\mathbf{M}$:

$$\mathbf{M} = \begin{pmatrix} -(\lambda f(y) + d_J + \Phi_{res}) & b(u_{mut})f(y) & b(u_{mut})f(y) \\ p(u_{mut})\lambda f(y) & -\delta_A & 0 \\ (1 - p(u_{mut}))\lambda f(y) & 0 & -\delta_A \end{pmatrix}, \quad (17)$$

where Φ_{res} is the total killing rate of the predatory adults of the resident species on juveniles of the mutant species. The invasion fitness is defined as the spectral abscissa of the mutant strategy's Jacobian matrix $\mathbf{M}$:

$$\alpha(\mathbf{M}) = \max \{\text{Re}(\lambda)\} \quad . \quad (18)$$

Results

TEI following exposure to *Novosphingobium* diet is associated with increase in brood size

In the nematode androdioecious hermaphrodites *C. elegans* and *P. pacificus*, total brood size after self-fertilization represents the most commonly used proxy for fitness. As the total offspring of a hermaphrodite is nearly exclusively hermaphroditic and can therefore reproduce itself, brood size is directly related to the reproductive value. In such hermaphrodites, this measurement is equivalent to the lifetime reproductive success (LRS), which represents the total number of viable offspring an individual produces throughout its entire life.

To explore the potential relationship between LRS – as a major component of fitness – and the dietary induction of the predatory (Eu) mouth form in the *P. pacificus* isolate RSC011, as well the subsequent TEI of this trait, we designed an experiment in which naive nematodes (P0) – previously propagated on the standard *E. coli* OP50 food – were grown on *Novosphingobium* L76 for 10 generations (Figure 2A). Two sets of reversal lines were established during the experiment, in which nematodes, having been propagated on *Novosphingobium* L76 for five or 10 generations were transferred back to the standard *E. coli* OP50 diet and maintained for multiple generations (Figure 2A). During this experiment, we singled out J4 hermaphrodites and used them to conduct

LRS assay, in which a single worm is transferred to a fresh plate every 24 hours and the viable progenies laid on the plates are counted (Figure 2A).

The LRS assays conducted during induction on *Novosphingobium* L76 – previously shown to induce an immediate and complete induction of the predatory mouth form in *P. pacificus* RSC011 (21) – indicated steady increase in LRS compared to naive worms (Figure 2B). Surprisingly, immediately after reversal to the standard *E. coli* OP50 diet following five generations of exposure to *Novosphingobium* L76, further increase in LRS was observed, which declined in subsequent generations on *E. coli* OP50 (Figure 2B), a decline that mimics the pattern of TEI of the predatory mouth form in *P. pacificus* RSC011 within a four-generations time frame (Figure 1C). A similar pattern was observed when conducting LRS assays during 10 generations of induction of *Novosphingobium* L76 and subsequent reversal to *E. coli* OP50 (Figure 2C).

TEI-associated increase in brood size is not dependent on the maternal mouth form

Given the pattern of change in LRS observed during reversals following exposure to *Novosphingobium* L76, which mirrored the pattern of TEI of the predatory mouth form, one could hypothesize that the effect on LRS is directly linked to the induction of the predatory mouth form. To test this hypothesis, we designed an experiment in which, following five generations of exposure to *Novosphingobium* L76, nematodes were transferred to *E. coli* OP50 diet for three generations. In each of the three generations during the reversal phase, predatory and non-predatory hermaphrodites were singled out and were used to conduct the LRS assay. This design allowed us to directly test if the mouth form of the mother determines its LRS following reversal (Figure 3A). The first generation during the reversal phase suggests minor difference in LRS between predatory and non-predatory mothers (Figure 3B). However, the paucity of non-predatory nematodes immediately following five generations of exposure to *Novosphingobium* L76 grants limited statistical power. In contrast, the second and third generation during the reversal phase of our experiment, when enough non-predatory mothers were collected, no credible difference was observed in LRS when comparing

mothers with different mouth forms (Figure 3C and D). These results suggest that the observed patterns of change in LRS cannot be directly linked to the development of the predatory mouth form and are more easily attributed to the broader effect of the bacteria-derived trigger molecule on the nematode.

Mouth-form polyphenism is characterized by a major tradeoff

Our experimental observations indicate an interplay between mouth-form polyphenism and fecundity, including the transgenerational plasticity in both traits. However, this comparison, conducted on a given isolate, does not elucidate why different wild isolates of *P. pacificus* reflect a diversity in the level of induction of the predatory mouth form in a given environment (11, 14). We have previously shown how the level of induction of the predatory mouth form is accompanied by a tradeoff in LRS (14). However, the scope of our attempt was limited to exploring the effect of mouth-form polyphenism on fecundity, and not the subsequent transgenerational plasticity in fecundity. To explore these aspects, we turn to a game-theoretical model based on the Darwinian dynamics framework (Figure. 4A and B). Specifically, this framework enables a natural inclusion of tradeoff between the probability of developing the predatory mouth form and fecundity (Figure 4C). Our goal to develop these theoretical framework is two-fold: firstly, to extend our single isolate experiment to many isolates and potentially species in order to provide insights that could inform future experimental designs, and, secondly, provide a theoretical framework to explore both phenotypic developmental plasticity and transgenerational plasticity, which remains under-explored.

Strategies that prefer lower probability of the predatory mouth form evolve in polymorphic communities

To explore how strategies that determine the probability of assuming the predatory mouth form evolve, we envisioned a polymorphic community in which 21 different *P. pacificus* isolates – each with a distinct strategy $u_i \in [-6, 6]$ – compete (Figure 4D). Strategies that win in this scenario are not necessarily evolutionary stable – since they can be susceptible to invasion. However, they indicate strategies that evolution favors when other strategies are available. Our polymorphic scenario illustrates to what extent

a winning strategy with respect to mouth-form polyphenism in *P. pacificus* depends on the strength of tradeoff between the effect of u_i on the proportion of predatory adults of an isolate and its negative effect on the fecundity. For example, weak tradeoff prefers obligate predators since the benefit gained via LHIP dominates (Figure 4E). However, as tradeoff strengthens, isolates with strategies that are biased towards the development of non-predatory adults outcompete others (Figure 4E – 3G). Interestingly, the effect of the strength of tradeoff with the relative fitness of a given isolate with its distinct strategy in a polymorphic community is nonlinear. To illustrate this point, we simulated dynamics in polymorphic communities over a wide range of κ , which determines the strength of tradeoff (Eq 13). For each simulation, we calculated the maximum value of per capita growth rate, reflecting maximum fitness of a given strategy during competition. This analysis revealed that weak tradeoff results in selection of the highest value of u_i – i.e., the isolate that develops only predatory adults (Figure 4H). However, stronger tradeoff favors a range of strategies that are biased toward the development of non-predatory adults (Figure 4H).

Strategies that prefer high probability of the predatory mouth form are resistant to invasion

An evolutionary stable strategy was originally defined as one that is resistant to invasion (25). Following this definition, the evolutionary stability of each mouth-form strategy can be explored by introducing a mutant strategy at low frequency to a monomorphic community, entirely composed of a resident isolate with a given strategy, which has reached steady state before the invasion. Invasion fitness is calculated using Eq. 18. Although strategies that are biased towards the development of the predatory mouth form only fix in polymorphic communities when tradeoff is weak (Figure 4H) – given the density-dependent nature of predation –, these strategies resist invasion regardless of the strength of tradeoffs (Figure 5 A – C). This observation is consistent with our previous finding using an agent-based model based on experimental observations (15).

Environmental variability does not select for strategies that prefer high probability of the predatory mouth form

It could be argued that environmental variability might overcome the cost of tradeoff, thus favoring strategies that prefer high-predation. To test this hypothesis, we designed a different experiment in which isolates in polymorphic community have identical strategies ($u_i = u^* \quad \forall i \in s$, default $u^* = -0.5$) in non-inducing environment $e = 0$. Once the environment changes to $e = 1$, each isolate assumes a distinct strategy (Figure 5D). Random switching between $e = 0$ and $e = 1$ can result in the fixation of different strategies (Figure 5E and F). To explore the interplay between environmental variability and the strength of tradeoffs, we simulated polymorphic communities as described above in randomly varied environment. These experiments were repeated for different values of κ . In order to quantify the level of environmental complexity during each simulation, we calculated Lempel–Ziv complexity for the vector of environmental values ($e \in \{0, 1\}$) during the simulation. In short, the Lempel–Ziv complexity indicates how easy a sequence can be compressed, with static or periodically changing sequences being less complex than sequences with non-repeated patterns of change (26). The Lempel–Ziv complexity provides an intuitive measure of how the environment in our model changes during a simulation. Our analysis indicates that environmental complexity does not favor strategies that result in higher predatory adults when tradeoff is considerable (Figure 5G). In a weak tradeoff regime, the highest value of u_i fixes in a polymorphic community regardless of environmental complexity (Figure 5G).

TEI can disappear or lengthen as a consequence of tradeoff between mouth-form polyphenism and fecundity

TEI in our model is defined as the gradual change in u_i in response to the change in an environment with a given target strategy $u_i^*(e)$ (Eq. 16) (Figure 6A). Given the observed pattern of TEI in *P. pacificus* RSC011 (Figure 1C), could the TEI potentially evolve or even be selected against due to the tradeoff between mouth-form plasticity and fecundity? To answer this question, we designed an experiment in which isolates *a* and *b*, both with the same strategy in $e = 0$ are transferred to $e = 1$, where both have the same target strategy ($u_a^*(e = 1) = u_b^*(e = 1)$). These isolates differ only in their TEI

strategy (Figure 6B). In this example, $\tau_{\text{down},a} > \tau_{\text{down},b}$, resulting in a slower decline in u_a as it approaches $u_a^*(e = 1)$. With weak tradeoff, extended TEI is the winning strategy (Figure 6B). However, with strong tradeoff, short TEI is favored (Figure 6C).

To explore the evolution of TEI as a function of the strength of tradeoff, we used the scenario described above and considered the maximum fitness of isolate b relative to isolate a , where isolate a has a fixed $\tau_{\text{down},a} = 50$ and in each simulation isolate b is assigned a different value of $\tau_{\text{down},b}$ (Figure 6D). Surprisingly, depending on the strength of tradeoff, extension or elimination of TEI is the winning strategy. Additionally, depending on the value of κ , a range of TEI can be selected for, where extended or shortened TEI strategies beyond this range have lower fitness (Figure 6D). This observation suggests that the observed pattern of TEI in *P. pacificus* RSC011 could be evolvable.

In summary, our theoretical results illustrate how strategies that favor lower probabilities of developing the predatory mouth form can evolve, which could explain the observed diversity with respect to mouth-form polyphenism in the wild isolates of *P. pacificus*. Additionally, how the tradeoff between mouth-form polyphenism and fecundity can shape the dynamics of TEI.

Discussion

Despite growing interest in non-genetic inheritance, especially TEI (27–30), much of existing research is mostly concerned with establishing cases of TEI— itself an arduous task given the rigor needed (1) – rather than exploration of the potential eco-evolutionary consequence of TEI. Our integrative approach, combining experimental study of TEI and its effect of fitness in a nematode and a theoretical framework, provides a unique approach to investigate the conditions under which different strategies with respect to mouth-form polyphenism in *P. pacificus* can evolve. Specifically, our results indicate how, in the presence of tradeoff, strategies that favor high probability of developing the predatory mouth form are less likely to evolve, even in a rapidly changing environment. This result seems consistent with previous observation with

respect to the isolate-specific probability of developing the predatory mouth form in *P. pacificus* under standard laboratory condition (11), but a broader survey of mouth-form polyphenism in *P. pacificus* isolates across multiple environments is yet to be conducted in order to clarify just how diverse these strategies are.

Additionally, our model indicates that the strength of tradeoff between mouth-form plasticity and fitness can select for extension, elimination, or stabilization of TEI. This is a novel observation, since *a priori* the observed duration of TEI in *P. pacificus* can be attributed to different constraints and not malleable to selection. The existing theory on transgenerational plasticity frames the evolution of non-genetic inheritance as an anticipatory response that is selected in environments with repeatable and extended phases of change (31, 32). In this context, it has been shown that selection can favor or eliminate transgenerational plasticity, depending on the predictive power of the environmental cues (33, 34). However, our results add another wrinkle to the evolution of TEI, by illustrating how, in principle, evolution can shape the extension of TEI, or eliminate it, as a consequence of tradeoff between plasticity and fitness, even when an environmental cue is assumed to be reliable. We previously documented tradeoff between phenotypic plasticity and fecundity, as a proxy for fitness, across multiple isolates of *P. pacificus* (14). Here, we suggest that the reverberation of the tradeoff between plasticity and fitness across generations via TEI can further complicate both the evolution of TEI and its eco-evolutionary consequence. Our results with respect to the potential effect of selection on the TEI can be further explored via experimental evolution using *P. pacificus*.

Most examples of transgenerational plasticity are assumed to be of no adaptive value or even deleterious (reviewed in (35)). Consequently, this form of non-genetic inheritance is viewed mostly as a source of variation (36), as an extension of the notion of plasticity-first evolution (37). However, the case of TEI in *P. pacificus* seems rather distinct, as its ecological consequence, the development of a morphology that enables intraguild predation, coupled with its dispersal–colonization–competition life cycle in the wild (9), suggest a direct advantage in assuming the predatory mouth form.

Much remains to be explored to fully understand how TEI has evolved in *P.*
pacificus. Our experimental results, where reversal to a non-inducing environment is
shown to be accompanied by a transient increase in LRS before reverting to the naive
state, is intriguing, as it suggests a need to extend our previous attempt at cataloging
the fitness cost of plasticity (14) to include the dynamics of TEI across multiple isolates.
Only then, it will be possible to understand if the many assumptions made in our
Darwinian dynamics model hold. Additionally, while this theoretical framework
provides an intuitive way to understand phenotypic evolution – making it apt in the
context of polyphenism and TEI – it does not consider the observed stochasticity in
mouth-form plasticity in *P. pacificus* and the observed variation in the LRS data.
Elsewhere (15), we addressed such concerns – at least with respect to variance in
life-history data – by taking advantage of agent-based modeling. However, such complex
models limit the extent to which evolutionary questions can be addressed. In our view,
polyphenism of a cannibalistic trait in *P. pacificus* and the associated TEI can serve as
a unique anchor for a range of theoretical tools designed to address the lingering
ecological and evolutionary puzzles about plasticity and non-genetic inheritance.

Data availability

Code and data are available on the Github repository associated with this manuscript
(<https://github.com/Kalirad/NematodeDarwinianGame>)

Authors' contributions

SPQ, AK, and RJS designed the experiments. SPQ conducted the experiments. SPQ
collected the data. The mathematical and statistical models were developed by AK. AK
analyzed and visualized the results. SPQ, AK, and RJS wrote the manuscript.

Conflict of interest declaration

We declare we have no competing interests.

Funding

385

This work was funded by the Max Planck Society.

386

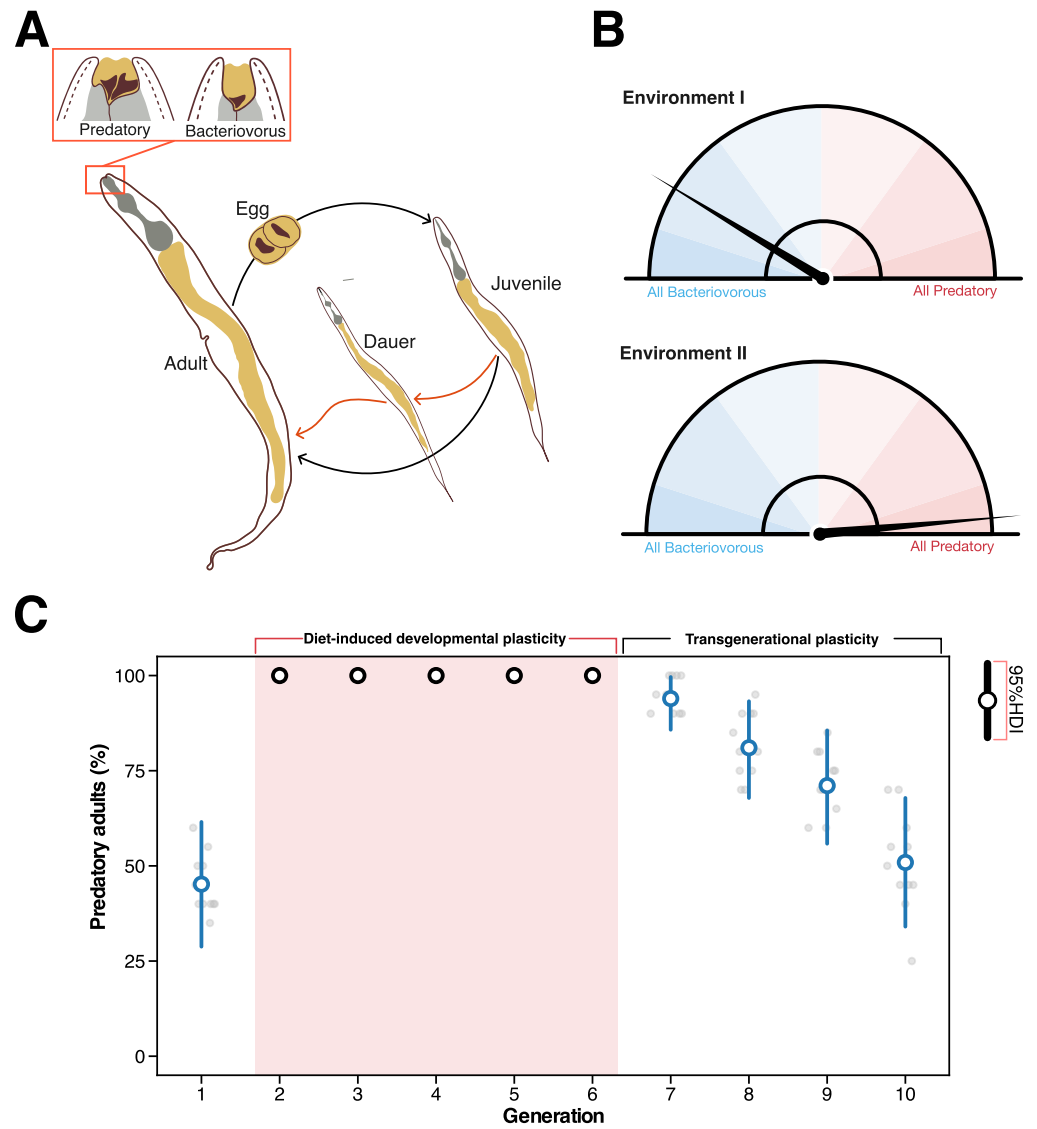


Figure 1

Figure 1 caption

(A) The nematode *Pristionchus pacificus* develops either of two mouth forms: a wide eurystomatous (Eu) form which enables predation of other nematodes in addition to bacterivory, and a narrow non-predatory stenostomatous (St) form. The irreversible decision to express a mouth form is taken during the development from juvenile to adult stage. (B) The probability of assuming either of the two mouth forms (shown as a needle) is dependent on the environment. In this example, it is very probable to assume the bacteriovorus form in environment I whereas environment II promotes the development of the predatory mouth form. The needle is used to emphasize the stochastic nature of the developmental decision taken in this context. (C) The mouth-form polyphenism in *P. pacificus* includes both the environmental induction of a trait and the transgenerational plasticity of the trait, where in the absence of the environmental cue, the parental plastic response reverberates across generations. The bar represent the 95% highest density interval (HDI) for the probability of assuming the predatory mouth form.

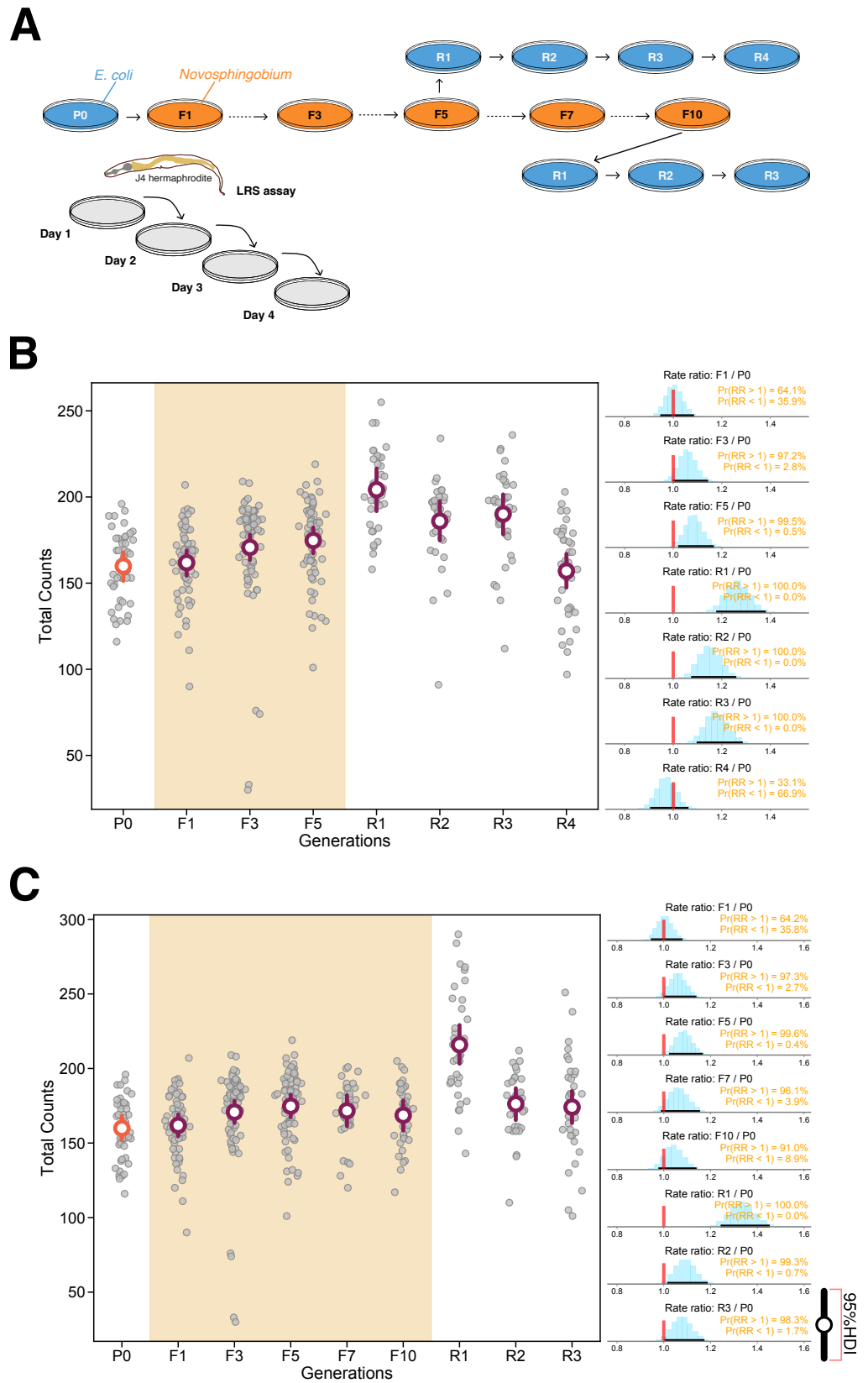


Figure 2

Figure 2 caption

(A) To assess the interplay between mouth-form polyphenism and fecundity as an indicator of fitness, we designed an experiment in which *P. pacificus* RSC011 were propagated on *Novosphingobium* L76 for ten generations. During the experiment, two reversal lines were established by propagating lines on *E. coli* OP50 after five or ten generations of propagation on *Novosphingobium* L76. In select generations, we conducted lifetime reproductive success (LRS) by transferring J4 hermaphrodites to fresh plates every 24 hours for 4 days and counting the number of viable progenies of each mother. (B) The change in fecundity during five generations of exposure to *Novosphingobium* L76 and the subsequent reversal on *E. coli* OP50 for four generations compared with the naïve *P. pacificus* on the *E. coli* diet (P0). The right column indicates the inferred rate ratio (RR) on LRS when comparing any given generation with P0. (C) The change in fecundity during ten generations of exposure to *Novosphingobium* L76 and the subsequent reversal on *E. coli* OP50 for four generations compared with the naïve *P. pacificus* on the *E. coli* diet (P0). The right column indicates the inferred RR on LRS when comparing any given generation with P0. The 95% HDI in fecundity data represent the inferred mean fecundity. In the RR panel, the horizontal black bar indicates 95% HDI for RR, with one (red vertical line) denoting no effect (See Figure S3 and S4 for trace plots and tables S1 and S2 for summary statistics associated with the statistical shown in this figure).

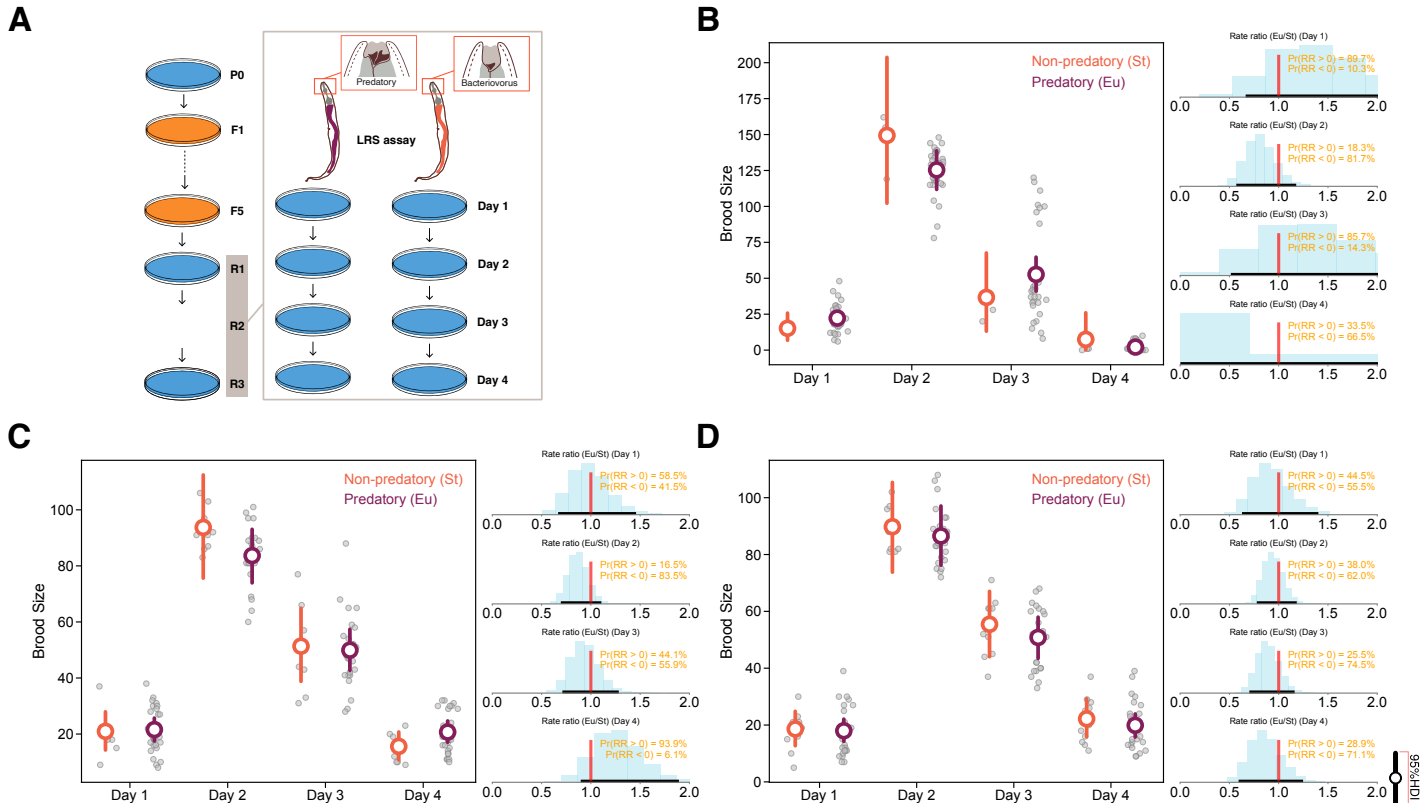


Figure 3

Figure 3 caption

(A) To explore if the observed increased in fecundity following exposure to *Novosphingobium* L76 is associated with mouth-form state of the mother, we designed an experiment in which *P. pacificus* RSC011 were propagated on *Novosphingobium* L76 for five generations, starting from the naïve *P. pacificus* grown on the *E. coli* diet (P0). After propagation on *Novosphingobium* L76, nematode were transferred on *E. coli* OP50 for three generations. In each generation during the reversal phase of the experiment, we selected J4 hermaphrodites with predatory (Eu) or non-predatory (St) mouth-form and use them to measure per-day fecundity by transferring them to fresh plates seeded with *E. coli* OP50 every 24 hours for 4 days and counting the viable progenies. (B) The results of LRS for non-predatory and predatory hermaphrodites selected from the first generation during the reversal phase. The right column indicates the inferred rate ratio (RR) on LRS when comparing the fecundity of predatory (Eu) hermaphrodites with non-predatory counterparts on a given day. (C) The results of LRS for non-predatory and predatory hermaphrodites selected from the second generation during the reversal phase. (D) The results of LRS for non-predatory and predatory hermaphrodites selected from the third generation during the reversal phase. The 95% HDI in fecundity data represent the inferred mean fecundity. In the RR panel, the horizontal black bar indicates 95% HDI for the effect, with one (red vertical line) denoting no effect (See Figure S5–S7 for trace plots and tables S3–S5 for summary statistics associated with the statistical shown in this figure).

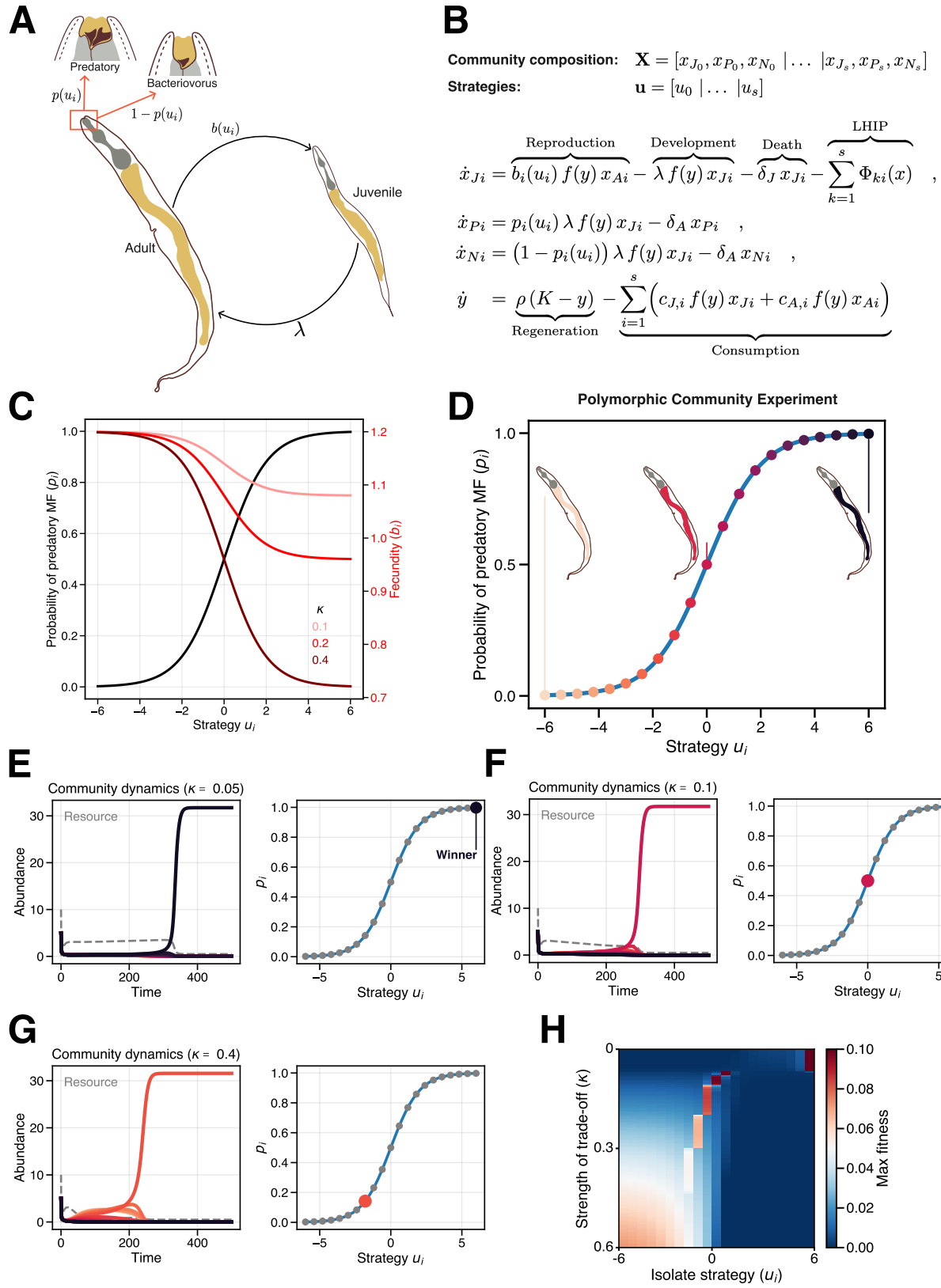


Figure 4

Figure 4 caption

(A) To investigate the effect of tradeoff between mouth-form polyphenism and fecundity, we considered a simplified life-cycle of *P. pacificus*, in which probability of assuming predatory (Eu) mouth form for isolate i (p_i) is determined by its strategy (u_i). The choice of the strategy simultaneously affect the probability of assuming either of the two mouth forms and the fecundity parameter (b_i), a relationship that capture a hypothesized tradeoff between plasticity and fecundity. (B) An abbreviated representation of the core of our model, which envisions a community as an assemblage of stage-structured populations belonging to s different isolates, each with its own distinct strategy. The ecological consequence of developing the predatory mouth form is reflected in our model as life-history intraguild predation (LHIP). (C) Strategy u_i determines the probability of assuming the predatory mouth form for species i (p_i). The intensity of tradeoff between strategy u_i and fecundity b_i is determined by parameter κ . (D) To test the variability different strategies, we designed an experiment in which a community starts with 21 *P. pacificus* isolates, each with its distinct strategy (circles). The set-up enables us to single out strategies that outcompete others in polymorphic community. (F) – (G) The outcome of competition starting from a polymorphic state with respect to strategy, is dependent on the strength of tradeoff, with weaker tradeoff ($\kappa = 0.05$) favoring isolate with a strategy that produces only predatory adults, while intermediate ($\kappa = 0.1$) and high ($\kappa = 0.4$) levels of tradeoffs increasingly favor strategies that are biased towards the development of non-predatory adults. In (F) – (G), the solid lines in the left panels represent the total biomass of each isolate, i.e., the abundance of juveniles and adults. The right panel in each subplot highlights the winning Strategy. (H) The maximum fitness of each isolate (calculated by inferring the per capita growth rate of all 21 isolates during competition starting from a polymorphic case) indicates how the strength of tradeoff affects the evolutionary viability of each strategy. Parameters used for the simulations are found in Table S6.

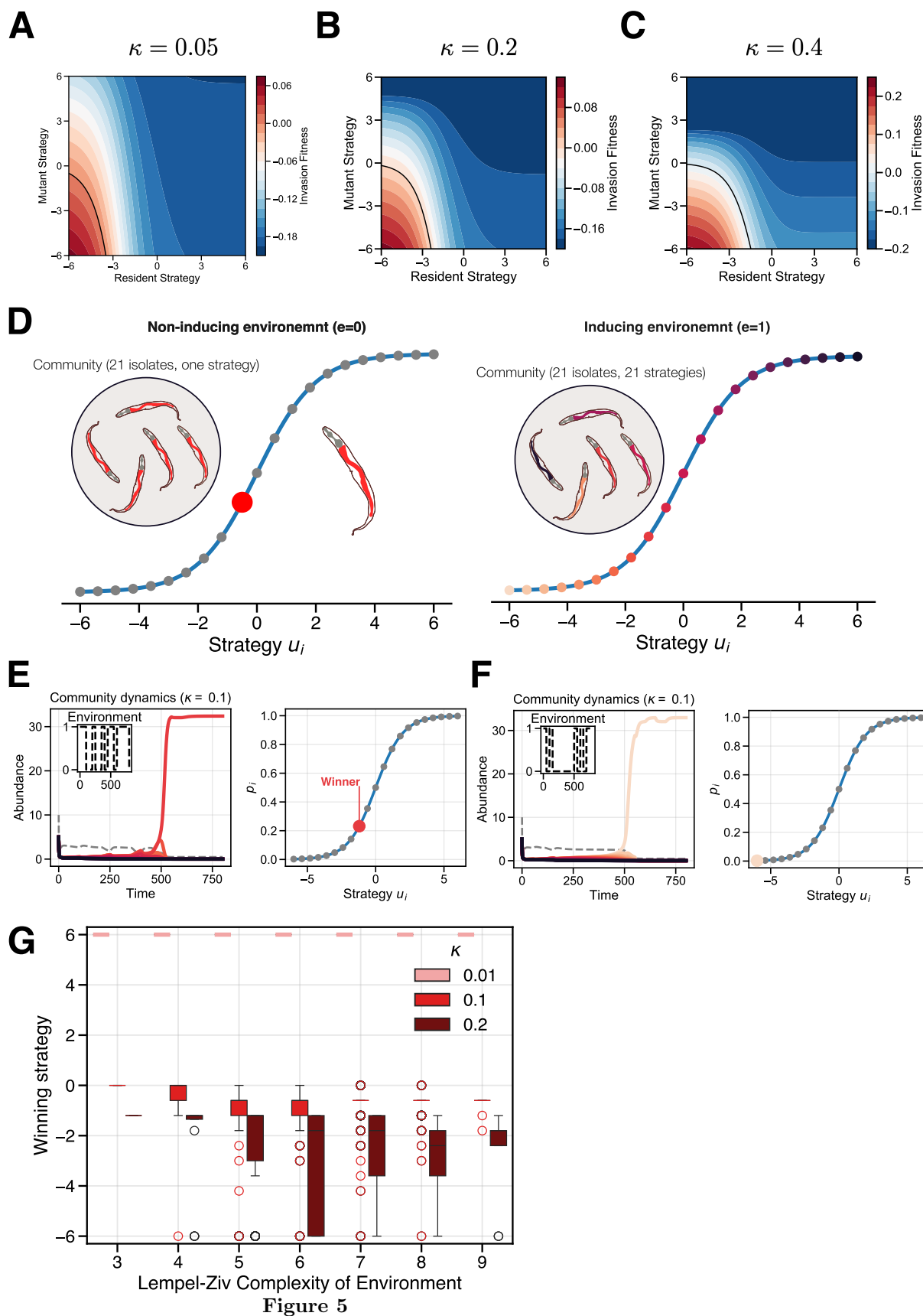


Figure 5 caption

(A) – (C) To explore the resistance of a given strategy to invasion, we conducted pairwise invasion assay, in which a *P. pacificus* isolate with a “mutant” strategy is introduced at low abundance to a community composed of a single resident isolate with its own strategy. The invasion fitness captures the initial per-capita growth rate of the mutant. Across three different trade-off levels, strategies that favor the production of predatory adults are resistant to invaders. (D) To illustrate the effect of environmental variability on the evolution of plasticity strategy in *P. pacificus*, we considered a simple scenario in which in environment $e = 0$, all isolates have the same strategy – which favors low probability of developing the predatory mouth form – but the same isolates assume distinct strategies in environment $e = 1$. (E) – (F) Simulation starting from a polymorphic state during which the environment randomly fluctuates for varying durations between $e = 1$ and $e = 0$. In each case, a different isolate outcomes the other 20 isolates that formed the community at the start. (G) For each value of κ , we simulated 1000 competition starting from a polymorphic state in environments that randomly fluctuated between $e = 1$ and $e = 0$. The Lempel–Ziv complexity indicates the regularity environmental change in each simulation. In the low tradeoff scenario ($\kappa = 0.01$), the strategy the exclusively produces predatory adults wins out regardless of the environmental complexity, while higher tradeoffs favor Strategies that are biased in favors of non-predatory adults. Parameters used for the simulations are found in Table S6.

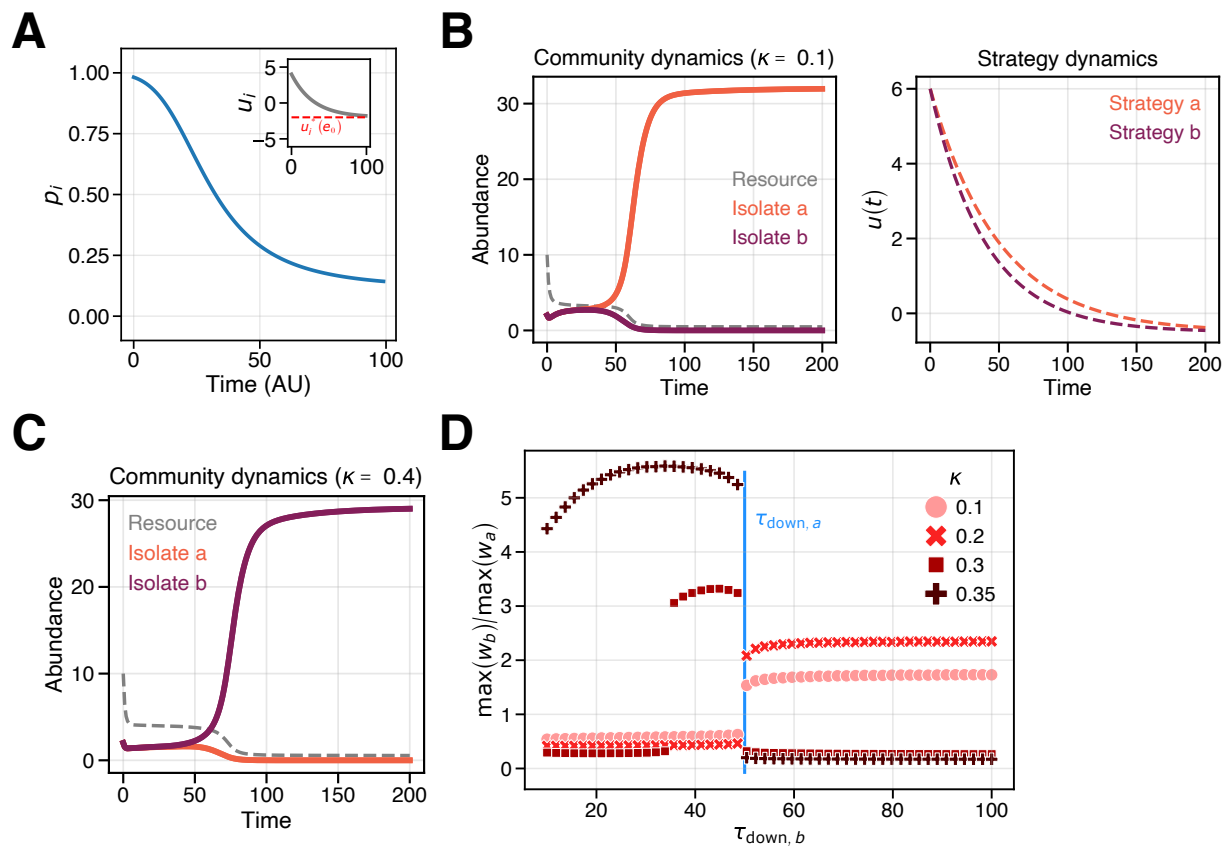


Figure 6

Figure 6 caption

(A) Our model enables the inclusion of TEI, where the strategy (u_i) and the corresponding probability of developing the predatory mouth form (p_i), decline to reach the target strategy for isolate i in environment e ($u_i^*(e)$). (B) To explore the affect of TEI strategy on competition, we simulated a scenario in which two isolates – starting from $u = 6.0$ – are competing in an environment with $u^* = -0.5$. The two isolates are identical except in their respective τ_{down} . In this case, isolate a has a TEI that returns to u^* more slowly than isolate b, resulting in the extinction of isolate b. However, stronger tradeoff ($\kappa = 0.4$) favors isolate b (C), since a shorter TEI results in higher comparative fecundity in the new environment. (D) We repeated this experiment by considering a scenario in which isolate a has a fixed $\tau_{\text{down}} = 50$ while the competing isolate b has a different τ_{down} in each simulation. We calculated the maximum fitness of isolate b relative to isolate a during each simulation. These simulations were repeated across a range of κ . Parameters used for the simulations are found in Table S6.

References

1. L Ryan Baugh and Troy Day. Nongenetic inheritance and multigenerational plasticity in the nematode *C. elegans*. *eLife*, 9:e58498, aug 2020. ISSN 2050-084X. doi: 10.7554/eLife.58498. URL <https://doi.org/10.7554/eLife.58498>.
2. Dustin J Marshall. Transgenerational plasticity in the sea: context-dependent maternal effects across the life history. *Ecology*, 89(2):418–427, Feb 2008. ISSN 0012-9658 (Print); 0012-9658 (Linking). doi: 10.1890/07-0449.1.
3. Tobias Uller, Shinichi Nakagawa, and Sinead English. Weak evidence for anticipatory parental effects in plants and animals. *Journal of evolutionary biology*, 26(10):2161–2170, 2013.
4. Leif Engqvist, Klaus Reinhold, and Matthew Davey. Adaptive trans-generational phenotypic plasticity and the lack of an experimental control in reciprocal match/mismatch experiments. *Methods in Ecology & Evolution*, 7(12), 2016.
5. Emilie C. Snell-Rood and Sean M. Ehlman. *Phenotypic Plasticity & Evolution: Causes, Consequences, Controversies*, chapter Ecology and Evolution of Plasticity. CRC Press, 1st edition, 2021.
6. Alison M Bell and Jennifer K Hellmann. An integrative framework for understanding the mechanisms and multigenerational consequences of transgenerational plasticity. *Annu Rev Ecol Evol Syst*, 50:97–118, 2019. ISSN 1543-592X (Print); 1545-2069 (Electronic); 1543-592X (Linking). doi: 10.1146/annurev-ecolsys-110218-024613.
7. Leah Houri-Zeevi, Yael Korem Kohanim, Olga Antonova, and Oded Rechavi. Three rules explain transgenerational small rna inheritance in *c. elegans*. *Cell*, 182(5):1186–1197.e12, 2020. ISSN 0092-8674. doi: <https://doi.org/10.1016/j.cell.2020.07.022>. URL <https://www.sciencedirect.com/science/article/pii/S0092867420309302>.
8. Titas Sengupta, Rachel Kaletsky, and Coleen T Murphy. The logic of transgenerational inheritance: timescales of adaptation. *Annual review of cell and developmental biology*, 39(1):45–65, 2023.

-
9. Ralf J. Sommer. Pristionchus – beetle associations: Towards a new natural history. *Journal of Invertebrate Pathology*, 209:108243, 2025. ISSN 0022-2011. doi: <https://doi.org/10.1016/j.jip.2024.108243>. URL <https://www.sciencedirect.com/science/article/pii/S0022201124001861>.
10. Gilberto Bento, Akira Ogawa, and Ralf J Sommer. Co-option of the hormone-signalling module dafachronic acid–daf-12 in nematode evolution. *Nature*, 466(7305):494–497, 2010.
11. Erik J Ragsdale, Manuela R Müller, Christian Rödelisperger, and Ralf J Sommer. A developmental switch coupled to the evolution of plasticity acts through a sulfatase. *Cell*, 155(4):922–933, 2013.
12. James W Lightfoot, Mohannad Dardiry, Ata Kalirad, Stefano Giaimo, Gabi Eberhardt, Hanh Witte, Martin Wilecki, Christian Rödelisperger, Arne Traulsen, and Ralf J Sommer. Sex or cannibalism: Polyphenism and kin recognition control social action strategies in nematodes. *Science Advances*, 7(35):eabg8042, 2021.
13. Gary A. Polis, Christopher A. Myers, and Robert D. Holt. The ecology and evolution of intraguild predation: Potential competitors that eat each other. *Annual Review of Ecology and Systematics*, 20:297–330, 1989. URL <http://www.jstor.org/stable/2097094>.
14. Mohannad Dardiry, Veysi Piskobulu, Ata Kalirad, and Ralf J Sommer. Experimental and theoretical support for costs of plasticity and phenotype in a nematode cannibalistic trait. *Evolution letters*, 7(1):48–57, 2023.
15. Ata Kalirad and Ralf J Sommer. The role of plasticity and stochasticity in coexistence. *Ecology Letters*, 27(2):e14370, 2024.
16. Sofia Casasa, Eleni Katsougia, and Erik J Ragsdale. A mediator subunit imparts robustness to a polyphenism decision. *Proceedings of the National Academy of Sciences*, 120(32):e2308816120, 2023.
17. Nicholas A Levis and Erik J Ragsdale. A histone demethylase links the loss of plasticity to nongenetic inheritance and morphological change. *Nature communications*, 14(1):8439, 2023.

-
18. Shelley Reich, Tobias Loschko, Julie Jung, Samantha Nestel, Ralf J Sommer, and Michael S Werner. Developmental transcriptomics in *pristionchus* reveals the environmental responsiveness of a plasticity gene-regulatory network. *Genome Research*, 35(7):1560–1573, 2025.
19. Michael S Werner, Tobias Loschko, Thomas King, Shelley Reich, Tobias Theska, Mirita Franz-Wachtel, Boris Macek, and Ralf J Sommer. Histone 4 lysine 5/12 acetylation enables developmental plasticity of *Pristionchus* mouth form. *nature communications*, 14(1):2095, 2023.
20. Audrey L Brown, Adriaan B Meiborg, Mirita Franz-Wachtel, Boris Macek, Spencer Gordon, Ofer Rog, Cameron J Weadick, and Michael S Werner. Characterization of the *pristionchus pacificus* “epigenetic toolkit” reveals the evolutionary loss of the histone methyltransferase complex *prc2*. *Genetics*, 227(1):iyae041, 2024.
21. Shiela Pearl Quiobe, Ata Kalirad, Waltraud Röseler, Hanh Witte, Yinan Wang, Christian Rödelsperger, and Ralf J Sommer. Ebax-1/zswim8 destabilizes mirnas, resulting in transgenerational inheritance of a predatory trait. *Science Advances*, 11(11):eadu0875, 2025.
22. Shiela Pearl Quiobe, Ata Kalirad, Raphaela Zurheide, Hanh Witte, Christian Rödelsperger, and Ralf J. Sommer. Diet-derived vitamin b12 induces transgenerational inheritance of nematode predation through elevated vitellogenin provisioning. *bioRxiv*, 2025. doi: 10.1101/2025.05.21.655301. URL <https://www.biorxiv.org/content/early/2025/05/26/2025.05.21.655301>.
23. Ronald A. Fisher. *The Genetical Theory of Natural Selection*. Clarendon Press, Oxford, 1930.
24. Oriol Abril-Pla, Virgile Andreani, Colin Carroll, Larry Dong, Christopher J. Fonnesbeck, Maxim Kochurov, Ravin Kumar, Junpeng Lao, Christian C. Luhmann, Osvaldo A. Martin, Michael Osthege, Ricardo Vieira, Thomas Wiecki, and Robert Zinkov. PyMC: A modern and comprehensive probabilistic programming framework in Python. *PeerJ Computer Science*, 9(e1516), 2023. doi: 10.7717/peerj-cs.1516.

-
25. Thomas L. Vincent and Joel S. Brown. *Evolutionary Game Theory, Natural Selection, and Darwinian Dynamics*. Cambridge University Press, 2005. 593
594
26. A. Lempel and J. Ziv. On the complexity of finite sequences. *IEEE Transactions on Information Theory*, 22(1):75–81, 1976. doi: 10.1109/TIT.1976.1055501. 595
596
27. Eva Jablonka and Marion J. Lamb. *Evolution in Four Dimensions: Genetic, Epigenetic, Behavioral, and Symbolic Variation in the History of Life*. MIT Press, Cambridge, MA, 2005. ISBN 9780262101073. 597
598
599
28. Alison M. Bell and Jennifer K. Hellmann. An integrative framework for understanding the mechanisms and multigenerational consequences of transgenerational plasticity. *Annual Review of Ecology, Evolution, and Systematics*, 50:97–118, 2019. doi: 10.1146/annurev-ecolsys-110218-024613. 600
601
602
603
29. Russell Bonduriansky and Troy Day. *Extended Heredity: A New Understanding of Inheritance and Evolution*. Princeton University Press, Princeton, NJ, 2021. 604
605
606
ISBN 9780691204147.
30. Caitlin McAndry, Alastair J. Wilson, Peter A. Cotton, and Manuela Truebano. Transgenerational plasticity research: Challenges and opportunities. *Functional Ecology*, 39(11):3083–3104, 2025. doi: <https://doi.org/10.1111/1365-2435.70139>. 607
608
609
URL <https://besjournals.onlinelibrary.wiley.com/doi/abs/10.1111/1365-2435.70139>. 610
611
31. Michael Lachmann and Eva Jablonka. The inheritance of phenotypes: An adaptation to fluctuating environments. *Journal of Theoretical Biology*, 181(1): 612
613
614
1–9, July 1996. doi: 10.1006/jtbi.1996.0109.
32. Stephen R. Proulx and Henrique Teotónio. What kind of maternal effects can be selected for in fluctuating environments? *The American Naturalist*, 189(6): 615
616
617
E118–E137, June 2017. doi: 10.1086/691423.
33. Olof Leimar and John M. McNamara. The evolution of transgenerational integration of information in heterogeneous environments. *The American Naturalist*, 185(3):E55–E69, March 2015. doi: 10.1086/679575. 618
619
620

-
34. John M McNamara, Sasha RX Dall, Peter Hammerstein, and Olof Leimar. 621
Detection vs. selection: integration of genetic, epigenetic and environmental cues 622
in fluctuating environments. *Ecology letters*, 19(10):1267–1276, 2016. 623
35. Russell Bonduriansky. Plasticity across generations. In *Phenotypic plasticity & 624
evolution*, pages 327–348. CRC Press, 2021. 625
36. Russell Bonduriansky, Amanda J. Crean, and Troy Day. The implications of 626
nongenetic inheritance for evolution in changing environments. *Evolutionary 627
Applications*, 5(2):192–201, February 2012. doi: 10.1111/j.1752-4571.2011.00213.x. 628
37. Ralf J. Sommer. Phenotypic plasticity: From theory and genetics to current and 629
future challenges. *Genetics*, 215(1):1–13, May 2020. doi: 630
10.1534/genetics.120.303163. 631

Supplementary information

Darwinian games in a cannibalistic polyphenism and its
transgenerational inheritance

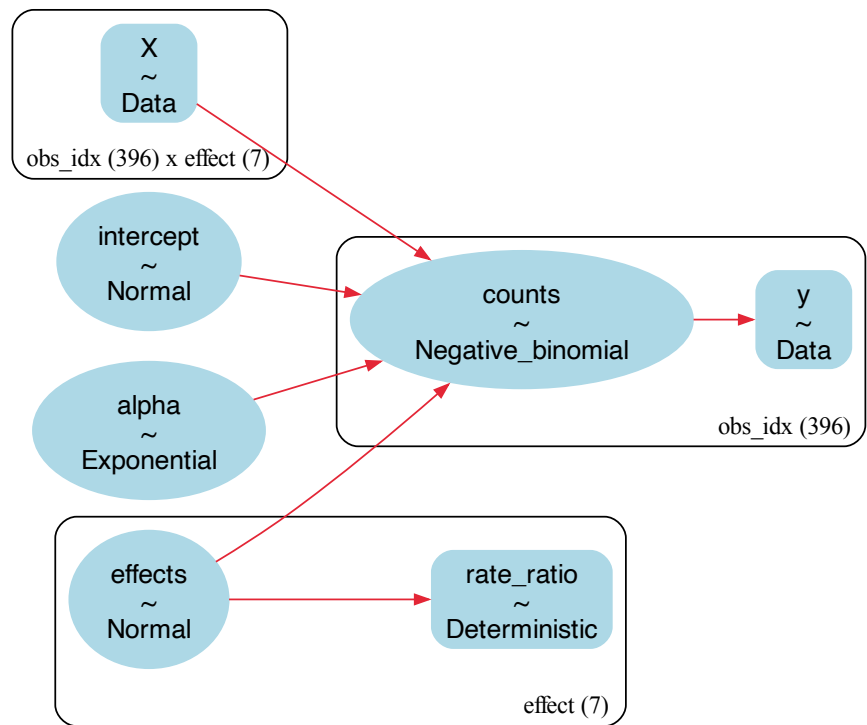


Figure S1. The structure of the model used in Figure 2.

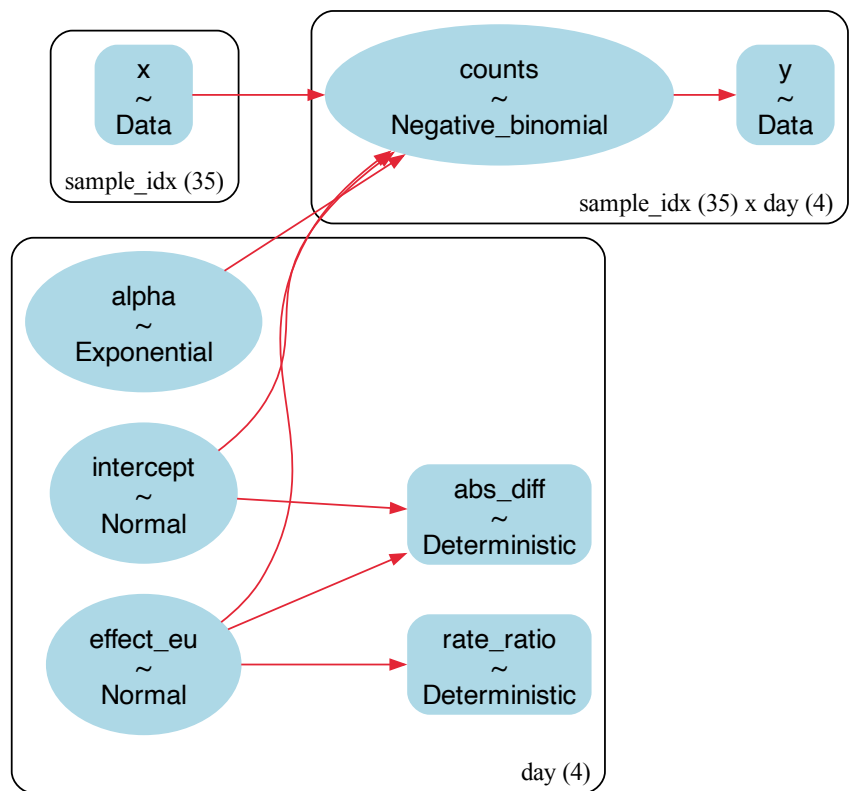


Figure S2. The structure of the model used in Figure 3.

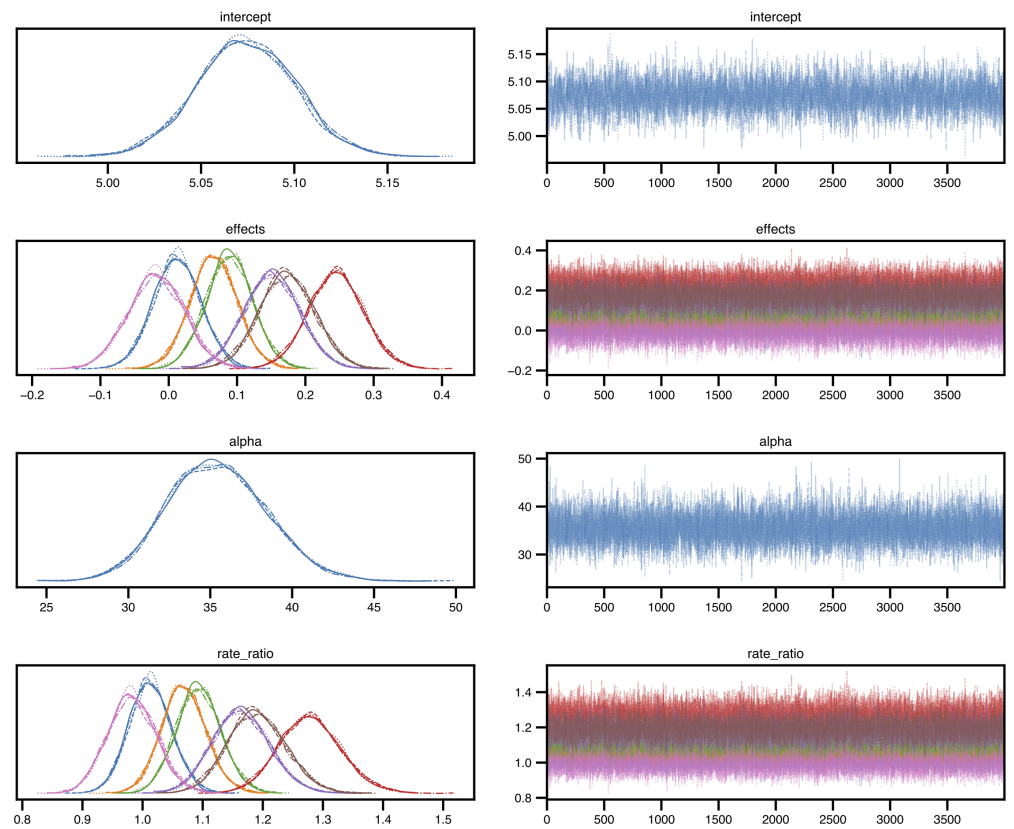


Figure S3. Trace plot for Figure 2B.

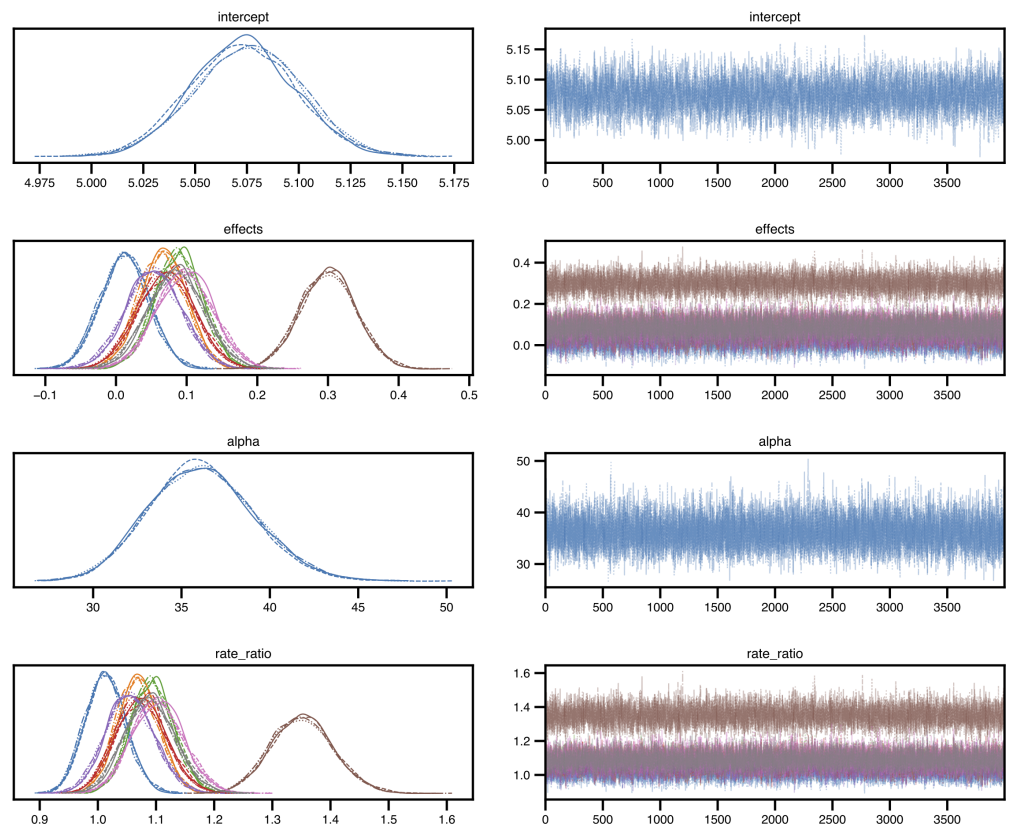


Figure S4. Trace plot for Figure 2C.

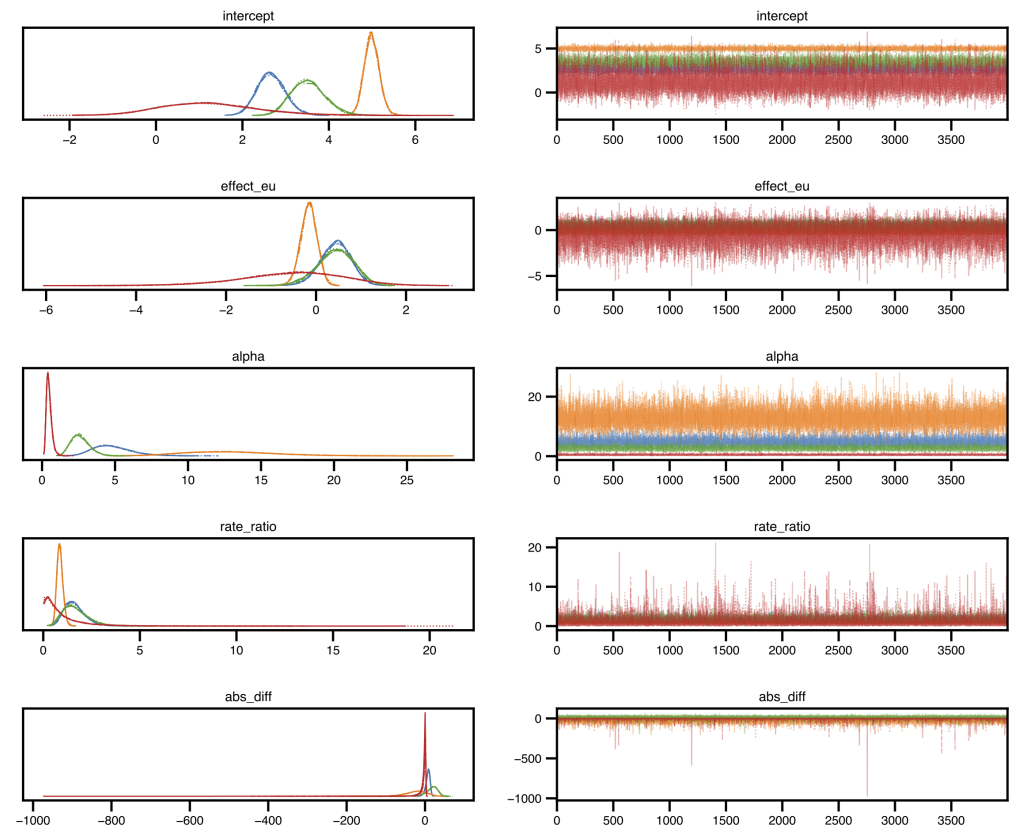


Figure S5. Trace plot for Figure 3B.

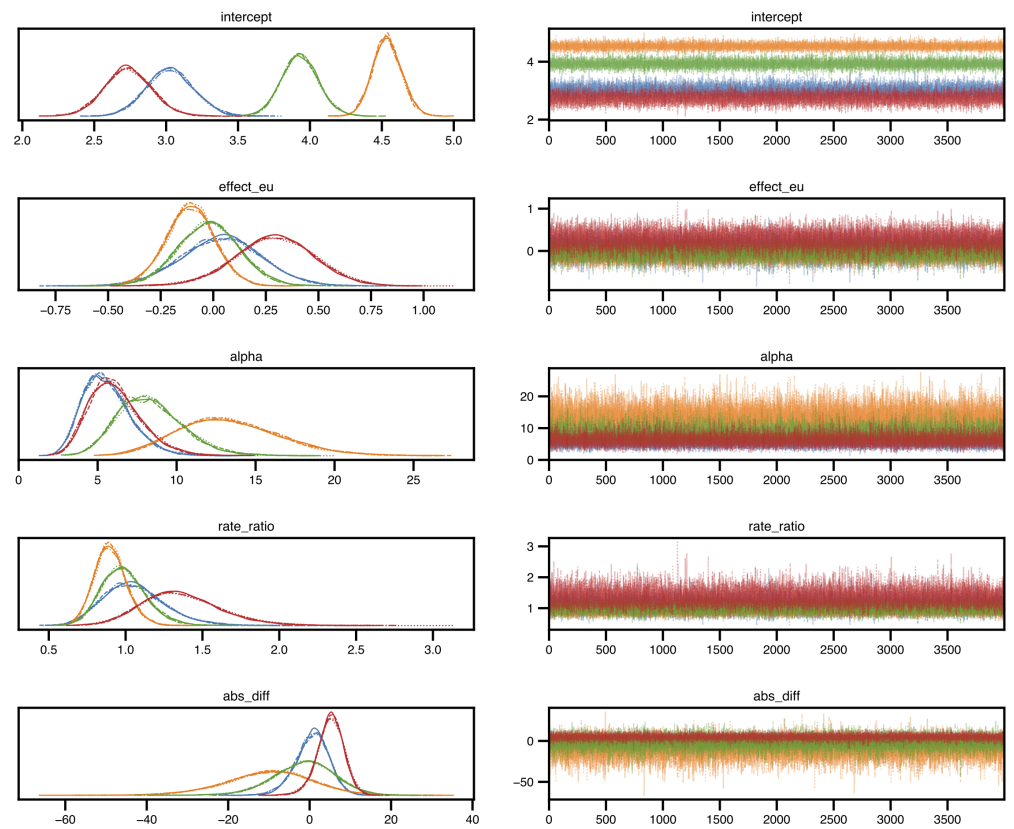


Figure S6. Trace plot for Figure 3C.

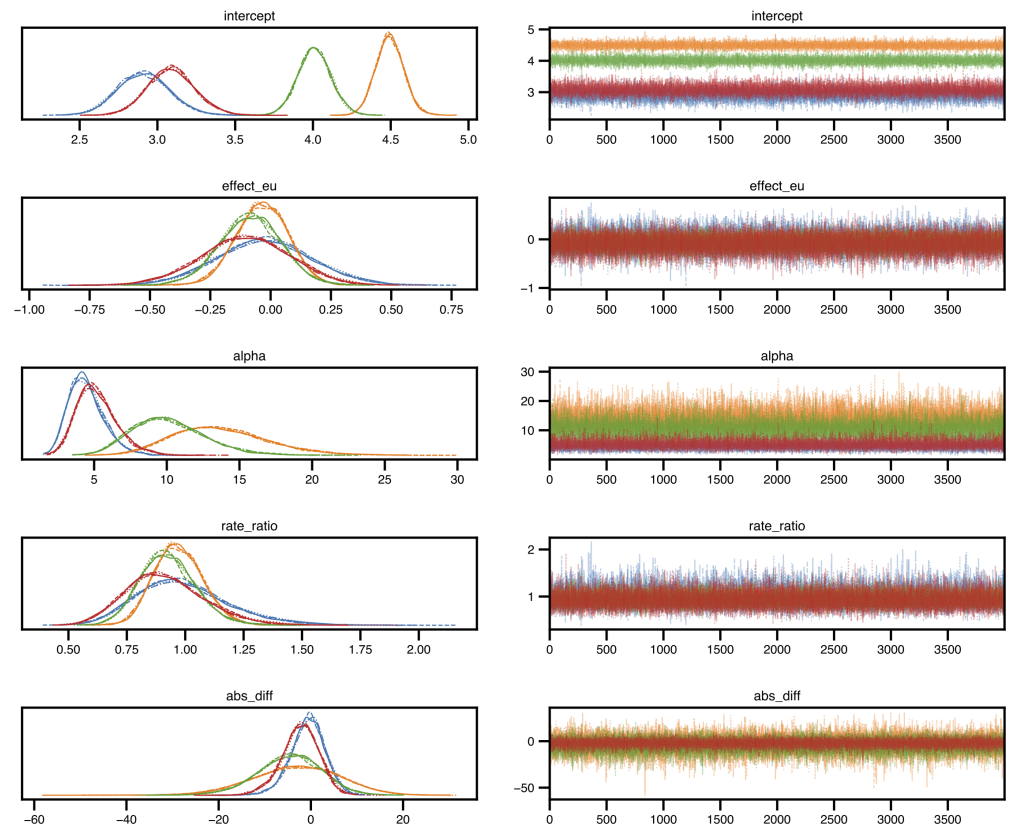


Figure S7. Trace plot for Figure 3D.

Table S1. Summary statistics for the analysis shown in Figure 2B.

	mean	sd	hdi_2.5%	hdi_97.5%	mcse_mean	mcse_sd	ess_bulk	ess_tail	r_hat
intercept	5.07	0.03	5.02	5.13	0.0	0.0	3816.06	5627.76	1.0
effects[F1N]	0.01	0.04	-0.06	0.08	0.0	0.0	5742.97	8449.65	1.0
effects[F3N]	0.07	0.03	-0.0	0.13	0.0	0.0	5656.11	8283.35	1.0
effects[F5N]	0.09	0.03	0.02	0.16	0.0	0.0	5184.07	8628.43	1.0
effects[F5R1]	0.24	0.04	0.16	0.32	0.0	0.0	6040.99	8872.21	1.0
effects[F5R2]	0.15	0.04	0.07	0.23	0.0	0.0	6126.05	9469.34	1.0
effects[F5R3]	0.17	0.04	0.09	0.25	0.0	0.0	6408.25	9378.72	1.0
effects[F5R4]	-0.02	0.04	-0.1	0.06	0.0	0.0	6823.25	9366.41	1.0
alpha	35.41	3.03	29.61	41.37	0.03	0.02	11076.39	10865.24	1.0
rate_ratio[F1N]	1.01	0.04	0.95	1.09	0.0	0.0	5742.97	8449.65	1.0
rate_ratio[F3N]	1.07	0.04	1.0	1.14	0.0	0.0	5656.11	8283.35	1.0
rate_ratio[F5N]	1.09	0.04	1.02	1.17	0.0	0.0	5184.07	8628.43	1.0
rate_ratio[F5R1]	1.28	0.05	1.18	1.38	0.0	0.0	6040.99	8872.21	1.0
rate_ratio[F5R2]	1.16	0.05	1.07	1.26	0.0	0.0	6126.05	9469.34	1.0
rate_ratio[F5R3]	1.19	0.05	1.1	1.29	0.0	0.0	6408.25	9378.72	1.0
rate_ratio[F5R4]	0.98	0.04	0.91	1.06	0.0	0.0	6823.25	9366.41	1.0

Table S2. Summary statistics for the analysis shown in Figure 2C.

	mean	sd	hdi_2.5%	hdi_97.5%	mcse_mean	mcse_sd	ess_bulk	ess_tail	r_hat
intercept	5.07	0.03	5.03	5.12	0.0	0.0	3642.06	5675.72	1.0
effects[F1N]	0.01	0.03	-0.06	0.08	0.0	0.0	5167.68	8590.92	1.0
effects[F3N]	0.07	0.03	-0.0	0.13	0.0	0.0	5211.56	8138.22	1.0
effects[F5N]	0.09	0.03	0.02	0.16	0.0	0.0	5072.63	8489.66	1.0
effects[F7N]	0.07	0.04	-0.01	0.15	0.0	0.0	5839.25	9426.19	1.0
effects[F10N]	0.05	0.04	-0.02	0.13	0.0	0.0	6209.46	8942.64	1.0
effects[F10R1]	0.3	0.04	0.22	0.38	0.0	0.0	5938.88	8708.9	1.0
effects[F10R2]	0.1	0.04	0.02	0.18	0.0	0.0	5813.06	9091.03	1.0
effects[F10R3]	0.08	0.04	0.01	0.17	0.0	0.0	6266.45	9475.08	1.0
alpha	36.15	2.95	30.42	41.83	0.03	0.02	10499.35	10818.36	1.0
rate_ratio[F1N]	1.01	0.03	0.94	1.08	0.0	0.0	5167.68	8590.92	1.0
rate_ratio[F3N]	1.07	0.04	1.0	1.14	0.0	0.0	5211.56	8138.22	1.0
rate_ratio[F5N]	1.09	0.04	1.02	1.17	0.0	0.0	5072.63	8489.66	1.0
rate_ratio[F7N]	1.07	0.04	0.99	1.16	0.0	0.0	5839.25	9426.19	1.0
rate_ratio[F10N]	1.06	0.04	0.98	1.14	0.0	0.0	6209.46	8942.64	1.0
rate_ratio[F10R1]	1.35	0.05	1.24	1.45	0.0	0.0	5938.88	8708.9	1.0
rate_ratio[F10R2]	1.1	0.04	1.01	1.19	0.0	0.0	5813.06	9091.03	1.0
rate_ratio[F10R3]	1.09	0.04	1.0	1.17	0.0	0.0	6266.45	9475.08	1.0

Table S3. Summary statistics for the analysis shown in Figure 3B.

	mean	sd	hdi_2.5%	hdi_97.5%	mcse_mean	mcse_sd	ess_bulk	ess_tail	r_hat
intercept[D1]	2.67	0.33	2.02	3.3	0.0	0.0	13118.12	9779.78	1.0
intercept[D2]	4.99	0.17	4.66	5.34	0.0	0.0	14030.33	10309.93	1.0
intercept[D3]	3.52	0.39	2.78	4.31	0.0	0.0	11080.22	8768.85	1.0
intercept[D4]	1.24	1.14	-0.94	3.51	0.01	0.01	11819.23	9009.73	1.0
effect_eu[D1]	0.43	0.34	-0.24	1.09	0.0	0.0	13109.06	9866.6	1.0
effect_eu[D2]	-0.16	0.18	-0.51	0.2	0.0	0.0	13856.4	10609.03	1.0
effect_eu[D3]	0.44	0.41	-0.33	1.27	0.0	0.0	11115.06	9082.95	1.0
effect_eu[D4]	-0.53	1.15	-2.84	1.67	0.01	0.01	11945.44	9031.44	1.0
alpha[D1]	4.81	1.3	2.5	7.46	0.01	0.01	21926.17	10907.84	1.0
alpha[D2]	12.84	3.11	7.26	19.1	0.02	0.03	19884.83	10188.7	1.0
alpha[D3]	2.61	0.62	1.51	3.91	0.0	0.01	21739.08	11359.08	1.0
alpha[D4]	0.48	0.19	0.18	0.84	0.0	0.0	17781.04	11066.47	1.0
rate_ratio[D1]	1.63	0.55	0.66	2.72	0.0	0.01	13109.06	9866.6	1.0
rate_ratio[D2]	0.87	0.16	0.57	1.18	0.0	0.0	13856.4	10609.03	1.0
rate_ratio[D3]	1.68	0.68	0.51	3.0	0.01	0.01	11115.06	9082.95	1.0
rate_ratio[D4]	1.06	1.37	0.0	3.4	0.01	0.04	11945.44	9031.44	1.0
abs_diff[D1]	7.09	5.67	-4.35	17.0	0.05	0.07	12990.55	9818.71	1.0
abs_diff[D2]	-24.02	27.48	-78.46	26.22	0.24	0.3	13859.13	10496.4	1.0
abs_diff[D3]	15.98	17.41	-17.89	45.2	0.18	0.35	11136.52	8850.69	1.0
abs_diff[D4]	-5.33	18.47	-24.36	3.33	0.2	1.95	11713.71	8865.31	1.0

Table S4. Summary statistics for the analysis shown in Figure 3C.

	mean	sd	hdi_2.5%	hdi_97.5%	mcse_mean	mcse_sd	ess_bulk	ess_tail	r_hat
intercept[D1]	3.03	0.17	2.7	3.35	0.0	0.0	13096.59	11249.51	1.0
intercept[D2]	4.54	0.1	4.34	4.73	0.0	0.0	16968.87	11150.46	1.0
intercept[D3]	3.93	0.13	3.68	4.19	0.0	0.0	13216.61	10425.03	1.0
intercept[D4]	2.73	0.16	2.41	3.06	0.0	0.0	12711.22	11156.05	1.0
effect_eu[D1]	0.04	0.19	-0.36	0.4	0.0	0.0	13106.65	11485.52	1.0
effect_eu[D2]	-0.11	0.12	-0.33	0.13	0.0	0.0	16833.64	11056.31	1.0
effect_eu[D3]	-0.02	0.15	-0.31	0.27	0.0	0.0	13058.57	10196.92	1.0
effect_eu[D4]	0.29	0.19	-0.08	0.66	0.0	0.0	12691.03	10185.8	1.0
alpha[D1]	5.58	1.53	2.82	8.59	0.01	0.02	25994.68	11623.85	1.0
alpha[D2]	13.36	3.22	7.22	19.57	0.02	0.03	22655.96	10971.95	1.0
alpha[D3]	8.34	2.09	4.39	12.37	0.01	0.02	24049.25	11002.8	1.0
alpha[D4]	6.13	1.67	3.14	9.48	0.01	0.02	25295.15	11495.57	1.0
rate_ratio[D1]	1.06	0.2	0.67	1.46	0.0	0.0	13106.65	11485.52	1.0
rate_ratio[D2]	0.9	0.1	0.69	1.11	0.0	0.0	16833.64	11056.31	1.0
rate_ratio[D3]	0.99	0.15	0.71	1.28	0.0	0.0	13058.57	10196.92	1.0
rate_ratio[D4]	1.36	0.26	0.9	1.89	0.0	0.0	12691.03	10185.8	1.0
abs_diff[D1]	0.61	4.14	-7.51	8.7	0.04	0.04	13005.54	11305.99	1.0
abs_diff[D2]	-10.05	10.67	-31.36	10.85	0.08	0.1	16931.55	10521.8	1.0
abs_diff[D3]	-1.43	7.62	-16.55	13.26	0.07	0.07	12973.06	9959.92	1.0
abs_diff[D4]	5.11	3.24	-1.5	11.28	0.03	0.03	12838.08	10185.78	1.0

Table S5. Summary statistics for the analysis shown in Figure 3D.

	mean	sd	hdi_2.5%	hdi_97.5%	mcse_mean	mcse_sd	ess_bulk	ess_tail	r_hat
intercept[D1]	2.91	0.17	2.6	3.25	0.0	0.0	13914.68	10854.89	1.0
intercept[D2]	4.49	0.09	4.32	4.67	0.0	0.0	17080.1	11034.2	1.0
intercept[D3]	4.01	0.11	3.81	4.22	0.0	0.0	15910.54	11419.8	1.0
intercept[D4]	3.09	0.15	2.79	3.39	0.0	0.0	15371.1	12005.17	1.0
effect_eu[D1]	-0.03	0.2	-0.43	0.36	0.0	0.0	14518.04	10363.71	1.0
effect_eu[D2]	-0.03	0.11	-0.25	0.17	0.0	0.0	17156.02	11658.07	1.0
effect_eu[D3]	-0.08	0.13	-0.35	0.16	0.0	0.0	15908.92	11841.17	1.0
effect_eu[D4]	-0.1	0.18	-0.47	0.26	0.0	0.0	15134.14	11302.46	1.0
alpha[D1]	4.47	1.23	2.24	6.89	0.01	0.01	22009.89	10537.68	1.0
alpha[D2]	13.68	3.27	7.84	20.4	0.02	0.03	20820.38	10771.21	1.0
alpha[D3]	10.13	2.53	5.58	15.18	0.02	0.02	25282.89	12768.7	1.0
alpha[D4]	5.18	1.39	2.66	7.97	0.01	0.01	22246.64	11628.29	1.0
rate_ratio[D1]	0.99	0.2	0.63	1.4	0.0	0.0	14518.04	10363.71	1.0
rate_ratio[D2]	0.97	0.11	0.78	1.18	0.0	0.0	17156.02	11658.07	1.0
rate_ratio[D3]	0.93	0.12	0.7	1.16	0.0	0.0	15908.92	11841.17	1.0
rate_ratio[D4]	0.92	0.17	0.59	1.25	0.0	0.0	15134.14	11302.46	1.0
abs_diff[D1]	-0.64	3.75	-8.05	6.64	0.03	0.03	14319.07	9715.54	1.0
abs_diff[D2]	-3.24	9.66	-22.45	15.07	0.08	0.09	17077.49	11143.15	1.0
abs_diff[D3]	-4.62	6.95	-18.54	8.82	0.06	0.06	15763.33	11258.2	1.0
abs_diff[D4]	-2.28	4.02	-10.5	5.41	0.03	0.04	15000.71	10815.9	1.0

Table S6. The parameters of the model

Category	Symbol	Description	Default Value
Resource	ρ	Resource regeneration rate	0.8
	K	Resource carrying capacity	5.0
	H	Half-saturation constant	1.0
Demography	b_0	Baseline fecundity coefficient	1.2
	m	Maturation rate constant	0.7
	d_J	Juvenile mortality rate	0.2
	d_A	Adult mortality rate	0.2
Consumption	c_J	Juvenile resource consumption coeff.	0.4
	c_A	Adult resource consumption coeff.	0.3
Strategy	σ	Logistic slope (Predatory adults $p(u_i)$, Eq. 12)	1.0
	κ	Fecundity tradeoff constant	0.4 ^a
	u_0	Strategy target (Env 0)	-0.5
	u_1	Strategy target (Env 1)	Variable ^b
Dynamics	τ_{up}	Relaxation time (upshift: $u^* > u$)	1.0
	τ_{down}	Relaxation time (downshift: $u^* \leq u$)	NA
	$\alpha_{k,i}$	Attack rate (LHIP)	0.8
	$h_{k,i}$	Handling time (LHIP)	0.1

^a Unless otherwise specified.

^b This parameter is assigned different values, depending on the design of experiment.

**A phenotypically anchored map of endogenous triggers
controlling miRNA destabilization in *Pristionchus pacificus***

Shiela Pearl Quiobe, Hanh Witte, Ralf J. Sommer

Ready for Submission

A phenotypically anchored map of endogenous triggers controlling miRNA destabilization in *Pristionchus pacificus*

Shiela Pearl Quiobe¹, Hanh Witte¹, and Ralf J. Sommer¹

¹Max Planck Institute for Biology Tübingen, Department for Integrative Evolutionary Biology, Max-Planck Ring 9, 72076 Tübingen, Germany

Abstract

Target-directed miRNA degradation (TDMD) is an important mechanism controlling miRNA stability, yet most known triggers have been identified through molecular signatures without clear organismal phenotypes. Here, we exploit the natural binary mouth-form plasticity of the nematode *Pristionchus pacificus* as a phenotypic readout to investigate TDMD *in vivo*. We show that the ubiquitin ligase *Ppa*-EBAX-1 regulates transgenerational inheritance of the predatory morph by controlling the stability of the expanded *miR-35/2235a* cluster. Through bioinformatic prediction, genetic perturbation, and small RNA profiling, we identify endogenous transcripts whose 3' UTR binding sites function as TDMD triggers. Disruption of these sites stabilizes cognate miRNAs and perturbs the dynamics of transgenerational inheritance, supporting a role for regulated miRNA turnover in maintaining developmental memory. Small RNA sequencing further reveals coordinated upregulation of multiple cluster members upon loss of individual triggers, consistent with co-transcription, shared turnover machinery, and feedback in miRNA homeostasis. Notably, while many predicted targets have orthologous genes in related species, their TDMD-relevant binding sites appear lineage-specific, suggesting rapid evolutionary turnover of this regulatory layer. Together, our findings establish a direct link between TDMD, miRNA cluster dynamics, and organismal phenotypes, and highlight the power of phenotypically anchored approaches to uncover functional TDMD triggers that may be missed by sequence- or biochemistry-based screens alone.

Introduction

MicroRNAs (miRNAs) are small noncoding RNAs that post-transcriptionally repress mRNAs through partial base pairing with sites in the mRNAs' 3' UTRs¹. Each miRNA associates with an Argonaute (Ago) protein to form the core of a miRNA-induced silencing complex (miRISC)². In most cases, complementarity between a target sequence and the miRNA seed region (nucleotides 2-8) is sufficient to predict target repression^{3,4}. Because these minimal targeting requirements allow each of the hundreds of conserved miRNAs to regulate numerous transcripts, miRNAs are widely regarded as master regulators of post-transcriptional gene expression.

Although miRNA biogenesis and function have been studied extensively, the mechanisms governing miRNA decay and its clear phenotypic and biological consequences remain comparatively poorly understood⁵. It has long been observed that certain targets with extensive 3' complementarity beyond the seed region can trigger rapid miRNA turnover, a process known as target-directed miRNA degradation (TDMD)⁶. When miRNAs bind to TDMD targets, hereafter referred to as TDMD "triggers", extensive base-pairing induces a conformational change in the Argonaute protein, allowing ZSWIM8 to ubiquitinate and target it for proteasomal degradation, which in turn exposes the miRNA to ribonuclease-mediated decay⁷⁻⁹. Endogenous TDMD in mammals was shown to be mediated by ZSWIM8, a Cullin-RING E3 ubiquitin ligase^{7,8}. Since this discovery, orthologs of ZSWIM8 have been shown to mediate TDMD in other systems, including Dora in *Drosophila* and EBAX-1 in *Caenorhabditis elegans*, indicating that this pathway is evolutionarily conserved^{8,10-12}.

Previously, we showed that ubiquitin ligase EBAX-1 is required for the transgenerational epigenetic inheritance (TEI) of the predatory mouth form following induction by a *Novosphingobium* diet in the nematode *Prisitonchus pacificus*¹³. Loss of EBAX-1 increase the abundance of the heavily-clustered *miR-35/2235a* locus which contains an extreme expansion of 44 *miR-2235a* and 42 *miR-8286b* copies, creating a ~10 kb miRNA-rich region within an otherwise gene-poor genomic region. Compared to *C. elegans*, the *miR-35/2235a* family in *P. pacificus* shows remarkable lineage-specific differences, being greatly expanded into multiple copies and dispersed across nearly all chromosomes rather than confined to a single cluster (Fig. 1a). The role of *Ppa*-EBAX-1 and *miR-2235a* in TEI of the predatory mouth form provides a unique

system in which TDMD can be studied at the organismal level using a clear, naturally occurring binary readout in *P. pacificus*. During postembryonic development, genetically identical *P. pacificus* individuals adopt one of two alternative morphs: the narrow-mouthed stenostomatous (St) form, with a single dorsal tooth, or the wide-mouthed eury stomatous (Eu) form, featuring a claw-like dorsal tooth and an opposing subventral tooth¹⁴. This discrete morphological choice offers a robust phenotypic indicator to probe how miRNA turnover influences transgenerational developmental outcomes.

Such a system suggests that *Ppa*-EBAX-1 may facilitate transgenerational inheritance by destabilizing miRNAs that would otherwise repress TEI of the predatory morph. Given the known functions of EBAX-1/ZSWIM8 proteins, this likely involves TDMD or a related mechanism acting on Argonaute-miRNA complexes, where environmental cues activate *Ppa*-EBAX-1, leading to *miR-2235a* destabilization and derepression of downstream targets required for transgenerational memory. Identifying these triggers is therefore critical to elucidate the molecular mechanisms of TDMD and its phenotypic consequences in *P. pacificus*.

Results

Genome-wide prediction of *mir-2235a* and *mir-8286b* targets

Identifying primary RNA triggers of EBAX-1/ZSWIM8/Dora-mediated miRNA degradation has proven challenging¹⁵. To date, studies in vertebrates have identified only four independent triggers^{16–18}, while work in *Drosophila* uncovered seven endogenous RNA triggers^{11,19}. Although TDMD-inducing targets typically show extensive base pairing with the miRNA 3' end^{7,8,11}, studies in *C. elegans* suggest that degradation of miR-35 family members can occur with seed-sequence pairing alone¹⁰. This raises the possibility that memory transmission of the predatory mouth form in *P. pacificus* may not require extended 3'-end complementarity.

In this context, canonical miRNA targets and classical TDMD triggers represent distinct regulatory modes. Canonical targets, typically containing complementary sites within the 3' UTR, are repressed by miRNAs via translational inhibition or mRNA degradation, directly reducing target expression^{1,20}. In contrast, TDMD triggers, which exhibit extensive complementarity including the miRNA 3' end, promote degradation of the

miRNA itself rather than the target transcript^{8,11,16}. Thus, while canonical targets are actively silenced by the miRNA, TDMD triggers indirectly modulate downstream gene regulation by controlling miRNA stability, providing a distinct mechanism for regulating *miR-2235a* levels in the context of mouth-form memory in *P. pacificus*.

Using established bioinformatic pipelines¹¹, we searched for candidate triggers and identified hundreds of predicted binding sites with full or partial complementarity to *miR-2235a* and *miR-8286b* within the 3' UTR regions of the *P. pacificus* RSC011 genome (Fig. 1b,c). In total, 375 unique *miR-35/miR-2235a* cluster binding sites were identified within 3' UTRs of mRNAs, including 210 sites for *miR-2235* and 165 for *miR-8286b*. As expected, relaxing complementarity criteria from 8-mer to 6-mer substantially increased the number of predicted seed matches.

Several predicted targets correspond to known components of the mouth-form gene regulatory network (Fig. 1c). For example, *lsy-12*, a histone acetyltransferase, contains predicted *miR-2235a* 6-mer and A1-7-mer seed matches, while *nas-6* shows both 6-mer and 7-mer pairing with *miR-8286b*. Additional mouth-form regulators were also predicted to harbor *miR-2235a* cluster binding sites, including *dpy-6*, which contains *miR-8286b* 6-mer and A1-7-mer matches^{21,22}, and *daf-25*²², which contains both 6-mer and A1-7-mer sites for *miR-8286b* and notably an extensive complementarity with *miR-2235a* predicted within the *chs-2* 3' UTR²³. Importantly, all of these predicted canonical targets are yet to be experimentally validated, and their functional relevance to miRNA-mediated repression remains to be tested.

***Ppa-unc-86* triggers *miR-2235a* destabilization**

To prioritize a top predicted TDMD trigger for *miR-2235a* based on minimum free energy, we considered genes with one-to-one orthology to *C. elegans* as well as *Pristionchus*-specific binding sites. A further criterium was that the orthologous genes only contained a *miR-2235a* binding site in the *P. pacificus* copy of the gene but not in *C. elegans* (Fig. 2a). Based on these criteria, I selected *Ppa-unc-86* as a leading candidate trigger for *miR-2235a*. In *P. pacificus*, *unc-86* contains a single predicted *miR-2235a* binding site in its 3' UTR. To distinguish whether *Ppa-unc-86* functions as a TDMD trigger or a conventional miRNA target, we generated four CRISPR-edited alleles affecting the 3' UTR, including insertions and deletions that disrupt miRNA

binding as well as an allele lacking the entire *miR-2235a* binding site (Fig. 2A). All four alleles were viable, exhibited normal growth, and displayed the wild-type RSC011 Eu mouth-form phenotype on *E. coli* (Fig. 2b). Upon exposure to *Novosphingobium* for 5 and 10 generations, all alleles showed full induction of the Eu morph, comparable to wild type (Fig. 2c,d). However, following reversal to *E. coli*, all *Ppa-unc-86* 3' UTR mutants exhibited a complete loss of TEI. While the F10R1 and F10R2 generations showed Eu mouth-form frequencies comparable to wild type, the and F10R3 generation reverted to baseline Eu frequencies, in contrast to wild-type animals, which maintained high Eu penetrance across generations. Thus, disruption of the *miR-2235a* binding site in the *Ppa-unc-86* 3' UTR abolishes transgenerational inheritance of the predatory mouth form. Notably, two independent *Ppa-unc-86* knockout alleles were also generated. However, these mutants exhibited a fully penetrant Eu mouth-form phenotype even when grown on *E. coli*, unlike wild type. This indicates that *unc-86* itself plays a major regulatory role in mouth-form determination. Note that only *Ppa-unc-86* knockout mutants showed the typical touch sensitivity defect, but not the *Ppa-unc-86* 3' UTR mutants.

To determine whether these phenotypes were associated with altered miRNA levels, we performed small RNA (sRNA) sequencing on F5R2 animals and found that, in all *Ppa-unc-86* 3' UTR mutant backgrounds, *miR-2235a*, *miR-8286b*, and other members of the *miR-35/2235a* cluster were significantly upregulated (Fig. 2e). Together, these results demonstrate that mutations disrupting *miR-2235a* binding to *Ppa-unc-86* lead to both miRNA accumulation and loss of TEI. This phenotype closely phenocopies *Ppa-ebax-1* mutants, supporting the conclusion that *Ppa-unc-86* functions as a TDMD trigger upstream of EBAX-1-mediated miRNA destabilization. The distinct phenotypes of *unc-86* knockout and 3'UTR binding-site mutants indicate that UNC-86 functions both as a developmental regulator of mouth form and as a TDMD trigger for *miR-2235a*, with the 3'UTR specifically required for miRNA destabilization and transgenerational inheritance.

An unusual result was observed for these *unc-86* binding-site mutations during the early exposures on *Novosphingobium*, after only three generations. Upon reversal to *E. coli*, an earlier onset of TEI was detected, with the Eu response remaining elevated above baseline from F3R1 to F3R3 relative to wild type (Supplementary Fig.

1). In these mutants, *miR-35/miR-2235a* levels showed a non-significant decrease. Conversely, in *miR-35/miR-2235a*-deficient animals, *unc-86* mRNA showed a non-significant increase, suggesting that any regulation of *unc-86* by this miRNA may be modest or primarily post-transcriptional, consistent with a potential canonical target role (Supplementary Fig. 1).

***Ppa-fkb-5* triggers *miR-8286b* destabilization**

Following analysis of the top predicted *miR-2235a* trigger, we next examined the leading predicted trigger for the other highly clustered miRNA, *miR-8286b*. Similar to *Ppa-unc-86*, *Ppa-fkb-5* contains a single predicted *miR-8286b* binding site within its 3' UTR but not in its *C. elegans* ortholog. To test its function, we introduced a mutation (*tu2220*) that disrupts *miR-8286b* binding at the 3'-end pairing region. Animals carrying the *fkb-5* binding-site mutation exhibited baseline mouth-form responses when grown on *E. coli*. Exposure to *Novosphingobium* resulted in a fully penetrant Eu phenotype that remained stable for 5 and 10 generations (Fig. 3a, b). Upon reversal to *E. coli*, the F5R1 generation showed Eu frequencies comparable to wild type, whereas the F5R2 and F5R3 generations reverted to baseline levels (Fig. 3a), indicating a loss of transgenerational inheritance.

To distinguish effects on TDMD function from potential roles of the protein itself, we generated an *fkb-5* knockout allele by introducing an 11 bp deletion (*tu2326*). Naïve knockout animals displayed mouth-form phenotypes similar to wild type on *E. coli*. Likewise, exposure to *Novosphingobium* for five generations induced a fully penetrant Eu response, followed by a decline to baseline levels in the F5R2 and F5R3 generations after reversal (Fig. 3c). A comparable trend was observed after ten generations of exposure, with the Eu response rapidly diminishing upon reversal and reaching baseline by F10R2 (Fig. 3d). sRNA-seq of animals carrying the *fkb-5* binding-site mutations revealed a significant increase not only in *miR-8286b* but also in *miR-2235a* along its other members (Fig. 3e). Together, these results indicate that the *miR-8286b* binding site within the *fkb-5* 3' UTR is specifically required for transgenerational inheritance of the predatory morph, consistent with a role for *fkb-5* as a TDMD trigger rather than a direct developmental regulator of mouth-form determination. Thus, testing two trigger candidates for their role in TDMD indicated that they are both

involved in miRNA degradation with an associated memory defect of the predatory mouth form.

Exploring the TDMD trigger landscape

Following the observation that binding-site mutations in the 3' UTR regions of *unc-86* and *fkf-5* influence transgenerational mouth-form inheritance, we next systematically explored the broader landscape of predicted TDMD triggers to identify additional transcripts capable of modulating miRNA turnover. Candidate TDMD triggers were prioritized based on the predicted binding stability, using minimum free energy calculations between miRNAs and their targets. The candidate TDMD triggers, *mnp-1*, *vab-1*, and *C05C8.7*, were selected to represent distinct biological modules encompassing environmental response, developmental signaling, and metabolic processes^{24–26}. Specifically, we deleted the entire 3' UTR binding sites of *miR-8286b* in *mnp-1* and *C05C8.7_NA*, and of *miR-2235a* in *vab-1*. Similar to wild-type animals, naïve mutants grown on *E. coli* displayed no mouth-form phenotype, and exposure to *Novosphingobium* for five generations induced a fully penetrant Eu response in all binding-site mutants (Fig. 5b-d). During reversal, mutants of *mnp-1* and *vab-1* retained TEI, with Eu responses overlapping wild type from F5R1 (*vab-1*, $0.81 \leq \text{HDI}(\theta_{R1}) \leq 0.989$; *mnp-1*, $0.787 \leq \text{HDI}(\theta_{R1}) \leq 0.981$) to F5R3 (*vab-1*, $0.651 \leq \text{HDI}(\theta_{R3}) \leq 0.937$; *mnp-1*, $0.548 \leq \text{HDI}(\theta_{R3}) \leq 0.88$) (Fig. 5b,c). In contrast, deletion of the *miR-8286b* binding site in *C05C8.7* abolished TEI, with the Eu morph returning to baseline by F5R2 ($0.403 \leq \text{HDI}(\theta_{R1}) \leq 0.771$) (Fig. 5d). These results indicate that multiple transcripts exhibit TDMD-trigger-like characteristics, with only a subset required for maintaining TEI, in addition to the previously tested triggers *unc-86* and *fkf-5*.

Engineering TDMD sites in EUD-1 reveals functional impact on TEI

Having identified functional TDMD triggers that modulate miRNA stability and transgenerational mouth-form inheritance, we next asked how miRNA-mediated regulation of downstream targets could directly shape developmental outcomes. To establish the epistatic relationship between the TDMD pathway, the miRNA, and the gene regulatory network in which *eud-1* functions as a central developmental switch

controlling mouth-form plasticity^{27,28}, we performed genetic interaction experiments using double mutants.

Double mutants of *eud-1* and the miRNA knockout phenocopied the *eud-1* single mutant, indicating that *eud-1* also acts functionally downstream of the miRNA pathway (Fig. 4a). These epistasis results highlight that downstream effectors mediate miRNA function. To directly test how miRNA regulation of a downstream target can shape developmental outcomes, we engineered extensive miRNA binding sites in the *eud-1* 3'UTR, creating canonical and TDMD-style interactions (Fig. 4b). This allowed us to probe the consequences of miRNA-mediated repression and degradation in a target that normally functions downstream of the miRNA-pathway, but without being an endogenous TDMD substrate under wild type conditions.

Specifically, we introduced one or eight *miR-2235a* binding sites into the *Ppa-eud-1* 3' UTR. While both alleles exhibited wild-type mouth-form phenotypes during exposure to *Novosphingobium*, loss of transgenerational inheritance was observed only in the allele carrying eight *miR-2235a* binding sites (Fig. 5c). This result suggests that the number and strength of miRNA interactions can modulate the efficacy of transgenerational regulation, demonstrating that engineered TDMD-style sites are sufficient to influence miRNA-dependent inheritance of developmental traits.

Conservation and lineage-specific expansion of TDMD triggers

Building on our functional characterization of TDMD triggers, we next explored the evolutionary conservation of predicted TDMD target genes across seven nematode species, including *P. exspectatus*, *P. arcanus*, *P. maxplancki*, *P. japonicus*, *P. entomophagus*, and *P. fissidentatus*. Although some of these genomes remain fragmented and not fully assembled at the chromosome level, searches for *miR-35/miR-2235a* copies are still feasible and reveal considerable variability in copy number across species. For instance, *P. exspectatus* has 33 copies each of *miR-2235a* and *miR-8286b*, *P. arcanus* contains 3 mature *miR-2235* copies located on different scaffolds, *P. maxplancki* has two mature *miR-2235a* copies, *P. japonicus* has 31 mature copies, *P. entomophagus* lacks exact *miR-2235a* copies but shows a major expansion of 302 copies of a related miRNA, *miR-2235b*, and *P. fissidentatus* has no exact *miR-2235a* copies. Given this variability in miRNA copy number across species,

we next asked how predicted TDMD target genes are conserved, revealing that while some candidates are broadly distributed, many exhibit a patchy or lineage-specific pattern (Fig. 5a; Supplementary Table 1).

Specifically, few candidates like ZK1307.6_*fzr-1*, F46A9.5_*skr-1*, and C34G2.2a_*bicd-1*, are present in multiple species but missing in others, showing a patchy conservation pattern. Genes like C05C8.7_*NA*, F18A12.8a_*nep-11*, and K01B6.1_*fozi-1* were found only in *P. pacificus* and absent in all other surveyed species, indicating lineage-specific evolution (Fig. 5a; Supplementary Table 1). Together, these results suggest that while some predicted TDMD trigger may reflect conserved elements of miRNA regulation, many have evolved rapidly within the *Pristionchus* lineage, potentially shaping species-specific traits such as transgenerational mouth-form plasticity.

Discussion

Traditional approaches to identifying TDMD triggers in mammalian and *Drosophila* systems have largely relied on molecular signatures of miRNA destabilization, such as miRNA up-regulation upon loss of the TDMD factor ZSWIM8, cross-species base-pairing predictions, or biochemical assays that detect extensive target:miRNA complementarity (e.g., AGO-CLASH)^{8,17,18}. Several studies have used such strategies to define TDMD-sensitive miRNAs and to pinpoint candidate trigger RNAs on the basis of sequence complementarity and ZSWIM8-dependent miRNA stabilization, often without direct phenotypic readouts^{7,8}. These efforts have successfully expanded the list of endogenous triggers in cultured cells and tissues, but their functional consequences in organismal phenotypes remain limited. In contrast, the binary mouth-form system in *P. pacificus* allows the identification of RNA triggers whose disruption not only stabilizes cognate miRNAs but also abolishes a measurable developmental trait, providing direct in vivo evidence that these triggers control both miRNA function and organismal outcomes. This phenotypically anchored strategy complements existing molecular screens and highlights the value of integrating physiological assays into TDMD discovery pipelines, particularly for uncovering triggers with noncanonical pairing or context-dependent effects that might be missed by sequence-based approaches alone.

275 In this study, we investigated the broader landscape of predicted RNAs that could act
276 as triggers or canonical targets of the *miR-35/miR-2235a* family and uncovered a
277 central role for target-directed miRNA degradation (TDMD) in regulating
278 transgenerational mouth-form inheritance in *P. pacificus*. By combining bioinformatic
279 prediction, genetic perturbation, and small RNA profiling, we show that specific 3' UTR
280 binding sites within endogenous transcripts function as TDMD triggers that control the
281 stability of *miR-35/2235a* cluster members. We further demonstrate that mutations
282 disrupting these sites abolish transgenerational inheritance of the predatory morph
283 and lead to stabilization of their cognate miRNAs, indicating that regulated miRNA
284 turnover, rather than canonical target repression alone, is essential for maintaining
285 developmental memory. Although most predicted targets possess one-to-one
286 orthologs in *Caenorhabditis elegans*, the majority of 3' UTR binding sites appear to
287 be *Pristionchus*-specific, suggesting lineage-specific evolution of TDMD-mediated
288 regulation.

289 sRNA-seq analysis of 3' UTR binding-site mutants revealed that disruption of TDMD
290 triggers in *Ppa-unc-86* or *Ppa-fkb-5* stabilized not only their cognate miRNAs (*miR-*
291 *2235a* and *miR-8286*) but also led to coordinated upregulation of other members of
292 the *miR-35/2235a* cluster. This pattern is consistent with TDMD for several reasons.
293 First, *miR-8286b* and *miR-2235a* may be co-transcribed as part of a polycistronic
294 cluster, and TDMD of one miRNA could indirectly influence the stability or processing
295 of other cluster members ^{8,16}. Second, miRNA turnover machinery, including
296 ZSWIM8/EBAX-1-mediated TDMD, may preferentially target a single miRNA for
297 degradation while indirectly affecting the availability or RISC loading of other co-
298 expressed miRNAs ^{7,11}. Third, feedback mechanisms in miRNA homeostasis can
299 stabilize other cluster members when one miRNA is no longer degraded, leading to a
300 coordinated increase in cluster-wide miRNA levels ^{10,16}. Importantly, the direct effects
301 of the *Ppa-fkb-5* 3' UTR on *miR-8286b* and the *Ppa-unc-86* 3' UTR on *miR-2235a* are
302 specific to their respective miRNAs, while the upregulation of other cluster members
303 likely represents a secondary, indirect consequence of cluster co-regulation rather
304 than a loss of TDMD function. Therefore, these observations remain fully consistent
305 with *Ppa-fkb-5* and *Ppa-unc-86* acting as TDMD triggers for *miR-8286b* and *miR-*
306 *2235a*, respectively.

307 Together, our findings illustrate how TDMD contributes to fine-tuning miRNA stability
308 and shaping organismal phenotypes in a lineage-specific context. By revealing both
309 conserved and species-specific TDMD triggers, our results underscore the
310 evolutionary flexibility of this mechanism and its potential to modulate organism-level
311 traits. More generally, this work illustrates the value of combining genetic, molecular,
312 and phenotypic analyses to uncover regulatory interactions that may be overlooked by
313 approaches relying solely on sequence or biochemical features.

314 **Methods**

315 **Experimental model and bacterial culture conditions**

316 The ancestral *P. pacificus* strain RSC011 used in this work was isolated from Coteau
317 Kerveguen on La Réunion. Worms were cultured under standard conditions on NGM
318 plates seeded with either *E. coli* OP50 or *Novosphingobium* L76 and maintained at 20
319 °C. To avoid confounding influences on mouth-form ratios, the stock population was
320 not exposed to bleaching, starvation, or large temperature shifts. Bacterial strains and
321 mutant lines were grown overnight in LB (Lysogeny broth), with kanamycin added to
322 a final concentration of 50 µg/ml when needed for food-reversal experiments. Cultures
323 were incubated at 30 °C or 37 °C depending on species requirements. For nematode
324 feeding, 300 µl of overnight cultures were spread onto 6-cm NGM plates and allowed
325 to grow for two days prior to use.

326 **Nematode culture and dietary reversal experiments**

327 Dietary exposure and reversal assays were conducted largely as described
328 previously^{13,29}. For diet-switch experiments, individual J4 larvae were transferred
329 to *Novosphingobium* L76 plates, with a brief pre-exposure step (~1 hour) on initial L76
330 plates to minimize OP50 carryover before placement onto final experimental plates.
331 For worms previously maintained on *Novosphingobium* L76, worms were first cultured
332 for one generation on NGM plates containing 50 µg/ml kanamycin and were then
333 transferred to OP50-seeded plates for subsequent generations.

334 **Statistical analysis of** mouth-form phenotype

335 Mouth-form was scored under a stereomicroscope (150× magnification) by examining
336 the buccal cavity according to established morphological criteria²⁷. Final frequencies
337 were calculated as the mean of at least 10-20 biological replicates, with each replicate
338 consisting of 20 animals assessed from a single plate. Statistical analysis to estimate
339 the probability of developing the Eu mouth form in *P. pacificus* (θ) were based on a
340 previously established hierarchical Bayesian model¹³.

CRISPR/Cas9-Induced Mutations

Mutations in target genes were generated using CRISPR/Cas9 according to established protocols³⁰. Specifically, Gene-specific crRNAs and universal trans-activating CRISPR RNA (tracrRNA) were combined, denatured and annealed. The mixture was complexed with Cas9 before microinjection into the germline of 40-50 young adult *P. pacificus* RSC011. Eggs from injected P0 animals were collected for up to 16 hours post-injection. After hatching and two days of growth, F1 progeny were isolated onto individual plates until they matured and produced sufficient eggs. F1 genotypes were determined by Sanger sequencing to identify mutations before establishing homozygous mutant lines. All sgRNA and primer sequences are listed in the supplementary table. All sgRNA and primer sequences are listed in the supplementary materials.

miRNA target site prediction and conservation analysis across species

The pipeline for identifying TDMD triggers was adapted from a previously established in silico method in *Drosophila*¹¹. Using the new and improved RSC011 annotations, All 3'UTR annotations for the RSC011 strain were loaded as input to initiate miRNA target searches. 3'UTR sequences from other *Pristionchus* species were defined as the sequence 100 nucleotide downstream of the annotated protein. The pipeline queried complementarity to the 3' region of *miR-2235a* (22 nt) and *miR-8286b* (extended to 22 nt), starting from the 13th nucleotide to the 3' end of the miRNA. Complementarity to the seed region (nucleotides 2-8) was also assessed within 30 nt upstream of the 3' region, allowing up to two mismatches in both seed and 3'-end analyses. Canonical miRNA-target pairing was assessed by restricting the query to the seed sequence. Multiple types of miRNA-target interactions were evaluated, including 8-nt, 7-nt, A1-7-nt, and 6-nt pairing. These searches were performed independently of expression datasets.

sRNA extraction and sequencing

Total RNA was extracted from mixed-stage RSC011 worms collected from three NGM plates cultured on either *Novosphingobium* or *E. coli* OP50. Worms were collected and pelleted after defined generations of dietary exposure. Total RNA was then

371 isolated using the Direct-Zol RNA MiniPrep kit (Zymo Research) following the
372 manufacturer's instructions. For sRNA processing, <4 µg of RNA was treated with 25 U
373 of TAP (Cap-Clip Acid Pyrophosphatase) in 5 µl of 10× reaction buffer, and PCR-grade
374 water was added to a final volume of 50 µl per sample. The reaction was incubated at
375 37 °C for 2 hours. RNA was then purified using the RNA Clean & Concentrator kit
376 (Zymo Research) following the manufacturer's instructions. sRNA-seq library
377 preparation and 50-nt single-end sequencing were performed by Novogene.

References

1. Bartel, D. P. Metazoan MicroRNAs. *Cell* **173**, 20–51 (2018).
2. Frédérick, P.-M. & Simard, M. J. Regulation and different functions of the animal microRNA-induced silencing complex. *WIREs RNA* **13**, e1701 (2022).
3. Friedman, R. C., Farh, K. K. H., Burge, C. B. & Bartel, D. P. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res.* **19**, 92–105 (2009).
4. Agarwal, V., Bell, G. W., Nam, J.-W. & Bartel, D. P. Predicting effective microRNA target sites in mammalian mRNAs. *Elife* **4**, e05005 (2015).
5. Treiber, T., Treiber, N. & Meister, G. Regulation of microRNA biogenesis and its crosstalk with other cellular pathways. *Nat. Rev. Mol. Cell Biol.* **20**, 5–20 (2019).
6. Buhagiar, A. F. & Kleaveland, B. To kill a microRNA: emerging concepts in target-directed microRNA degradation. *Nucleic Acids Res.* **52**, 1558–1574 (2024).
7. Han, J. *et al.* A ubiquitin ligase mediates target-directed microRNA decay independently of tailing and trimming. *Science* (1979). **370**, (2020).
8. Shi, C. Y. *et al.* The ZSWIM8 ubiquitin ligase mediates target-directed microRNA degradation. *Science* (1979). **370**, 1–10 (2020).
9. Han, J. & Mendell, J. T. MicroRNA turnover: a tale of tailing, trimming, and targets. *Trends Biochem. Sci.* **48**, 26–39 (2023).
10. Donnelly, B. F. *et al.* The developmentally timed decay of an essential microRNA family is seed-sequence dependent. *Cell Rep.* **40**, (2022).
11. Kingston, E. R., Blodgett, L. W. & Bartel, D. P. Endogenous transcripts direct microRNA degradation in *Drosophila*, and this targeted degradation is required for proper embryonic development. *Mol. Cell* **82**, 3872–3884 (2022).
12. Stubna, M. W., Shukla, A. & Bartel, D. P. Widespread destabilization of *Caenorhabditis elegans* microRNAs by the E3 ubiquitin ligase EBAX-1. *RNA* **31**, 51–66 (2025).
13. Quiobe, S. P. *et al.* EBAX-1/ZSWIM8 destabilizes miRNAs resulting in transgenerational memory of a predatory trait. *Sci. Adv.* **11**, 1–17 (2025).
14. Wilecki, M., Lightfoot, J. W., Susoy, V. & Sommer, R. J. Predatory feeding behaviour in *Pristionchus* nematodes is dependent on phenotypic plasticity and induced by serotonin. *Journal of Experimental Biology* **218**, 1306–1313 (2015).
15. Wang, P. Y. & Bartel, D. P. A statistical approach for identifying primary substrates of ZSWIM8-mediated microRNA degradation in small-RNA sequencing data. *BMC Bioinformatics* **24**, 1–18 (2023).
16. Bitetti, A. *et al.* MicroRNA degradation by a conserved target RNA regulates animal behavior. *Nat. Struct. Mol. Biol.* **25**, 244–251 (2018).

17. Ghini, F. *et al.* Endogenous transcripts control miRNA levels and activity in mammalian cells by target-directed miRNA degradation. *Nat. Commun.* **9**, (2018).
18. Li, L. *et al.* Widespread microRNA degradation elements in target mRNAs can assist the encoded proteins. *Genes Dev.* **35**, 1595–1609 (2021).
19. Hiers, N. M. *et al.* An endogenous cluster of target-directed microRNA degradation sites induces decay of distinct microRNA families. *Cell Rep.* **44**, (2025).
20. Jonas, S. & Izaurralde, E. Towards a molecular understanding of microRNA-mediated gene silencing. *Nat. Rev. Genet.* **16**, 421–433 (2015).
21. Sun, S., Theska, T., Witte, H., Ragsdale, E. J. & Sommer, R. J. The oscillating Mucin-type protein DPY-6 has a conserved role in nematode mouth and cuticle formation. *Genetics* **220**, iyab233 (2022).
22. Lenuzzi, M. *et al.* Influence of environmental temperature on mouth-form plasticity in *Pristionchus pacificus* acts through daf-11-dependent cGMP signaling. *J. Exp. Zool. B Mol. Dev. Evol.* **340**, 214–224 (2023).
23. Sun, S., Witte, H. & Sommer, R. J. Chitin contributes to the formation of a feeding structure in a predatory nematode. *Current Biology* **33**, 15–27 (2023).
24. Tucker, M. & Han, M. Muscle cell migrations of *C. elegans* are mediated by the α -integrin INA-1, Eph receptor VAB-1, and a novel peptidase homologue MNP-1. *Dev. Biol.* **318**, 215–223 (2008).
25. Chin-Sang, I. *et al.* The Ephrin VAB-2/EFN-1 Functions in Neuronal Signaling to Regulate Epidermal Morphogenesis in *C. elegans*. *Cell* **99**, 781–790 (1999).
26. Santillan, E. M., Cormack, E. D., Wang, J., Rodriguez-Steube, M. & Cochella, L. An ancient and essential miRNA family controls cellular interaction pathways in *C. elegans*. *Sci. Adv.* **11**, 1–11 (2025).
27. Ragsdale, E. J., Müller, M. R., Rödelisperger, C. & Sommer, R. J. A developmental switch coupled to the evolution of plasticity acts through a sulfatase. *Cell* **155**, 922–933 (2013).
28. Kieninger, M. R. *et al.* The Nuclear Hormone Receptor NHR-40 Acts Downstream of the Sulfatase EUD-1 as Part of a Developmental Plasticity Switch in *Pristionchus*. *Current Biology* **26**, 2174–2179 (2016).
29. Quiobe, S. P. *et al.* Diet-derived vitamin B12 induces transgenerational inheritance of nematode predation through elevated vitellogenin provisioning. *bioRxiv* 2025.05.21.655301 (2025).
30. Witte, H. *et al.* Gene inactivation using the CRISPR/Cas9 system in the nematode *Pristionchus pacificus*. *Dev. Genes Evol.* **225**, 55–62 (2014).

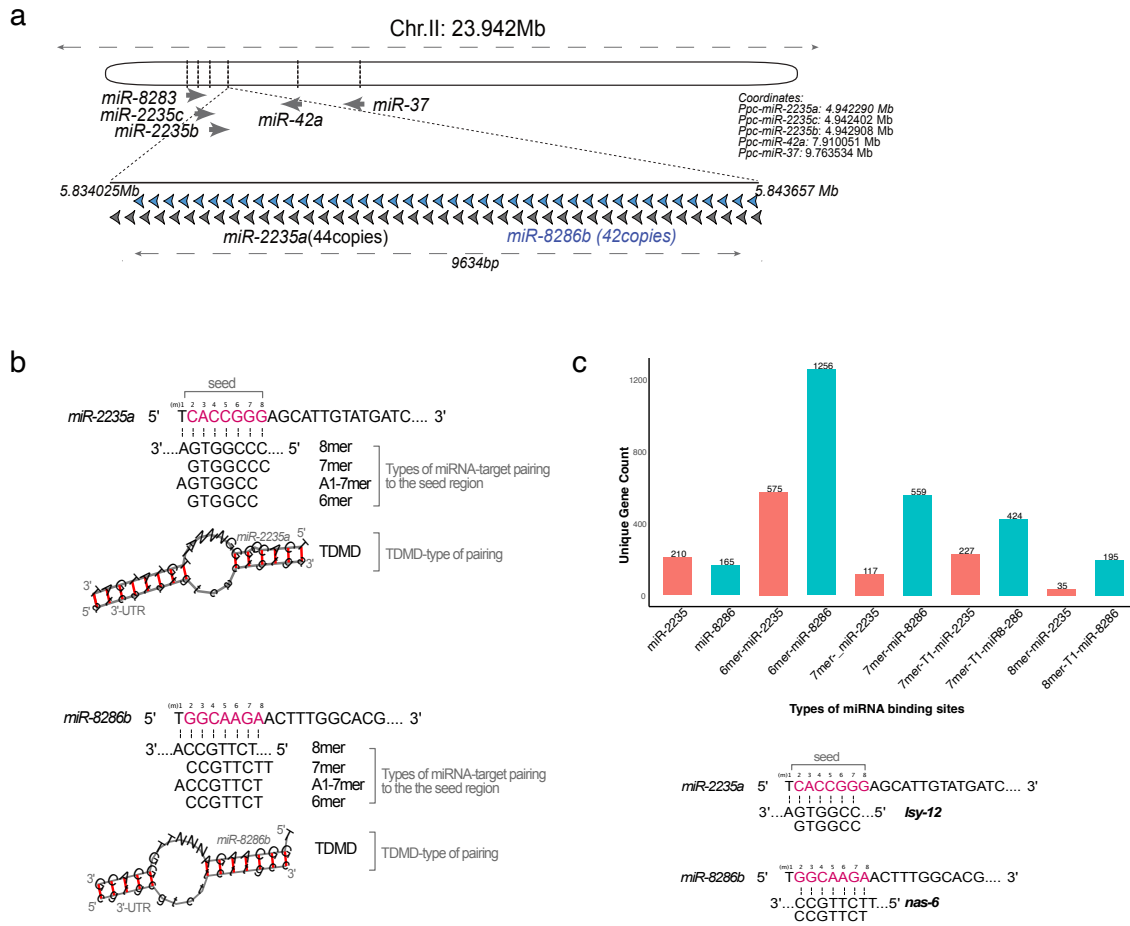


Fig. 1. **a**, The *miR-35/miR-2235a* family members found in chromosome II. With *miR-2235a* and *miR-8286b* being the most heavily expanded member, with 44 and 42 copies respectively. Gray arrowheads, *miR-2235a* including other members; blue arrowheads, *miR-8286b*. **b**, miRNA sequences of *miR-2235a* and *miR-8286b* with the different types pairing towards the target RNA. **c**, Total number of unique predicted target sites in the 3'UTR regions of the *P. pacificus* RSC011 genome. Visualisation of the type of miRNA-target pairing that exists in the genes found in the gene-regulatory network, like *Ppa-lsy-12* and *Ppa-nas-6*.

the previous experimental data. Yellow area highlights the RSC011 baseline response averaged across 101 generations on *E. coli*, as previously established¹³.

e, Differential small-RNA expression analysis of *tu226* vs the wild type during F10R2 reversal. The upregulated *miR-2235a* family members and their isoforms (*) are labeled.

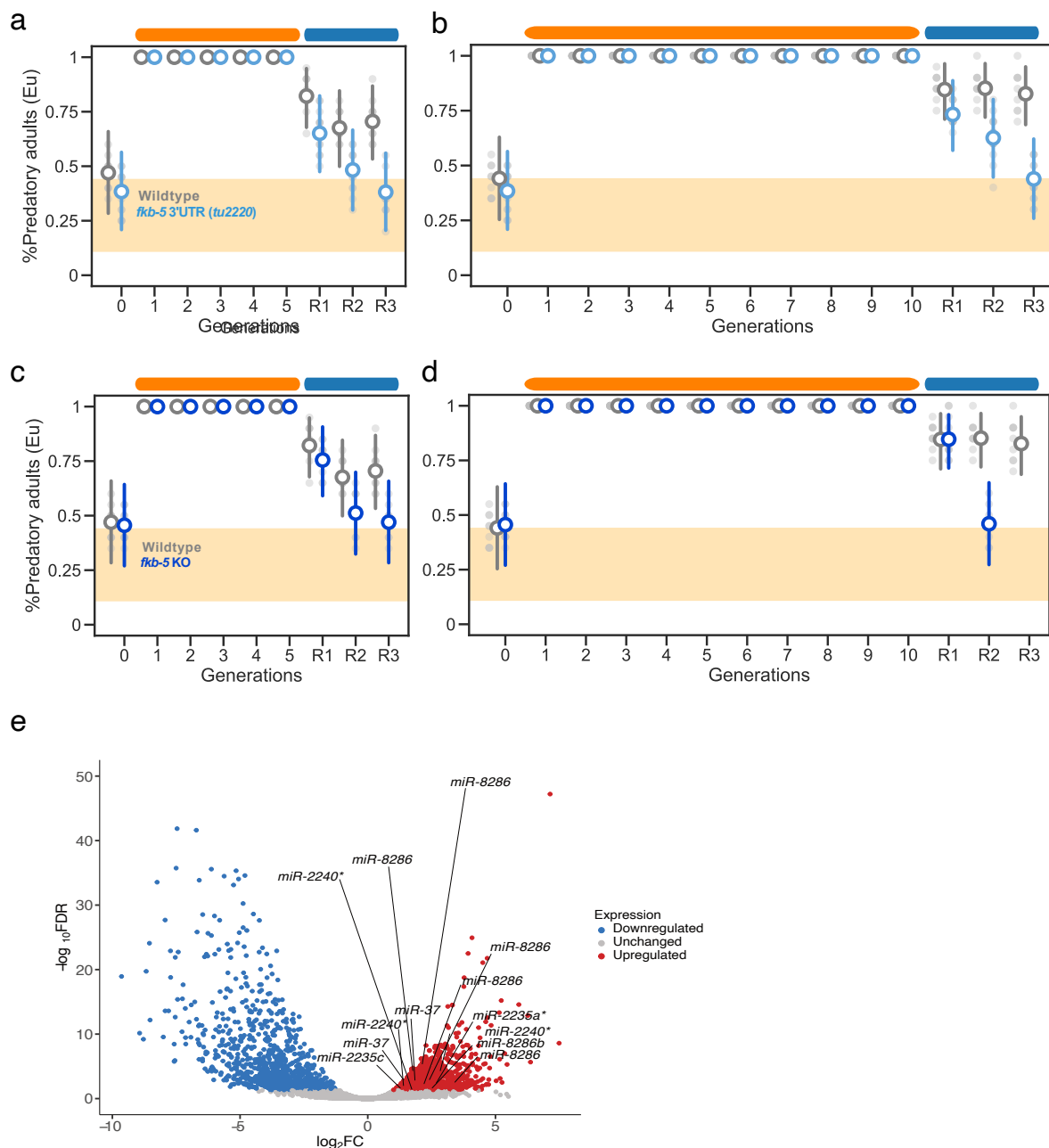


Fig. 3. a, Mean probability of the predatory mouth form in the 3' UTR region of *Ppa-fkf-5*, a top predicted trigger candidate of *miR-8286b*, during an exposure of 5

generations and **b**, 10 generations on *Novosphingobium* and reversal to *E. coli*. **c**, Mean probability of the predatory mouth form in the *fkf-5* knockout mutants for 5 and, **d**, 10 generations on *Novosphingobium* and reversal to *E. coli*. **e**, Differential small-RNA expression analysis of *tu2220* vs the wild type during F10R2 reversal. The upregulated *miR-2235* family members and their isoforms (*) are labeled.

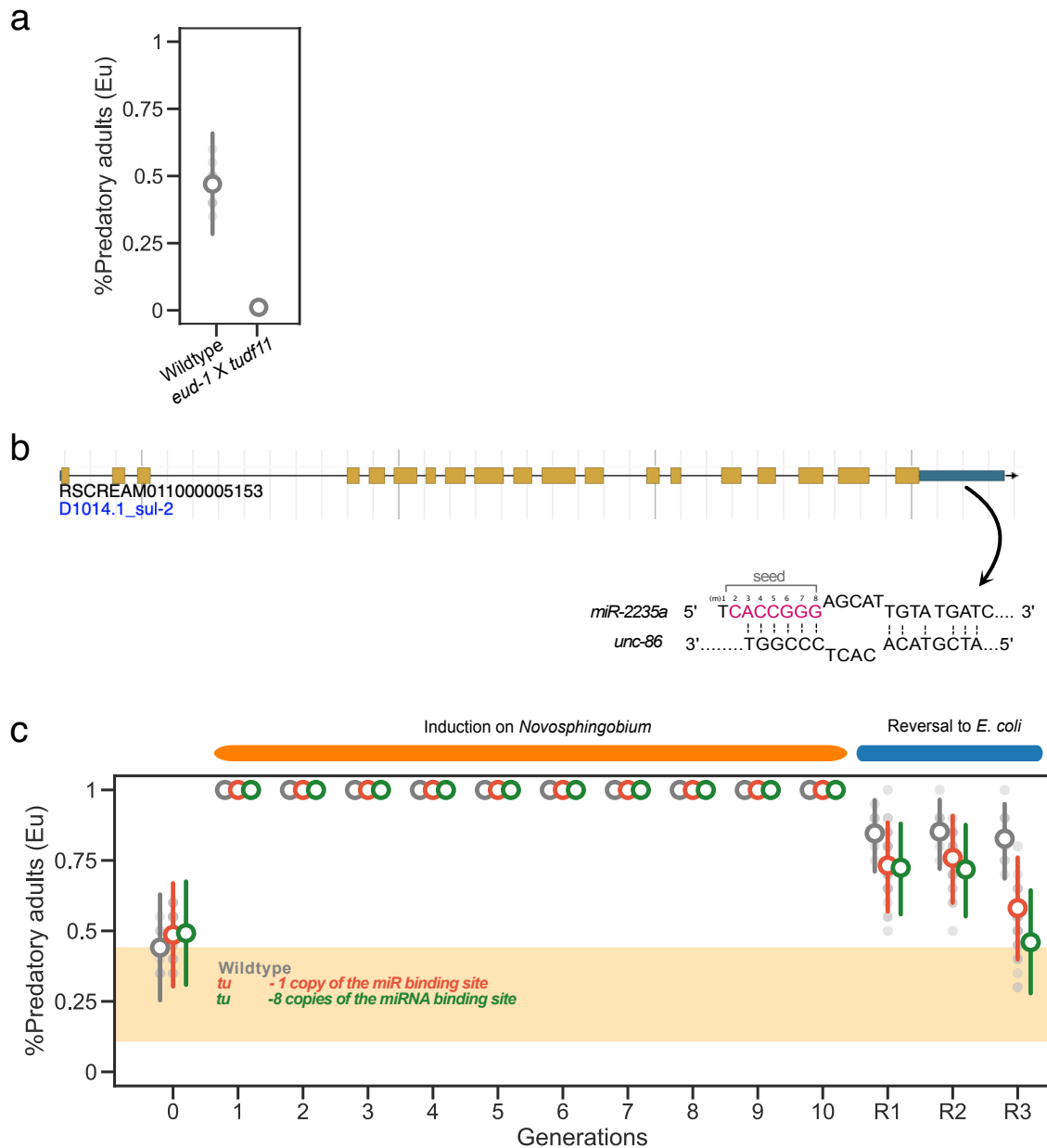


Fig. 4. **a**, Mean probability of the predatory mouth form of the RSC011 wildtype and the double mutant from crossing between *Ppa-eud-1(tu1695)* and miRNA-deficient allele (*tuDf11*).

b, A graphical representation of *Ppa-eud-1* gene model. The arrow indicates the 3'UTR region where the miR-2235 binding sites were introduced by CRISPR/Cas9.

c, Mean probability of the predatory mouth form of the two alleles with the engineered miR-2235 binding sites for 10 generations on *Novosphingobium* and reversal to *E. coli*.

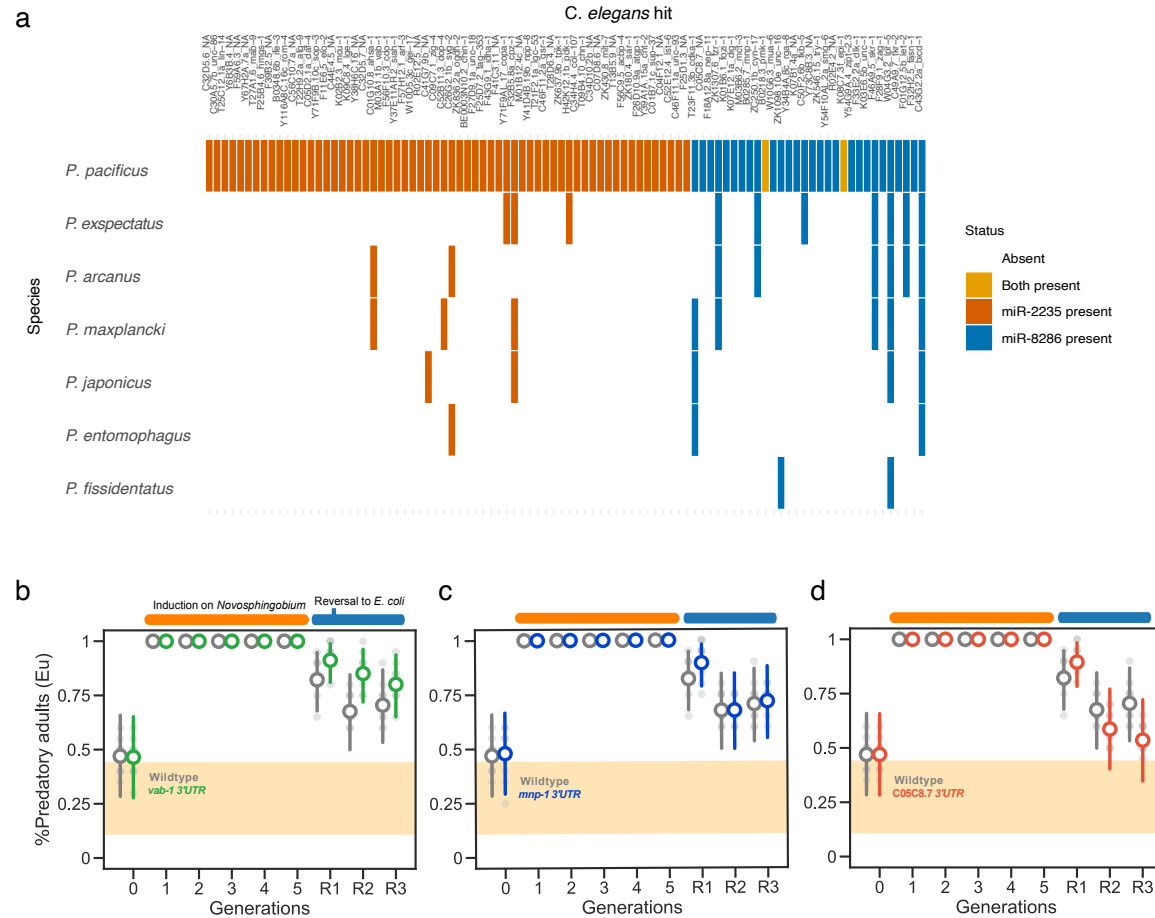


Fig. 5. a, The evolutionary conservation of top predicted TDMD triggers for both *miR-2235a* and *miR-8286b* was examined across seven nematode species. Predicted TDMD triggers display mixed conservation patterns, with some broadly shared across species and others showing lineage-specific distributions. **b**, Mean probability of the predatory mouth form during the *Novosphingobium* exposure of 10 generations and its reversal to *E. coli*. The assay was done using animals having mutations in the binding sites of *miR-2235a* (*Ppa-vab-1*) and of *miR-8286b* (*Ppa-mnp-1* and the uncharacterized C05C8.7).

**Genetic analysis of the Weismann Barrier in *Pristionchus*
*Pacificus***

Shiela Pearl Quiobe, Hanh Witte, Christian Rödelsperger, Ralf J. Sommer

Ready for Submission

Genetic analysis of the Weismann Barrier in *Pristionchus pacificus*

Shiela Pearl Quiobe¹, Hanh Witte¹, Christian Rödelberger¹, Ralf J. Sommer¹

¹Max Planck Institute for Biology Tübingen, Department for Integrative Evolutionary Biology, Max-Planck Ring 9, 72076 Tübingen, Germany

Abstract

Transgenerational epigenetic inheritance (TEI) challenges the classical Weismann barrier by demonstrating that environmentally induced somatic signals can influence germline transmission. Here, we investigate the role of maternal provisioning in mediating this process using the nematode *Pristionchus pacificus*. We show that vitellogenins act as essential soma-to-germline carriers required for the stable inheritance of a diet-induced predatory mouth-form phenotype. Progressive deletion of vitellogenin genes reduces the efficiency of environmental induction and completely abolishes TEI, phenocopying loss of the vitellogenin receptor RME-2. Although bacterial vitamin B12 is sufficient to trigger the predatory morph and its TEI, vitamin B12 supplementation cannot rescue inheritance in vitellogenin or *rme-2* mutants, demonstrating that germline delivery depends on yolk-mediated transport. Genetic analyses further reveal that RME-2 functions downstream of microRNA pathways, indicating that vitellogenins cooperate with small RNAs to sustain heritable memory. Together, our findings identify vitellogenins as key mediators of maternal-to-progeny communication, providing a mechanistic framework for how environmental information crosses the soma-germline barrier.

Introduction

At the end of the nineteenth century, when the discovery of DNA and the molecular mechanisms underlying development and epigenetic regulation still lay in the future, August Weismann proposed the germ plasm theory of heredity. While this concept

correctly identified sperm and eggs as the carriers of heritable information responsible for forming the developing embryo, other aspects proved incomplete -particularly the notion of a strict separation between somatic tissues and the germline, later termed the Weismann barrier, and the idea that somatic cells could not contribute heritable information to germ cells ^{1,2}.

In recent decades, the discovery of epigenetic mechanisms such as DNA methylation, chromatin modifications, and small RNA transmission has challenged the traditional view of heredity. However, the role of maternal provisioning, particularly vitellogenins, as major conduits for transmitting somatic information into the germline, and thereby bypassing the Weismann barrier, remains largely unexplored ^{3,4}. These processes illustrate that environmentally induced states can influence progeny phenotypes across generations, yet the mechanisms by which somatic signals cross into the germline are still poorly understood.

Maternal deposition of vitellogenin represents a direct and substantial flow of material from soma to germline in oviparous species, with the potential to influence progeny phenotypes ⁴. In the nematode *Pristionchus pacificus*, long-term dietary induction experiments have shown that dietary induction to *Novopshingobium* triggers transgenerational epigenetic inheritance (TEI) of the predatory morph. This process requires the ubiquitin ligase EBAX--1/ZSWIM8 by destabilizing the clustered miR-2235a/miR-35 family, which normally acts as a repressor of this trait ^{5,6}. Following this, we previously showed that bacteria-derived vitamin B12 is both necessary and sufficient to induce transgenerational memory and goes through the methionine synthase ⁶. Disruption of microbial vitamin B12 synthesis abolishes TEI of the predatory morph, while supplementation restores it. Interestingly, the induction of the predatory morph is inherited through increased vitellogenin transcription, and requires the major vitellogenin receptor *Ppa-rme-2*, indicating maternal nutrient provisioning as the transmission mechanism ⁶.

In *Caenorhabditis elegans*, the six vitellogenins are among the most highly expressed genes in the adult hermaphrodite intestine, producing copious yolk to provision developing eggs ⁴. Beyond their nutritional role, these yolk proteins provide a well-characterized system for studying how metabolic and environmental cues influence energy balance, aging, and progeny physiology. Building on this framework, studies

in *Pristionchus pacificus* suggest that vitellogenins may also mediate transgenerational epigenetic inheritance, linking maternal nutrient provisioning to stable, environmentally induced phenotypes in the next generation. Here, we investigate conserved pathways involved in maternal deposition via vitellogenins in the context of mouth-form plasticity in *P. pacificus*, aiming to understand how nutrient and small RNA-mediated mechanisms contribute to TEI.

Results

Deletion of eight and nine *vit*-genes showed absence of TEI

Pristionchus pacificus exhibits a more complex vitellogenin repertoire compared with *C. elegans*. Whereas the *C. elegans* N2 genome contains six vitellogenin genes, the *P. pacificus* RSC011 genome harbors nine genes, all of which are most closely related to *Cel-vit-6* (Fig. 1a). Accordingly, these genes were designated *Ppa-vit-6-A* through *Ppa-vit-6-I* based on their chromosomal positions (Fig. 1b). Although the *Ppa-vit* genes are more similar to one another than the *Cel-vit* genes, where *Cel-vit-6* is the single divergent member (Spieth et al., 1985), there is still sufficient sequence divergence among the *Ppa-vit* genes to allow them to be individually distinguished in gene expression analyses (Quiobe et al., 2025).

To evaluate the role of vitellogenins in transgenerational epigenetic inheritance (TEI) of the Eu morph, we designed guide RNAs (gRNAs) that simultaneously targeted multiple *Ppa-vit* genes due to their high sequence similarity. Although this similarity complicated the design of gene-specific gRNAs, subsequent validation of homozygous mutations was achieved using gene-specific primers for Sanger sequencing. Using this approach, we generated and cryopreserved mutant lines carrying quadruple (*tu2348*), pentuple (*tu2349*), octuple (*tu2273*), and nonuple (*tu2354*) mutations across the *Ppa-vit* gene family. Mutations in these backgrounds were confirmed by Sanger sequencing, with whole-genome sequencing further validating the octuple and nonuple mutants. Although frameshift mutations were generated in most *Ppa-vit* genes, *Ppa-vit-6E* contained an in-frame deletion of three base pairs, which may retain partial protein function. To assess the functional

significance of *Ppa-vit* genes, all mutant lines were subjected to *Novosphingobium* exposure and reversal assays. Quadruple and pentuple mutants displayed complete Eu morph induction across five generations on *Novosphingobium*, similar to wild type (Fig. 1c,d). In contrast, octuple and nonuple mutants grown on *E. coli* showed no mouth-form phenotype on their own, but exhibited a high yet incomplete Eu induction when exposed to *Novosphingobium* across five generations (Fig. 1e,f). These results indicate that Eu morph induction remains largely intact despite extensive loss of vitellogenins, although the magnitude of induction decreases with increasing numbers of deleted genes.

Strikingly, upon reversal to *E. coli*, all vitellogenin mutant backgrounds failed to maintain transgenerational memory. Eu morph frequencies returned to baseline by the F5R2 generation in quadruple mutants and dropped immediately in the F5R1 generation in octuple and nonuple mutants (Fig. 1c-f). Notably, the octuple and nonuple vitellogenin knockouts phenocopied the *Ppa-rme-2* mutant, indicating that vitellogenin provisioning is essential for transgenerational epigenetic inheritance of the predatory mouth form (Fig. 1e,f). While Eu morph induction can still occur in the absence of multiple vitellogenins, its magnitude progressively declines with increasing gene loss, suggesting that vitellogenins contribute in a dosage-dependent manner to the efficiency of environmental induction but are strictly required for the stable transmission of the response across generations.

***rme-2* is downstream of the miRNA pathway**

We previously demonstrated that the *miR-2235/miR-35* cluster in *P. pacificus* functions as a repressor TEI⁶. To investigate whether yolk provisioning contributes to the accelerated TEI phenotype observed upon deletion of this miRNA cluster, we examined the role of the major vitellogenin receptor *rme-2*. In nematodes, vitellogenins are synthesized in the intestine and transported to the germline via receptor-mediated endocytosis, a process that depends exclusively on the low-density lipoprotein receptor (LDLR) encoded by *rme-2*^{4,7}.

We previously analyzed the *Ppa-rme-2* (*tu2155*) allele, which carries a 16 bp insertion resulting in a premature stop codon, using a five-generation *Novosphingobium* induction assay⁶. Across all generations, worms

harboring this mutation exhibited only incomplete induction of the Eu morph and after the reversal to *E. coli*, *Ppa-rme-2* (*tu2155*) did not show transgenerational memory of the predatory morph⁶. To determine whether *rme-2* is epistatic to *miR-2235* or vice versa, we generated double mutants carrying *rme-2* (*tu2155*) and *miR-2235* (*tuDf11*) through genetic crosses (Fig. 2a). If *miR-2235* functions upstream of *rme-2*, loss of *rme-2* would be expected to abolish the prolonged transgenerational inheritance observed in miRNA-deficient mutants. Consistent with this prediction, double mutant animals exposed to *Novosphingobium* for a single generation exhibited a high but incomplete Eu mouth-form induction relative to wild type, resembling the phenotype of *Ppa-rme-2* (*tu2155*) single mutants (Fig. 2a). This elevated yet incomplete response persisted across five generations. Moreover, upon reversal to *E. coli*, Eu morph frequencies rapidly returned to baseline by the F5R1 generation (Fig. 2a). Together, these results indicate that *rme-2* acts downstream of the *miR-2235* family in regulating transgenerational inheritance of the predatory mouth form.

Vitamin B12 supplementation tests limiting germline uptake in RME-2-mediated vitellogenin transport

In *P. pacificus*, the inherited effect of vitamin B12 acts through vitellogenin, and exposure to *Novosphingobium* induces vitellogenin expression at the transcription level⁶. To test whether vitamin B12 could rescue the *rme-2* or octuple/nanuple vitellogenin knockout phenotypes, we performed vitamin B12 supplementation experiments. Although *Novosphingobium* naturally produces vitamin B12^{6,8}, its delivery to the germline in wild type worms could depend on vitellogenins and the RME-2 receptor. The supplementation experiments had two main objectives: first, to assess whether germline vitamin B12 uptake is limiting and whether increased availability can bypass defects in the RME-2 or vitellogenin pathways; and second, to distinguish nutrient effects from carrier-mediated delivery, testing whether the RME-2-vitellogenin system is strictly required or if excess B12 alone is sufficient. Ideally, supplementation should not rescue the *rme-2* phenotype, as other vitellogenin-associated factors, including vitamins and lipids, cannot be efficiently transported into the germline without RME-2^{4,7}. However, phenotypic rescue would suggest that vitamin B12 availability is normally limiting and that vitellogenin uptake mediated by

149 RME-2 functions to concentrate critical factors in the germline to support
150 transgenerational inheritance.

151 To test these hypotheses, diets were supplemented with vitamin B12 at a final
152 concentration of 1,500 nM, a level established in earlier work ⁶. To test whether vitamin
153 B12 alone could induce TEI of the predatory mouth form, we performed a mini-assay
154 consisting of five generations of exposure to a B12-supplemented *E. coli* diet followed
155 by three generations on an unsupplemented diet. In wild type animals, vitamin B12
156 supplementation caused an immediate and strong, though incomplete, induction of the
157 Eu mouth form that persisted throughout the five generations of exposure (Fig. 2b).
158 Notably, TEI of the Eu morph was maintained for three subsequent generations after
159 removal of supplementation. In contrast, supplementation failed to rescue TEI in *rme*-
160 2 mutants, *rme*-2; miRNA-deficient double mutants, or nanuple vitellogenin mutants
161 (Fig. 2b-d). Together, these results indicate that increasing vitamin B12 availability is
162 insufficient to rescue TEI and that its effect strictly depends on vitellogenin-mediated
163 maternal provisioning and RME-2-dependent transport into the germline.

Discussion

In *C. elegans*, vitellogenins are synthesized in the intestine of adult hermaphrodites and transported to the germline. This process starts with yolk complexes being secreted into the pseudocoelom, traversing the gonadal basal lamina and sheath cell pores, and finally being internalized by maturing oocytes via receptor-mediated endocytosis^{7,9,10}. This pathway ensures efficient maternal provisioning of lipids, vitamins, and other factors critical for progeny development. Our results in *P. pacificus* indicate that vitellogenins serve as the primary conduits for transmitting environmentally derived signals, with the initial dietary induction of the predatory Eu mouth form depending on the number of vitellogenin genes disrupted, and complete transgenerational inheritance being abolished upon deletion of multiple vitellogenin genes or disruption of the RME-2 receptor. Moreover, vitamin B12 supplementation cannot bypass this requirement, indicating that maternal transport of other vitellogenin-associated factors into the germline is essential for initiating TEI. These findings position vitellogenins as a key soma-to-germline conduit in oviparous species, including *P. pacificus*, capable of transporting other factors to initiate and sustain transgenerational memory.

Deposition of vitellogenin alone cannot be attributed to long-lasting TEI effects, but could be due to other wide range of vitellogenin-associated factors^{11,12}. Among these, small RNAs are particularly likely to coordinate diverse transgenerational epigenetic effects by surveying and interacting with both the transcriptome and genome. In *C. elegans*, heritable small RNAs are strongly enriched for vitellogenin targets, and antisense transcription across these yolk genes may generate dsRNA precursors that initiate small RNA amplification^{11,13}. Small RNA-mediated regulation is uniquely suited for this role because of its sequence-specific targeting via base pairing and the existence of dedicated machinery that enables non-cell-autonomous activity, distinguishing it from other epigenetic processes such as DNA methylation or histone modification^{14,15}. Consequently, small RNAs provide a powerful molecular framework for epigenetic communication across cells, generations, and even between organisms, allowing inherited small RNAs to be propagated and to exert long-lasting transgenerational effects^{14,16}. Consistently, in *P. pacificus*, TEI of the predatory mouth form strictly requires both maternal vitellogenin provisioning via RME-2 and functional

196 group of microRNAs, as RME-2 and miRNA deficient mutants completely abolish
197 inheritance. Similarly in insects, maternally deposited vitellogenin mediates
198 transgenerational immune priming by binding pathogens and pathogen-associated
199 molecular patterns, transporting these immune elicitors into developing oocytes to
200 prime offspring immunity ¹². These results highlight vitellogenins as key mediators of
201 maternal-to-progeny communication, capable of transporting diverse effectors across
202 the soma-germline barrier with the *P. pacificus* plastic system providing a concrete
203 demonstration of how environmental signals shape offspring physiology and behavior.

204 **Methods**

205 **Worm strain and culture conditions**

206 *Pristionchus pacificus* RSC011, originally isolated from Coteau Kerveguen, La
207 Réunion, was maintained at 20 °C on NGM plates seeded with *E. coli* OP50
208 or *Novosphingobium* L76. Stock populations were not exposed to bleaching,
209 starvation, or temperature shifts to avoid influencing mouth-form ratios. Bacterial
210 strains and mutants were grown overnight in LB, with kanamycin (50 µg/ml) added
211 as needed, and plated (300 µl per 6-cm NGM plate) 48 h prior to worm exposure.

212 **Dietary induction and reversal assays**

213 Diet-switch experiments followed established protocols ^{5,6}. J4 larvae were briefly pre-
214 exposed (~1 h) to L76 to minimize OP50 carryover before placement on
215 experimental plates. Worms previously maintained on *Novosphingobium* L76 were
216 propagated for one generation on kanamycin-supplemented NGM plates before
217 transfer to OP50 for subsequent generations during reversal experiments.

218 **Mouth-form scoring and analysis**

219 Eu mouth-form phenotypes were assessed under 150× magnification based on
220 buccal cavity morphology ¹⁷. Frequencies represent the mean of ≥10 biological
221 replicates (20 worms per replicate). The probability of developing the Eu morph (θ)
222 was estimated using a hierarchical Bayesian model ⁵.

223 **CRISPR/Cas9-Mediated gene editing**

224 Targeted mutations were generated in candidate genes using CRISPR/Cas9 ¹⁸.
225 Gene-specific crRNAs and tracrRNA (Integrated DNA Technologies) were mixed,
226 denatured at 95 °C for 5 min, and annealed at room temperature. Cas9 protein (New
227 England Biolabs) was added, and the mixture was adjusted with TE buffer to final
228 concentrations of 18.1 µM sgRNA and 2.5 µM Cas9. This complex was microinjected
229 into the germline of 40-50 young adult *P. pacificus* RSC011 hermaphrodites. Eggs
230 were collected up to 16 h post-injection, and F1 progeny were isolated onto
231 individual plates. Mutations were identified by Sanger sequencing, and homozygous
232 mutants were subsequently established. Null alleles were confirmed based on
233 frameshift mutations introducing premature stop codons. For both octuple and

234 nonuple vitellogenin mutants, whole-genome sequencing was performed to verify all
235 targeted mutations within these backgrounds.

236 **Vitamin B12 Supplementation**

237 Vitamin B12 (methylcobalamin, CAS No. 13422-55-4) was dissolved in water as a
238 fresh stock before each experiment. NGM agar was supplemented with B12, poured
239 into 6 cm plates, dried for two days, and seeded with *E. coli* OP50. Based on prior
240 testing ⁶, 1,500 nM was used throughout supplementation experiments, as this
241 concentration reliably induced transgenerational inheritance of the predatory mouth
242 form.

References

1. Jablonka, E. Epigenetic inheritance and plasticity: The responsive germline. *Prog. Biophys. Mol. Biol.* **111**, 99–107 (2013).
2. Weismann, A. *The Germ-Plasm: A Theory of Heredity*. (Scribner's, 1893).
3. Perez, M. F. & Lehner, B. Intergenerational and transgenerational epigenetic inheritance in animals. *Nat. Cell Biol.* **21**, 143–151 (2019).
4. Perez, M. F. & Lehner, B. Vitellogenins - Yolk Gene Function and Regulation in *Caenorhabditis elegans*. *Front. Physiol.* **10**, 1–17 (2019).
5. Quiobe, S. P. *et al.* EBAX-1/ZSWIM8 destabilizes miRNAs resulting in transgenerational memory of a predatory trait. *Sci. Adv.* **11**, 1–17 (2025).
6. Quiobe, S. P. *et al.* Diet-derived vitamin B12 induces transgenerational inheritance of nematode predation through elevated vitellogenin provisioning. *bioRxiv* 2025.05.21.655301 (2025).
7. Grant, B. & Hirsh, D. Receptor-mediated endocytosis in the *Caenorhabditis elegans* oocyte. *Mol. Biol. Cell* **10**, 4311–4326 (1999).
8. Akduman, N. *et al.* Bacterial vitamin B12 production enhances nematode predatory behavior. *ISME Journal* **14**, 1494–1507 (2020).
9. Kimble, J. & Sharrock, W. J. Tissue-specific synthesis of yolk proteins in *Caenorhabditis elegans*. *Dev. Biol.* **96**, 189–196 (1983).
10. Hall, D. H. *et al.* Ultrastructural Features of the Adult Hermaphrodite Gonad of *Caenorhabditis elegans*: Relations between the Germ Line and Soma. *Dev. Biol.* **212**, 101–123 (1999).
11. Rechavi, O. *et al.* Starvation-induced transgenerational inheritance of small RNAs in *C. elegans*. *Cell* **158**, 277–287 (2014).
12. Salmela, H., Amdam, G. V. & Freitak, D. Transfer of Immunity from Mother to Offspring Is Mediated via Egg-Yolk Protein Vitellogenin. *PLoS Pathog.* **11**, (2015).
13. DePina, A. S. *et al.* Regulation of *Caenorhabditis elegans* vitellogenesis by DAF-2/IIS through separable transcriptional and posttranscriptional mechanisms. *BMC Physiol.* **11**, 11 (2011).
14. Rechavi, O., Minevich, G. & Hobert, O. Transgenerational inheritance of an acquired small RNA-based antiviral response in *C. elegans*. *Cell* **147**, 1248–1256 (2011).
15. Chitwood, D. H. & Timmermans, M. C. P. Small RNAs are on the move. *Nature* **467**, 415–419 (2010).
16. Gu, S. G. *et al.* Amplification of siRNA in *Caenorhabditis elegans* generates a transgenerational sequence-targeted histone H3 lysine 9 methylation footprint. *Nat. Genet.* **44**, 157–164 (2012).

17. Ragsdale, E. J., Müller, M. R., Rödelberger, C. & Sommer, R. J. A developmental switch coupled to the evolution of plasticity acts through a sulfatase. *Cell* **155**, 922–933 (2013).
18. Witte, H. *et al.* Gene inactivation using the CRISPR/Cas9 system in the nematode *Pristionchus pacificus*. *Dev. Genes Evol.* **225**, 55–62 (2014).

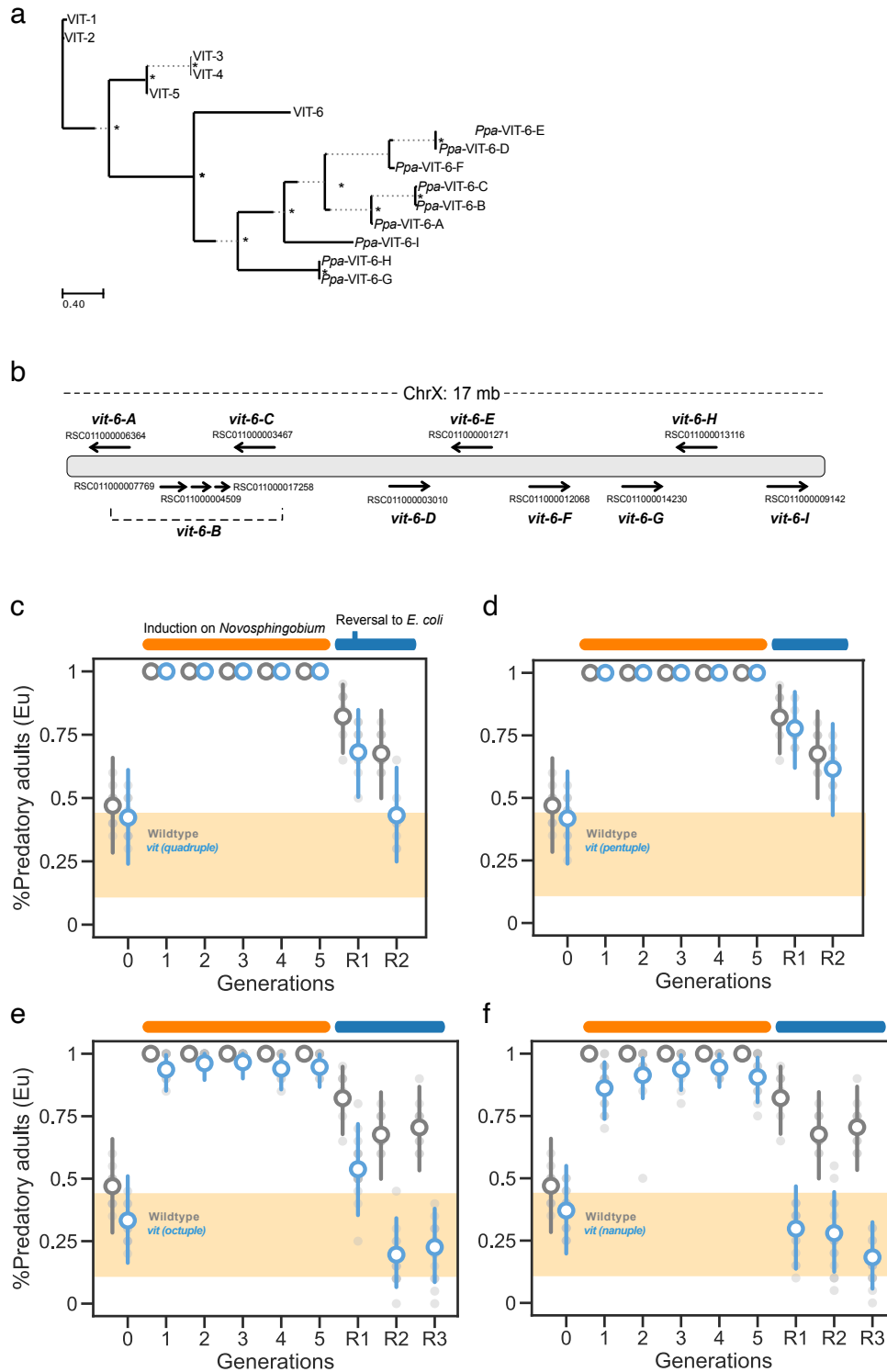


Fig.1 **a**, *P. pacificus* RSC011 possesses nine vitellogenin genes, all located on the X chromosome and spanning ~17 Mb. These genes are designated *Ppa-vit-6-A* to *Ppa-vit-6-I* according to their chromosomal order. Notably, three transcripts encode *Ppa-vit-6-B*. **b**, Phylogenetic analysis of vitellogenin genes from *P. pacificus* and *C. elegans*. In *C. elegans*, the six vitellogenin genes exhibit greater

sequence divergence, with *Cel-vit-6* representing the most divergent copy. Despite this, *Cel-vit-6* is the closest homolog to individual *P. pacificus* vitellogenin genes and to those of other nematodes. Nodes with bootstrap support ≥ 90 are marked with asterisks (*). Note that these phylogenetic analysis and graphical representations of *vit* genes were from previous report⁶. **c-f**, Mean probability of the predatory morph in vitellogenin (*vit*) transcripts with mutations in quadruple (c), pentuple (d), octuple (e) and nanuple (f) *vit* genes. Exposure to *Novosphingobium* was done for 5 generations followed by reversal to *E. coli*. Note that the R3 generations for both quadruple and pentuple mutants are excluded due to contamination and lethality. The 95% HDI for the means is based on Bayesian model and shows the probability of developing the Eu morph inferred from the previous experimental data⁵. Yellow area highlights the RSC011 baseline response averaged across 101 generations on *E. coli*, as previously established⁵.

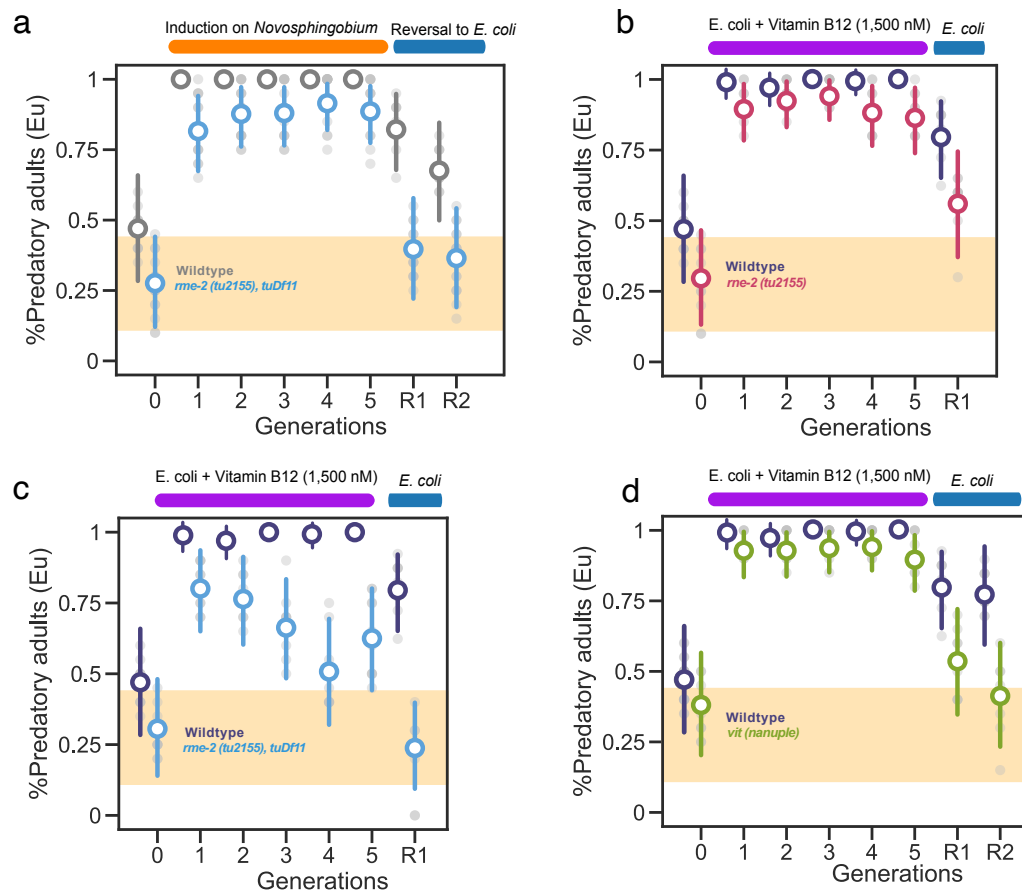


Fig. 2. **a**, Probability of the predatory morph in the *rme-2 (tu2155)* mutant crossed with a miRNA-deficient allele (*tuDf11*). Exposure on *Novopshingobium* was done for 5 generations, followed by reversal to *E. coli*. **b-d**, Vitamin B12 supplementation of the *E. coli* diet for 5 generations followed by exposure to un-supplemented *E. coli*. Probability of the predatory morph during and after vitamin B12 supplementation in the *rme-2* (b), *rme-2* with miRNA deficient mutations (c) and *vit* genes with nine mutations. Alleles that underwent two or fewer generations (R2 or less) during the reversal and/or *E. coli* exposure phases did so because lethality was observed. The 95% HDI for the means is based on Bayesian model and shows the probability of developing the Eu morph inferred from the previous experimental data ⁵. Yellow area highlights the RSC011 baseline response averaged across 101 generations on *E. coli*, as previously established ⁵.