The Dynamics of Vital Polyphenisms Dauer and Mouth Form of *Pristionchus* Nematodes in a Natural Context

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"IT SAYS NOTHING AGAINST THE RIPENESS OF A SPIRIT THAT IT HAS A FEW WORMS."

"ES SPRICHT NICHT GEGEN DIE REIFE EINES GEISTES, DAß ER EINIGE WÜRMER HAT."

Friedrich Nietzsche, *Menschliches, Allzumenschliches: Ein Buch für freie Geister*

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I Abstract

The dynamic fight for resources is a common theme throughout the kingdoms, with myriad factors affecting the outcomes of multifaceted battlegrounds of life. Competition has been a force in the evolution of phenotypic plasticity, an incredible phenomenon defining the capacity of an organism to develop different phenotypes from the same genotype, depending on environmental influences. Thus, phenotypic plasticity plays an imperative role in maintaining competitive advantages, though the details of such significance is only gradually being elucidated. Nematode *Pristionchus pacificus* has proven an instrumental model organism in furthering our comprehension of the genetic, molecular, and evolutionary bases of phenotypic plasticity. This ubiquitous, scarab beetle-associated roundworm displays two vital polyphenisms, dauer and mouth form. The latter has been extensively studied, with numerous accounts describing the genetic and environmental factors that regulate the development of two discrete, irreversible mouth forms: stenostomatous (St) and eurystomatous (Eu). St worms have one tooth, restricting feeding to solely bacteria, while Eu worms are equipped with two teeth, enabling predation on other worms, thus expanding their dietary range to include the remnants of predaceous escapades. The other essential plastic trait *P. pacificus* displays is dauer, a stress-resistant, long-living dispersal stage that serves as an alternative to a juvenile stage in the direct lifecycle. Dauer is especially critical in the ecology of *P. pacificus*, as it is found exclusively in this stage on insect hosts; post host-death, worms exit dauer and feed on the microbial blooms devouring the insect carcasses. I investigated the relevance of mouth form and its potential cross-talk with dauer in a natural context by exploring *Pristionchus* dynamics in the wild and lab on expansive temporal and spatial scales. I identified different dispersal strategies that reflect either presence of competitors or bacterial food abundance, but surprisingly not microbial succession, and determined that the formation of exclusively the predatory morph is restricted to post-dauer wild worms, not post-dauer domesticated strains. Though, our laboratory experiments revealed an adult-specific, density-dependent pheromone that induces the predatory morph in progeny. Altogether, my work reveals the intricacies of ecological forces influencing phenotypic plasticity, necessitating further detailing of worm competition that serves as a model for the battleground of life.

II Zusammenfassung

Der dynamische Kampf um natürliche Ressourcen ist ein grundlegender Bestandteil der Biologie aller Organismen und unzählige Faktoren beeinflussen den Ausgang der Auseinandersetzungen, die auf den facettenreichen Schlachtfeldern des Lebens ausgefochten werden. Diese Form der Konkurrenz war und bleibt eine treibende Kraft in der Evolution phänotypischer Plastizität, ein unfassbares Phänomen, das die Fähigkeit eines Organismus beschreibt, als Reaktion auf verschiedene Umwelteinflüsse unterschiedliche Phänotypen von ein und demselben Genotyp zu produzieren. Folgerichtig spielt phänotypische Plastizität zwar eine unerlässliche Rolle in der Aufrechterhaltung von Konkurrenzvorteilen, allerdings werden die wichtigen Details dieses Phänomens nur sehr graduell aufgedeckt und viele Aspekte verbleiben größtenteils unklar. Der Nematode *Pristionchus pacificus* hat sich als essenzieller Modelorganismus zur Erforschung der genetischen, molekularen und evolutionären Grundlagen phänotypischer Plastizität herausgestellt. Dieser omnipräsente, und Blatthornkäfer-assoziierte Fadenwurm weist zwei entscheidende Polyphänismen auf: Dauer-Larven und alternative Mundformen. Letztere wurden in den vergangenen Jahren intensiv studiert und eine Vielzahl von Untersuchungen beschreibt die genetischen und ökologischen Faktoren, welche die Entwicklung zweier unterschiedlicher, irreversibler Mundformen – eurystomat und stenostomat – regulieren. Stenostomate Würmer haben einen einzelnen Zahn und sind auf eine strikt bakterielle Nahrung eingeschränkt, wohingegen eurystomate Würmer mit zwei Zähnen ausgestattet sind, die es ihnen ermöglichen Jagd auf andere Würmer zu machen, was das Nahrungsspektrum dieser Tiere erweitert. Der andere essentielle Polyphänismus von *P. pacificus* ist die Dauer-Larve: ein stressresistentes, langlebiges Verbreitungsstadium, welches als Alternative zum dritten Larvenstadium des direkten Lebenszyklus darstellt. Die Dauer-Larve ist besonders kritisch für die Ökologie von *P. pacificus*, denn das Wirtsinsekt trägt ausschließlich Würmer dieses Stadiums auf (bzw. in) sich. Sobald das Insekt stirbt, treten die Würmer aus dem Dauerstadium aus, woraufhin sie beginnen sich von der Bakterienblüte, die den Insektenkadaver verdaut, zu ernähren. In meiner Doktorarbeit untersuchte ich die Relevanz der Mundformen und deren potentielle Vernetzung mit dem Dauerstadium, in einem natürlichen Kontext. Hierzu erforschte ich die Dynamik von *Pristionchus* in der Wildnis und im Labor, sowohl über ausgedehnte Zeiträume als auch über weitreichende räumliche Gebiete. Ich identifizierte unterschiedliche Verbreitungsstrategien, die entweder die Präsenz von Konkurrenten oder einen Überfluss von bakterieller Nahrung widerspiegeln, überraschenderweise jedoch nicht die mikrobielle Sukzession auf dem Kadaver. Außerdem ermittelte ich, dass die Entwicklung ausschließlich räuberischer Morphen für wilde Würmer zu beobachten ist, die das Dauerstadium durchliefen; gleiches gilt jedoch nicht für domestizierte Würmer. Interessanterweise zeigen unsere Laborexperimente allerdings, dass die Tiere ein Adult-spezifisches, Populationsdichte-abhängiges Pheromon produzieren, welches die Ausbildung der räuberischen Mundform in ihren Nachkommen induziert. Zusammengenommen demonstriert meine Doktorarbeit die Feinheiten der ökologischen Faktoren, die phänotypische Plastizität beeinflussen können, und sie zeigt, dass weitere Untersuchungen zum Konkurrenzverhalten dieser Modellorganismen notwendig sind, um die Wettkämpfe auf den Schlachtfeldern des Lebens zu verstehen.

III List of Publications

Sommer, R.J., Dardiry, M., Lenuzzi, M., Namdeo, S., **Renahan, T**., Sieriebriennikov, B. and Werner, M.S. (2017). The genetics of phenotypic plasticity in nematode feeding structures. *Open Biology*, *7*(3), p.160332.

Werner, M.S., Sieriebriennikov, B., Loschko, T., Namdeo, S., Lenuzzi, M., Dardiry, M., **Renahan, T**., Sharma, D.R. and Sommer, R.J. (2017). Environmental influence on *Pristionchus pacificus* mouth form through different culture methods. *Scientific Reports*, *7*(1), pp.1-12.

Werner, M.S.*, Claaßen, M.H.*, **Renahan, T***., Dardiry, M. and Sommer, R.J. (2018). Adult influence on juvenile phenotypes by stage-specific pheromone production. *iScience*, *10*, pp.123-134.

* co-first author

Renahan, T. and Sommer, R.J. (2020). Mechanism of murderous mushrooms paves path for parasitic helminth halt. *Proceedings of the National Academy of Sciences*, *117*(13), pp.6974-6975.

Dulovic, A., **Renahan, T**., Röseler, W., Rödelsperger, C., Rose, A.M. and Streit, A. (2020). *Rhabditophanes diutinus* a parthenogenetic clade IV nematode with dauer larvae. *PLoS Pathogens*, *16*(12), p.e1009113.

Renahan, T., Lo, W.S., Werner, M.S., Rochat, J., Herrmann, M. and Sommer, R.J. (2021). Nematode biphasic 'boom and bust' dynamics are dependent on host bacterial load while linking dauer and mouth-form polyphenisms. *Environmental Microbiology*. *23*(9). pp. 5102-5113.

Highlighted by journal editors (Kanzaki, 2021)

Cover image

Renahan, T. and Sommer, R.J. (2021). Nematode interactions on beetle host indicate a role of mouth-form plasticity in resource competition. *Frontiers in Ecology and Evolution. 9:752695*.

IV Introduction

A. Why worms?

"I DO NOT WANT TO BE A FLY,
I WANT TO BE A WORM!"

Charlotte Perkins Gilman, "A Conservative"

Scientists are not alone in their obsession with these ubiquitous, motley worms; prominent philosophers and poets for centuries have included worms in their preeminent, powerful prose. All yearn for a profound understanding of the captivating organism, whether it through meticulous, detailed experimentation and observation, or effecting the mentality of and thus vicariously living the life of a worm, as exemplified by US American humanist Charlotte Perkins Gilman. Indeed, the diversity of enthrallment, from both Charlotte's desire to embody a worm and scientists' rapt interests, reflects the widespread abundance and distribution of nematodes, roundworms composing the phylum Nematoda.

A described (approximately) 27,000 (one million estimated, though debated) species of nematodes (Platt *et al*. 1984; Lambshead and Boucher 2003; Lambshead *et al.* 2004; Blaxter 2011) occupy an incredible scope of environments, ranging from extreme tropical heat to icy tundra, saline to fresh waters, high to low altitudes, and in association with both invertebrate and vertebrate hosts, as well as with plentiful plants (Van Den Hoogen *et al*. 2019). This outstanding array of surroundings strongly indicates a multitude of ecologically niches that roundworms occupy (Figure 1). Nematodes certainly have copious, distinguished roles in their communities, and their various behaviors, capabilities, and functions have been thoroughly studied and explored (Ferris 2010; Procter 1990), though much remains to be elucidated on the intricacies of nematode developmental and molecular evolution associated with these characteristics.

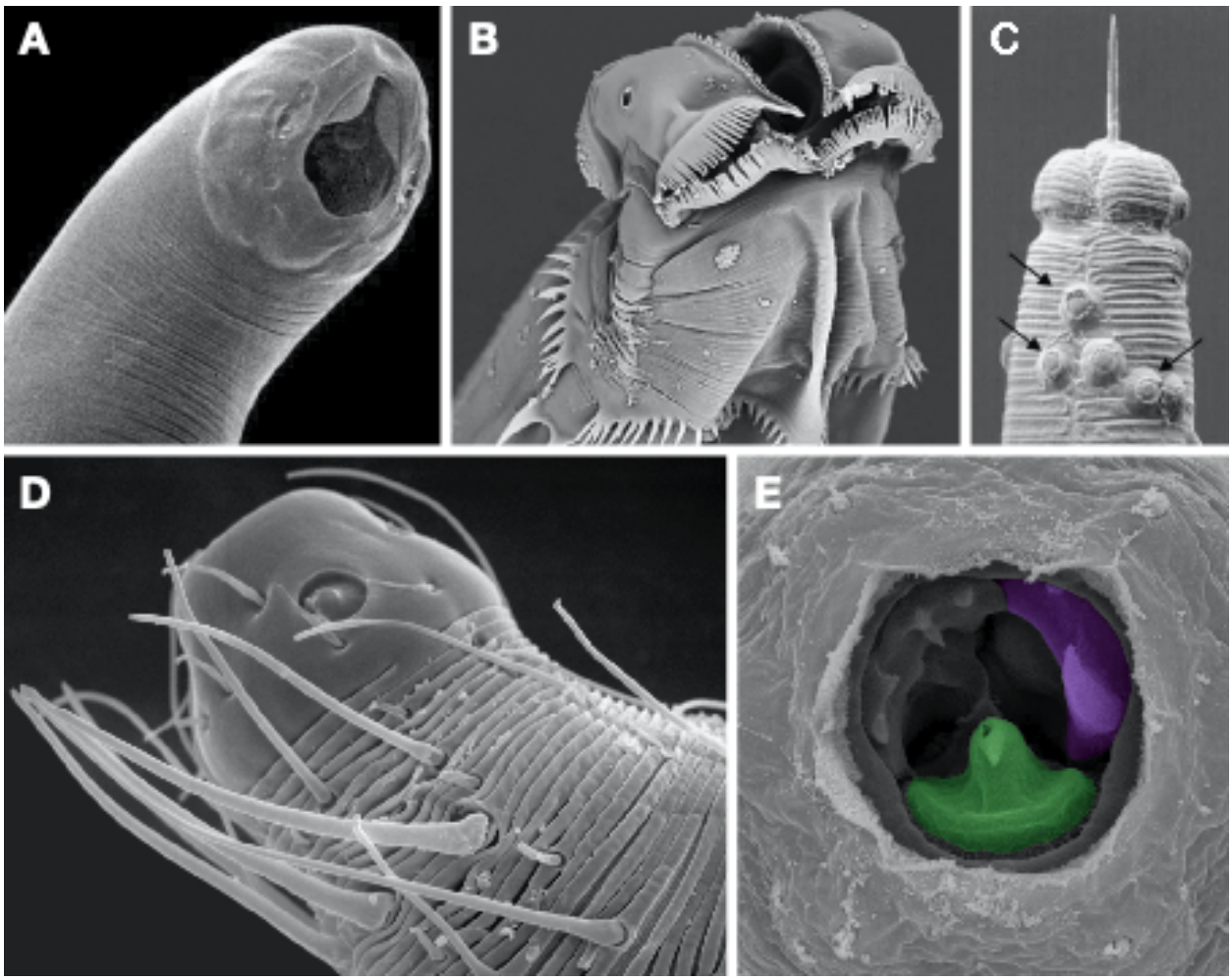

Figure 1. The astonishing array of remarkable roundworms.
(A) SEM of human parasitic hookworm *Necator americanus* presenting its ventral cutting plates (modified from Mehlhorn 2008). **(B)** SEM of millipede-associated *Heth pivari*, sublateral view of female anterior region (modified from Phillips *et al*. 2020). **(C)** LTSEM of head region of grass parasite *Belonolaimus longicaudatus*, covered in bacterial endospores and sporting a stretched suckling stylet (modified from Giblin-Davis *et al*. 2001). **(D)** SEM of "walking nematode" *Prochaetosoma marisalbi* from the White Sea, lateral view of the head (modified from Fedyaeva *et al*. 2016). **(E)** Pristine *Pristionchus pacificus* tantalizes us with its threatening teeth (green: dorsal; purple: subventral) in this SEM of a predatory (eurystomatous) head (modified from Lightfoot *et al*. 2019).

Many of these characteristics are affected by the environment and are subject to co-evolutions with worms' hosts and bacterial microbiomes. Numerous nematodes have tight symbiotic relationships, including commensal, mutualistic, and parasitic, and predator-prey associations, with plants, fungi, and animals ranging from beetles to humans (Vieira and Gleason 2019). These types of cross-kingdom interactions are not specific to nematodes, though reputable model organism *Caenorhabditis elegans* and satellite model *Pristionchus pacificus* have proven incredibly useful in studying myriad

probing biological inquiries, the relative ease due in part to short generation times, simple husbandry, and well-established forward and reverse genetic tools. *Pristionchus pacificus* in particular warrants singular attention, as in addition to its laboratory manipulability, it maintains tight associations with sundry insects globally (Herrmann *et al.* 2006; Herrmann *et al.* 2007).

The ubiquitous *P. pacificus* and its reliable affiliation with beetles have provided unique opportunities to study not only nematode-insect relationships, but tripartite interactions of nematodes, insects, and bacteria. Thus, *P. pacificus* thrives in diverse, distinctive environments, distinguished by motley factors including host type, microbiomes, and external abiotic and biotic conditions. These various environmental conditions can influence the expression of certain phenotypes stemming from the same genotype, a phenomenon referred to as phenotypic plasticity (West-Eberhard 2003). In *P. pacificus*, two plastic traits are critical to success on a carcass and are heavily impacted by the surroundings: developmental trajectory and mouth form. My work explores the lives of nematodes, focusing on *P. pacificus*, on beetle cadavers, and how the assorted environmental conditions affect the two phenotypically plastic characters, and whether there is any crosstalk between these two developmental decisions.

B. The environment

"WORM EATEN, DECKED WITH DUST,
SURROUNDED BY SMOKE-BLACKENED PAPER,
GLASS VIALS, BOXES ROUND ME, HURLED,
STUFFED WITH INSTRUMENTS THROWN TOGETHER,
PACKED WITH ANCESTRAL LUMBER-
THIS IS MY WORLD! AND WHAT A WORLD!"

"DEN WÜRME NAGEN, STAUB BEDECKT,
DEN, BIS ANS HOHE GEWÖLB' HINAUF,
EIN ANGERAUCHT PAPIER UMSTECKT;
MIT GLÄSERN, BÜCHSEN RINGS UMSTELLT,
MIT INSTRUMENTEN VOLLGEPFROPFT,
URVÄTER-HAUSRAT DREIN GESTOPFT –
DAS IST DEINE WELT! DAS HEIßT EINE WELT!"

Johann Wolfgang von Goethe, *Faust*

For decades prevalent model organism *Caenorhabditis elegans* has dominated molecular and developmental studies, with laboratories creating new worlds for worms. The establishment of *Pristionchus pacificus* as a satellite model organism opened not just comparable investigations utilizing a more intricate nematode, but also evolutionary and ecologically studies in a natural context. In addition to flourishing in vastly different biomes, *P. pacificus* and the other 48 *Pristionchus* species occupy various hosts, thus providing ample opportunities to study how nematodes develop depending on their environments.

B.1 The island

"BEETLES AND OTHER SMALL CREATURES WOULD MAKE THEIR WAY UP THROUGH THE CRACKS BETWEEN THEM, WORSE AFTER A RAIN, AND ONE MORNING I FOUND A LIVE WORM."

Margaret Atwood, *Alias Grace*

In the glistening turquoise waters of the Indian Ocean, just over 500 km east of Madagascar, stands the majestic volcanic island La Réunion (Figure 2A). The small (~2,500 km^2), impressive island is the youngest of its neighbors (Mauritius and Rodriguez, collectively the three make up the Mascarene islands), having formed only 2-3 mya (Wallace 1880; McGaughran and Morgan 2015). Anchored above a hotspot, La Réunion is composed of an active volcano, Piton de la Fournaise, and the inactive Piton de Neiges, towering at a staggering 3,000 m. The resulting range of altitudes, along with differing microclimates and geographic coordinates, gives rise to an island replete with disparate habitats. These multifarious ecozones are home to an assortment of flora and fauna, including bountiful beetles and their associated numerous nematodes, providing plentiful opportunities to study evolutionary ecology, population genetics, and host-symbiont interactions, as extensively done utilizing the Sommer Lab container on the island (Morgan *et al.* 2014; McGaughran *et al.* 2016; Moreno *et al.* 2016; Meyer *et al.* 2017). Due to multiple beetle invasion events, a worldwide haplotype diversity of *P. pacificus* is established on the island (Herrmann *et al.* 2010; Morgan *et al.* 2012). While the worm is affiliated with several beetles on the island, its reliable occurrence on the abundant

rhinoceros beetle *Oryctes borbonicus* (Figure 2B) (Morgan *et al.* 2012) has been utilized to study the natural ecology of *P. pacificus*, including the intricate interactions of the worms and bacteria on the beetle host (Meyer *et al.* 2017). Recent exploration in previously Max-Plank-uncharted territory on the island resulted in identification of a locality crawling with *Gymnogaster bupthalma*, a cockchafer formerly considered rare (Figure 2C). This discovery opened avenues to explore bi-genera nematode dynamics in a natural context.

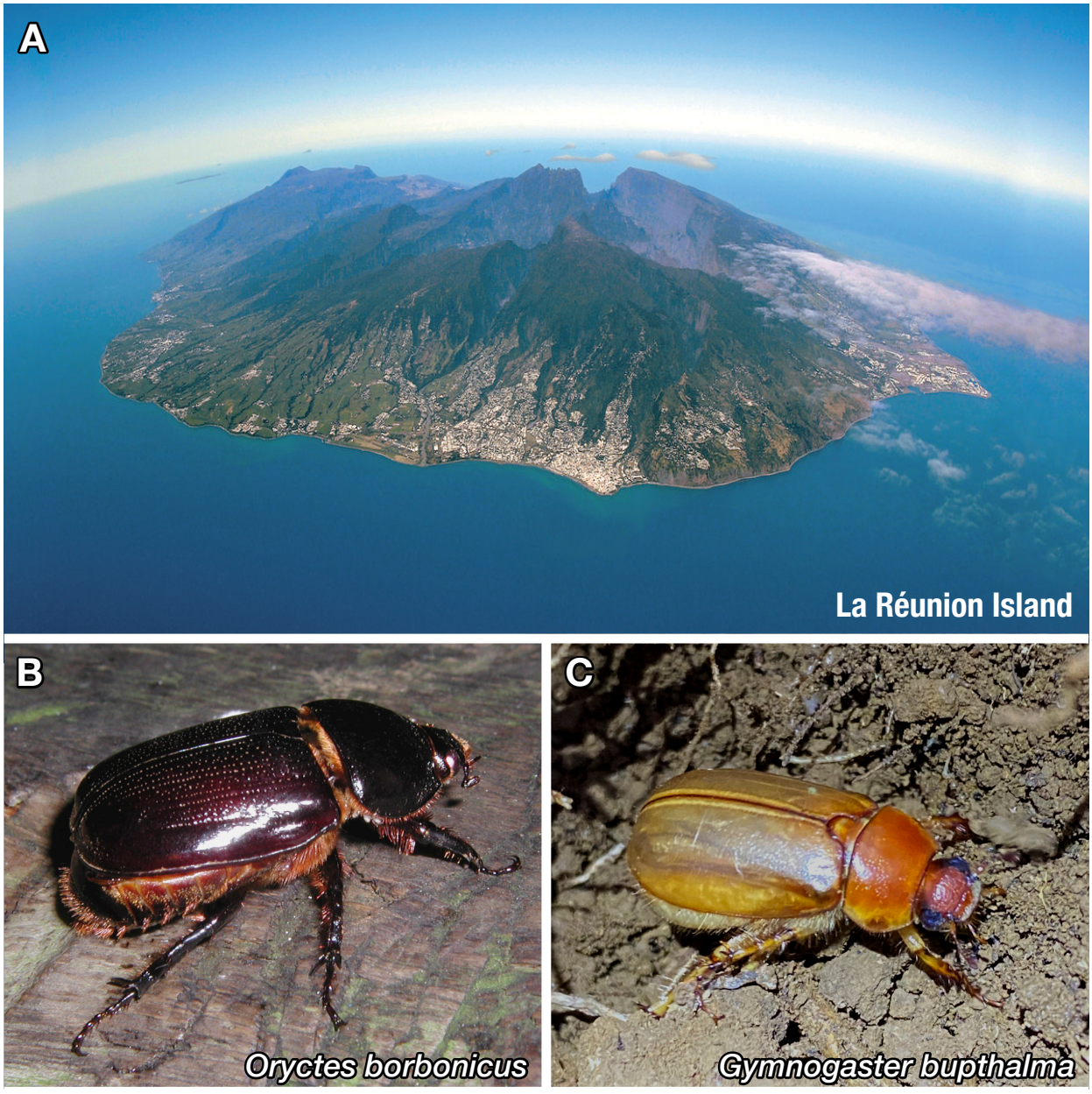

Figure 2. The wonderous worm world.
(A) Aerial view of La Réunion Island in the Indian Ocean (© Diving Réunion Tribloo 2021), which harbors the worldwide diversity of *Pristionchus pacificus* and is home to nematode-associated endemic **(B)** rhinoceros beetle *Oryctes borbonicus* and **(C)** cockchafer *Gymnogaster bupthalma*. (Images by Christian Weiler and Matthias Herrmann.)

B.2 The insect carcass

"THE KNELL, THE SHROUD, THE MATTOCK, AND THE GRAVE,
THE DEEP DAMP VAULT, THE DARKNESS AND THE WORM."

Edward Young, "Night Thoughts"

The grandiose order Coleoptera classifies an astounding ~400,000 beetle species that maintain diverse environmental ranges, omnifarious morphologies, and assorted associations with other organisms, in particular nematodes (Bovien 1937; Sudhaus 2008; Bouchard *et al.* 2009). Dauer nematodes (details in section C.1) are transported phoretically or maintained as parasites either internally or externally in different developmental stages of the host (Downes and Griffin 1996; Eng *et al.* 2005; Lee *et al.* 2017). Many of these associations are tight and specific, while others transpire when a hitchhiking opportunity arises. Phylum Nematoda is also flush with sundry species, many of which retain symbioses with beetles, though how frequently these associations occur is unsettled, as species descriptions for both beetles and nematodes are underreported (Lorenzen 1994; Peters 1996; Bardgett and Van Der Putten 2014; Stork *et al.* 2015). Yet, narratives of commensal, mutualistic, and parasitic relationships between nematodes and their beetle hosts are well-investigated and detailed.

Entomopathogenic nematodes (EPNs) are obligate and uncommonly facultative parasites of insects that utilize symbiotic bacteria to induce host death (Bedding *et al.* 1983; Kaya *et al.* 1993). The most prevalent of these are the genera *Heterorhabditis* and *Steinernema*, which have been thoroughly studied and consistently observed (Poinar 1990; Burnell and Stock 2000). In contrast, mutualistic relationships between nematodes and insects are considered scarce, but have been documented: *Diplogastrellus monhysteroides* is tightly associated with its dung beetle host *Onthophagus taurus*, and creates better access of augmented bacterial and fungal surroundings to beetle larvae, positively impacting their growth (Ledón-Rettig *et al.* 2018). Both of these types of associations are in contrast to necromenic *P. pacificus*, which based on current evidence maintains a commensal symbiosis with its beetle hosts. *Pristionchus pacificus* lives

externally on its host in an arrested developmental stage, dauer, presumably inflicting no harm on the insect. Though, insights from studies of *P. pacificus* with the oriental beetle *Exomala orientalis* reveal a *P. pacificus*-specific antagonism from the beetle, indicating a potential co-evolution towards a parasitic relationship (Cinkornpumin *et al.* 2014; Renahan and Hong 2017). Most reports of *P. pacificus* and its affiliated scarab host depict an innocuous worm waiting, without impacting, the death of its host. Thus, the varied relationships between nematodes and their hosts are dynamic and everchanging, necessitating constant and comprehensive investigations.

A decaying beetle carcass teems with microbial blooms readily feasted upon by nematodes both emerging from their recently deceased host and ones in the surrounding soil sensing whiffs of the bacterial spread. The success of each nematode hinges on a multiplicity of factors, ranging from initial population size on the insect, co-infestation of the insect, insect microbiome, and environmental influences at the grave site: temperature, acidity, humidity, soil bacteria, and soil nematodes (Molyneux 1986; Alatorre-Rosas and Kaya 1990; Kaya and Koppenhöfer 1996; Koppenhöfer and Kaya 1996; Koppenhöfer *et al.* 2006; Félix and Duveau 2012; Meyer *et al.* 2017).

The more thoroughly detailed beetle grave sites are those of EPNs. In addition to stimulating insect mortality, these bacteria also often produce antibiotics that are the demise to potential fungal and bacterial competitors (Kaya and Gaugler 1993; Dillman and Sternberg 2012). Thus, EPNs are able to cement their preferred bacteria as the dominating food source, establishing a competitive advantage from the drop of their host. Though, often their nematode competitors are also able to consume that particular nosh, so other strategies must be employed (Koppenhöfer *et al.* 1995; Blanco-Pérez *et al.* 2017). *Pristionchus pacificus* is subject to similar scenarios in which it fights for resources with nematodes co-infesting the host or ones joining the feast from the surrounding soil after host death. Thus, our nematode must be equipped with tactics to out-compete allospecifics in the thrilling, deep vault that is the insect grave swarming with worms.

In addition, *P. pacificus* is exposed to various microbiomes that may differ depending on host species, location, and local soil properties. These microbes have the

potential to influence characteristics of our favorite nematode, including two vital phenotypes subject to plasticity, dauer and mouth form.

C. Phenotypic plasticity

"AND STRIVING TO BE MAN, THE WORM
MOUNTS THROUGH ALL THE SPIRES OF FORM."

Ralph Waldo Emerson, "May-Day"

The copious spires of form organisms are able to develop depending on their environments is a riveting phenomenon termed "phenotypic plasticity." Multitudinous living beings across the domains display the capacity to respond dynamically to certain environmental conditions by induction of various phenotypes from the same genotype, affecting different characteristics, including behavior, morphology, and physiology (Schlichting and Pigliucci 1998; West-Eberhard 2003). The range of conditions that can trigger a plastic response, of the organisms susceptible to triggers, and of the types of responses are indeed all-encompassing, from temperature to microbiomes to molecular signaling, from bacteria to plants to humans, from developmental speed to sex type to dietary scope, respectively (West-Eberhard 1989; DeWitt and Scheiner 2004; Pfenning 2021).

Pristionchus pacificus is an established model organism to uncover the genetic factors, molecular mechanisms, and evolutionary origins of phenotypic plasticity (Hong and Sommer 2006; Sommer 2015). In particular, two plastic traits have captivated worm lovers for decades, stimulating thorough inquiries and investigations of the arrested developmental stage dauer and the enthralling mouth forms.

C.1 Dauer

"NICHT DIE STÄRKE, SONDERN DIE DAUER DER HOHEN EMPFINDUNG MACHT DEN HOHEN MENSCHEN."

"IT IS NOT THE STRENGTH, BUT THE DURATION OF GREAT SENTIMENTS THAT MAKES GREAT MEN."

Friedrich Nietzsche

Persisting through harsh environmental conditions and evading unsuitable surroundings to locate resources are imperative characteristics of innumerable nematodes, and longevity is often more beneficial than strength. Under stress, *C. elegans*, *P. pacificus*, and other nematodes can enter an alternative, indirect developmental pathway and form dauers, non-feeding arrested juveniles that are resistant to environmental stresses, including high temperatures, high population density, and limited food (Cassada and Russell 1975). Dauer is regulated by four distinct pathways: TGFβ-like, insulin-like, steroid hormone, and guanylyl cyclase pathways (Riddle *et al.* 1981; Thomas *et al.* 1993; Gerisch *et al.* 2001).

Under non-stressful environmental conditions, *P. pacificus* develops from embryo to adult in four days, growing through four juvenile stages defined by molting events (Figure 3A) (Sommer *et al.* 1996). Dauer is an alternative to the third juvenile stage and longevity is species and context dependent. Dauer exit is triggered by improved environmental settings with dauers resuming development into a reproducing adult, preceded by the final juvenile stage (Cassada and Russel 1975; Ogawa and Sommer 2009). In addition to persevering with inadequate resources, dauers are specialized for dispersion and are the stage most often associated with hosts (Poinar 1983). Dispersion and dispersion regulation mechanisms are largely conserved throughout nematode species.

Dauers are morphologically distinguished from other juvenile stages, epitomizing the necessary modifications required to survive in harsh conditions. The more conspicuous alterations include a thickened cuticle and closed stoma (Figure 3B), reflecting the need for protection against detrimental environmental factors (desiccation, pathogens, chemicals) and the lack of feeding, respectively (Cassada and Russell 1975; Riddle *et al.* 1981). Given the specialized cuticle and buccal plug (accompanied by a lack of pharyngeal pumping), dauers can clearly be identified utilizing dissecting microscopes. Indeed, these characteristics not only aid in pervading through hostile settings, but equip the worm with potentially useful armor when dispersing through environments with unknown conditions, including a dearth of food.

Free-living nematodes involved in phoretic interactions and necromenic nematodes are often found as dauers in soil and on their hosts. The analogous stage of dauers in parasitic and entomopathogenic nematodes, infective juveniles (IJs), are responsible for dispersion, identify and infect their hosts, are non-feeding, and occur when food is limited (Osche 1955; Ishibashi and Kondo 1990; Sudhaus 2008). A well-established mechanism for dispersion is seen in free-living *C. elegans* (Frézal and Félix 2012). Naturally, *C. elegans* is found in all developmental stages (dauers and direct developmental stages) in various habitats. When food becomes scarce, dauers leave the immediate environment in search of more abundant resources nearby. Various nematodes utilize insects as vehicles for dispersion (or require hosts for completing their life cycles). Dauers and IJs can be cruisers, actively pursuing their hosts, or they can be ambushers, standing relatively stationary and waiting for their hosts to sweep them up. Some ambushers will exhibit nictation, in which the worms stand on their tails and wave (Campbell and Gaugler 1993). Standing increases the likelihood that a passing insect will pick up the nictating worm, especially if these worms form "dauer towers" in which they stack vertically using a dauer-specific wax ester (Figure 3C) (Brown *et al.* 2011; Penkov *et al.* 2014). Some entomopathogenic IJs respond to air movement and volatile signals by jumping to attach to their hosts (Kaya and Campbell 2000). Dauers respond to various signals, including small-molecule signaling among worms themselves, both within the same strains and among other species. These pheromones influence a variety of characteristics, including mating behavior, aggregation/avoidance, mouth form, and dauer.

Signaling plays a role in both dauer induction and recovery. Since the identification of the *C. elegans* dauer-inducing pheromone, many scientists have been rapt with interest in the various pheromonal cocktails that can influence a variety of behaviors (Golden and Riddle 1982; Jeony *et al.* 2005). Both entry and exit of dauer are influenced by population density, alongside food and temperature. The state of population density is signaled by the dauer pheromone in a dose-dependent manner (Golden and Riddle 1984a). Thus, when density is high and a threat of food scarcity

looms, the more concentrated the dauer pheromone is, inducing young juveniles to enter the arrested stage and hindering dauer exit. Though, high food abundance can work against dauer entry (Golden and Riddle 1984b). Thus, interpreting and processing contrasting signals especially in a highly variable natural setting make for a complicated study.

The dauer pheromone consists of various ascarosides, derivates of dideoxysugar ascarylose (Figure 3A), that vary species to species and also have different effects; one strain's dauer cocktail is not often specific to only itself (Bose *et al.* 2014; Butcher 2017). In dispersal assays using the *C. elegans* ascaroside-rich dispersal pheromone, IJs of *Steinernema feltiae* and mobile juveniles of root knot nematodes were found to disperse in response to the *C. elegans'* pheromone (Kaplan *et al.* 2012). Infective juveniles of certain EPNs (*Steinernema* and *Heterorhabditis*) were found to have cross-species stimulation of dispersal depending on the brew of pheromones released (Hartley *et al.* 2019). While there are similarities among the dispersal pheromones in these species, the compositions of the pheromones are unique and specific to each one. Pheromones are also secreted to hinder dauer exit. Entomopathogenic *H. bacteriophora* releases an ascaroside when inside its host to prevent IJs from developing, so that the host will become saturated with IJs (Noguez *et al*. 2012). Intriguingly, a *C. elegans* ascaroside with a similar structure could not prevent IJ recovery; even conserved molecules influence differently, depending on the species.

Thus, worms not only communicate among kin but also engage in interspecific signaling. *Pristionchus pacificus*, along with other *Pristionchus* species, maintain an astonishing array of diverse molecules, expanding their repertoires to include not only ascarosides but more complex dimeric ascarosides and paratosides (Butcher 2017; Markov *et al.* 2016; Dong *et al.* 2020). These pheromones play an indispensable role in dauer and dispersal, and can also influence other vital plastic phenotypes, including mouth form.

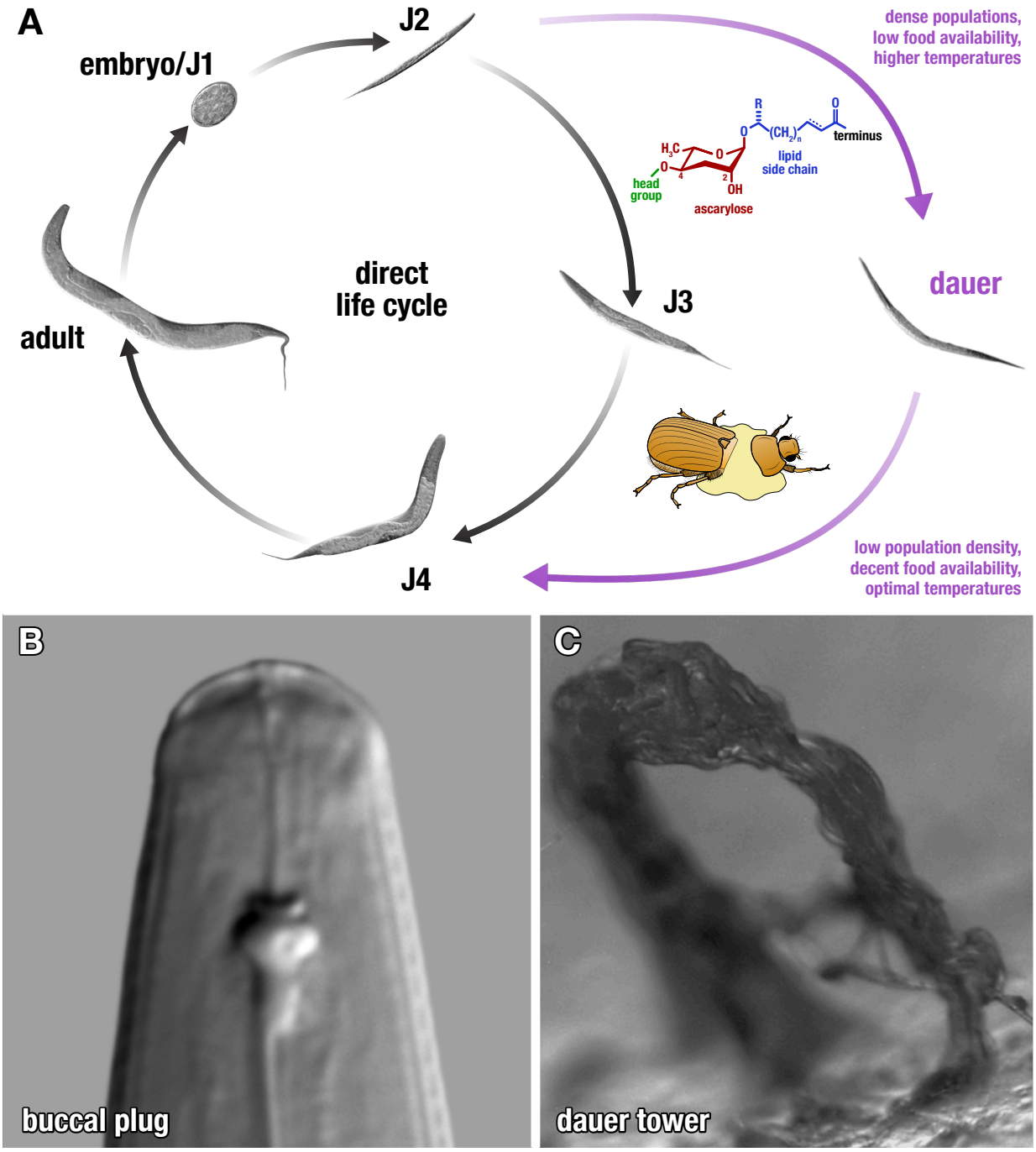

Figure 3. Phenotypic plasticity determines development: deploying dauer for dispersal
(A) The environment influences which of two alternative developmental trajectories is pursued by *Pristionchus pacificus*. The direct lifecycle takes roughly four days to complete at 20°C. Under adverse conditions, young worms can enter dauer, a durable stress-resistant dispersal stage, often triggered by dauer pheromones (lifecycle adapted from Renahan *et al.* 2021; chemical structure of ascarosides adapted from Panda *et al.* 2017). **(B)** Dauers are equipped with modified morphological features to ensure survival in harsh environments, including a buccal plug and thickened cuticle. **(C)** An impressive dauer tower, a collection of nictating dauers, which boosts host-finding and thus dispersal (image from Penkov *et al.* 2014).

C.2 Mouth form

"MEN HAVE DIED FROM TIME TO TIME, AND
WORMS HAVE EATEN THEM, BUT NOT FOR LOVE."

William Shakespeare, *As You Like It*

While assorted aspects of *P. pacificus* have been conserved since its divergence from *C. elegans* approximately 100 million years ago (Rota-Stabelli *et al.* 2013), our favorite nematode is now equipped with an evolutionary novelty, denticles that essentially function as teeth (Von Lieven and Sudhaus, 2000; Susoy *et al.* 2015; Susoy and Sommer 2016). In general, nematodes display an array of differing mouth parts that correspond to various dietary ranges, including bacteria, protists, fungi, and nematodes along with other metazoans; these expanded dietary ranges in turn reflect the expansion of nematode-inhabited niches. Plant parasitic nematodes are armed with stylets in their mouths, spears used for both injecting secretions and suction-feeding (Hussey 1989; Williamson and Gleason 2003). Free-living nematodes of the genus *Tylopharynx* rip apart cell walls with their two teeth and feast on fungal hyphae (Fürst von Lieven 2002). Hookworms are outfitted with specialized buccal cavities for conquering their hosts; canine-parasitic nematode *Ancylostoma caninum* has three sets of teeth for anchoring into host tissue while human parasite *Necator americanus* has both teeth and cutting plates (Burrows 1962; Chang *et al.* 2020). Comparatively, measly *C. elegans* lacks elaborate mouth parts that restricts its diet to only bacteria, wherein imposing *P. pacificus* with its two teeth can not only feast on bacteria and fungi, but can also predate on other nematodes, including paltry *C. elegans*, and not out of love.

Three fig-associated *Pristionchus* species display five beautiful morphs, an astonishing mélange and uncommon number in the genus (Susoy *et al.* 2016). In most mouth-form plastic worms, one of two distinct mouth forms can be developed: either a strict bacterial-feeding morph, stenostomatous (St) (Figure 4A), with a single blunt, elongated dorsal tooth and narrow mouth or eurystomatous (Eu) (Figure 4B), a wide mouth with two teeth, a claw-like dorsal tooth and subventral tooth that enables

predation (Figure 4C) (Ragsdale *et al.* 2013; Wilecki *et al.* 2015). This irreversible decision is made during an early juvenile stage and greatly impacts the livelihood of the adult worm, as its dietary range depends on the form developed.

While the capacity for the dimorphism varies in diplogastrids, even to the extent that in larger species, St animals can also predate, certain characteristics are maintained. Namely that the development of certain forms can be influenced by environmental factors, including pheromones from the worms themselves (Bento *et al.* 2010; Bose *et al.* 2012). These pheromones reflect an expansion of complex ascaroside dimers, built of ascarylose sugars and fatty acid side chains, not observed in *C. elegans*. An Eu-inducing pheromone is thus imperative in a competitive environment, and can alter the dynamics of nematodes striving to thrive on an insect carcass and altering the mode of interaction with predatory behavior.

Predation is prevalent throughout the kingdoms and has originated multiple times at various levels of interactions, with the introduction of predation wreaking drastic modifications to ecosystems and facilitating co-evolutions with symbiotic organisms. Indeed, predators have developed a range of specializations to excel in prey capture, destruction, and consumption. Various bacteria predate on other microbes by cell-to-cell attachment and secreting enzymes to degrade and assimilate prey molecules, carnivorous plants like the infamous Venus fly trap have specialized trapping mechanisms that distinguish between living prey and non-prey stimuli, malicious fungi ensnare, penetrate, and demolish innocent nematodes utilizing differing adhesive-related techniques, and our familial feline fellow has narrowly-spaced canine teeth specific for small rodent prey (Martin 2002; Hedrich and Fukushima 2021; Pramer 1964; Smith and Tchernov 1992). The teeth of Eu *P. pacificus* can rupture the cuticle of various nematodes, though the worms refrain from tearing apart the skin of their own kin.

The ability to discriminate between own and foreign tissue is a key necessity of many organisms. Self-recognition is observed and utilized across the kingdoms, including bacteria, fungi, and insects. Bacterium *Proteus mirabilis* builds boundaries between different strains but not within single strains, nonself-recognition in

filamentous fungi results in heterokaryon formation rejection, and predatory larvae of *Dytiscus* beetles avoid cannibalism even when prey density is low (Gibbs *et al.* 2008; Glass and Kaneko 2003; Inoda 2012). This cannibalism avoidance is especially critical as cannibalism is an inherent problem in predation. *Pristionchus pacificus* also distinguishes between kin and non-kin, and avoids predating on juveniles at a strain-specific level. The first elements determined to be involved in self-recognition of nematodes includes sensing of hypervariable small peptides on the worm cuticle (Lightfoot *et al.* 2019).

As the intricacies of mouth form, predation, and self-recognition are thoroughly being detailed both genetically and molecularly, their ecological relevancies must also be explored in parallel. The plastic capacity to become predacious and the ability to differentiate progeny from those of closely related strains are vital to ensure perseverance and dominance in the competitive insect-carcass environment. This power for phenotypic plasticity exemplifies Darwin's notion that competition drives evolutionary novelty.

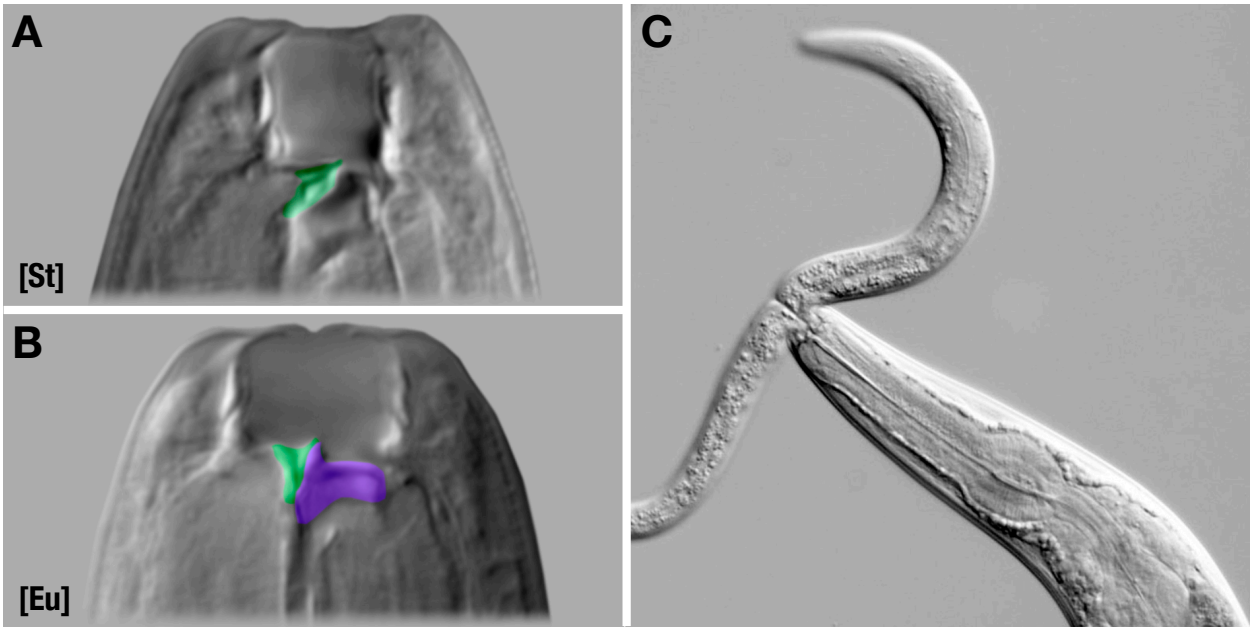

Figure 4. Phenotypic plasticity provides predation: two teeth to thrash
(A) The narrow stenostomatous (St) mouth lacks the subventral tooth and maintains only a thin, elongated dorsal tooth (green), restricting feeding to bacteria. **(B)** The predatory mouth form eurystomatous (Eu) is wide and armed with two teeth, an imposing claw-like dorsal tooth (green) and a threatening subventral tooth (purple), facilitating an omnivorous diet. **(C)** Predaceous Eu *P. pacificus* tears into *C. elegans* utilizing its two teeth (image from Dan Bumbarger).

D. The interplay

"THE SMALLEST WORME WILL TURNE, BEING TRODDEN ON"

William Shakespeare, *Henry VI*

With the incredible scope of environments and plethora of beetles with motley bacteria, there are many factors that can influence plastic phenotypes of nematodes. In addition, nematodes face competition from other worms, all of which are conforming to and attempting to control its surroundings to benefit their own kind. Indeed, during these raging wars on insect carcasses, worms can plastically turn when trodden on.

D.1 Competition

"I LIKE THE LAD, WHO WHEN HIS FATHER THOUGHT
TO CLIP HIS MORNING NAP BY HACKNEYED PHRASE
OF VAGRANT WORM BY EARLY SONGSTER CAUGHT
CRIED, "SERVED HIM RIGHT! IT'S NOT AT ALL SURPRISING
THE WORM WAS PUNISHED, SIR, FOR EARLY RISING!"

John Godfrey Saxe, "Early Rising"

Competition is ubiquitous in life, often over two major driving reasons to live, food and sex. "The struggle for existence," popularized by Darwin, describes the competition of living beings as a basis for natural selection (Darwin 1859). This struggle is observed everywhere, and the battle amid worms is dynamic, complex, and readily available to scrutinize. The fights for resources among various nematodes take place on insect cadavers, with the environments varying from carcass to carcass. The presence and abundance of certain microbes and nematodes, and their own interactions, greatly influence the structure of the insect community. Entomopathogenic, free-living, and necromenic nematodes make up myriad combinations that can be found in decaying hosts. These groups not only have to compete for resources with other families of nematodes, but within their own species. Advantages are largely species-specific, including broader bacterial preference ranges (or multiple food sources) and developmental time. With fluctuating and unpredictable microbial blooms, nematodes

with restricted diets may be outcompeted by nematodes that exploit several food types. In addition, nematodes that emerge more quickly from carcasses may establish both their group first and their preferred microbe. The latter is a characteristic that EPNs exercise; they release preferred symbiont bacteria that produce antibiotics, taking out potential fungal and bacterial competitors and establishing their primary food source (Bedding *et al.* 1983; Kaya *et al.* 1993). This, in combination with the different antibiotic release times (dependent on both the EPN species and host species), can drastically change the microbial environment on the cadavers and thus the population structure of emerging nematodes. While it appears that EPNs would have the upper hand, EPNs struggle to outcompete other EPN species, allowing free-living nematodes to take advantage, as seen with *Oscheius* in the presence of competing EPNs. Interspecific killing occurs in gonochoristic EPNs; males kill both virgin and mated males, and occasionally females (O'Callaghan *et al.* 2014), implying fighting for not just mates but also for resources. A study showed that if only one EPN species needs to compete with *Oscheius,* the EPN species produces the same number of juveniles as they would with no competitors present (Blanco-Pérez *et al.* 2017).

Another study, on the other hand, recorded decreasing juvenile production in co-occurring EPNs and *Oscheius* species compared to single populations (Koppenhöfer *et al.* 1995). The initial population of each group also determines success; the species with the higher initial population is reproductively favored. The successes of *Oscheius* largely depends on the reproductive strategy of the competing EPNs; hermaphroditic EPNs are quick to colonize and dominate while gonochoristic EPNs must have a significantly larger initial population to ensure survival over *Oscheius* (Campos-Herrera *et al.* 2015). The nematode community structure on decaying hosts depends on numerous factors that are both hard to control in a laboratory setting and impractical to determine in the field. What we think are initial population sizes and presence of certain species may be far off; estimates of EPN infestation are thought to be underestimates due to the likely displacement of EPNs by both free-living and necromenic nematodes (Duncan *et al.* 2002). The conflicting results of which nematode group emerges as victor reflects the

highly variable environments the nematode communities live in; initial population size, developmental rate, reproductive strategy, and combinations of nematodes are just some factors that influence competition among nematodes. Unsurprisingly, the host itself matters, as do abiotic and biotic factors in the host's environment, manipulation of which would change the dynamics of the fight for resources.

Feasibly, the first worm to exit dauer and establish a population on the carcass is not the one with a competitive advantage, but indeed will be the one punished for early rising. Naturally, the presence or absence of certain microbes can have an impact not anticipated by our worms.

D.2 Cross-kingdom interactions

"THAT WRETCHED NEMATODE!"

Carl Hiaasen, *Tourist Season*

The establishment of the hologenome theory of evolution crucially incorporates the microbial metagenome as an imperative factor in interactions affecting traits (Zilber-Rosenberg and Rosenberg 2008; Bordenstein and Theis 2015). Many genotype-environment interactions do not act in isolation; the host microbiome is often involved in the plasticity of certain phenotypes (Carrier and Reitzel 2018; Kolodny and Schulenburg 2020). The significance of the microbiome is well-established, with its recent general notoriety founded on the revelation that cascades of changes initiated by perturbations of the human microbiome can influence the capacity to digest and utilize certain foods and affect obesity (Suez *et al.* 2014; Sonnenburg and Bäckhed 2016). Across organisms, alterations of the microbiome can also stimulate changes in life history, behavioral traits, development, and morphology.

Life history traits in *Drosophila* can be modified by perturbations in microbiome compositions, as shown in various studies demonstrating how different bacteria can induce changes in fecundity, lifespan, and developmental rate. In one study that manipulated combinations of five key gut bacterial species, it was determined that

different microbiomes influence life history strategies towards either longevity (not necessarily developmental time) or reproduction (Gould *et al.* 2018). With flies following the former strategy, the authors attempted, but were unsuccessful, to increase reproduction by providing the flies with fecundity-promoting bacteria. While the authors note that changes in microbiome in late life stages cannot increase the reproductive window, they do not consider attempting the reproduction rescue with flies that began with microbiome compositions affecting developmental rate. Previously, one key bacterial species was determined to be integral in development, *Acetobacter pomorum*. Mutants of *A. pomorum,* while able to colonize the fly gut, hindered development. Developmental abnormalities varied depending on the bacterial mutation, with one mutant inducing decreased developmental rate (Shin *et al.* 2011). Perhaps a host with an initial microbiome cocktail of this mutant with the combination that increases longevity may be susceptible to reproduction restoration.

Behavioral changes have been observed in mice with inadequate microbiomes. Maternal diet affects and alters the gut microbiome of offspring, which corresponds to neurodevelopmental disorders. While some of the disorders (namely certain social behaviors) can be rescued by microbiome reconstitution, others, such as abnormal repetitive behaviors, cannot be (Buffington *et al.* 2016). The authors speculate that the latter behavior is not affected by gut microbiome changes; but perhaps it is, though unlike the social behaviors, it cannot be reversed.

Increasing interest in the natural ecology of nematode *C. elegans* has aided in elucidating the interaction between microbiomes and hosts. While the model organism is found worldwide in habitats with innumerable bacterial communities, its basic microbial composition is conserved (Dirksen *et al.* 2016). Since bacterial taxonomic groups are shared among isolates regardless of the environment, it is likely that the *C. elegans* microbiome either is a consequence of bacterial colonization efficiency or is selected for. Unsurprisingly, the habitat bacteria, aside from the backbone microbiome bacteria, can either contribute to or hinder the worms' fitness. Some bacteria support worm growth without inducing stress, while detrimental, though not necessarily

pathogenic, bacteria can halt growth and induce stress by activating the immune system. Of course, some of the microbes in the biome aid in host immunity and protect against infection (Montalvo-Katz *et al.* 2013).

The microbial communities can also affect the plastic developmental trajectory of the worm; juveniles can either develop directly into reproducing adults or indirectly via the arrested larval stage dauer. In populations with high levels of Alphaproteobacteria (a beneficial bacterium), *C. elegans* is quite prolific; in Bacteroidetes-rich communities, dauers are frequent (Samuel *et al.* 2016). In the presence of either or both detrimental and pathogenic bacteria, dauer entry is beneficial to the worm; dauers can either wait for more favorable bacteria to colonize the habitat or they can disperse to find beneficial bacteria. The impact the bacteria have depends on their relative abundances; Bacteroidetes must be in high abundance to be harmful, while pathogenic and beneficial bacteria affect the worms at low abundances.

It may appear that the microbiome is then simply an environmental factor that can affect certain plastic traits, but in fact it is not an independent entity but acts as a unit with its host. Microbiomes are susceptible to external factors, and they also aid in resilience to their hosts during environmental changes. In plants, assorted microbes provide defense against infections and extend adaptation to various environmental perturbations including drought and availability of nutrients (Vandenkoornhuyse *et al.* 2015). Furthermore, the microbiome of some hosts changes with host morphological plasticity. In three echinoid species whose larval morphology changes when experiencing low food abundance, the microbial community shifts with the morphological alterations. One echinoid species, *Strongylocentrotus droebachiensis*, when returned to an environment with plentiful food, shows a reversal back to its original microbiome (Carrier and Reitzel 2018). The specificity of microbiomes and hosts further suggest that the two can act as a unit. Not only is the *C. elegans* microbiome conserved across populations in different habitats, but it is also distinct from both the habitat microbial community and from other local *Caenorhabditis* species (Berg *et al.* 2016). Bacteria conferring resistance to pathogenic strains are also species-specific; the

bacterium that protects *C. elegans* does not protect *C. briggsae*, and vice versa. In addition, in experiments investigating the potential effects of temperature on microbial communities, Berg and colleagues found that the microbiome often altered in the opposite direction from the environmental bacteria. Thus, it is difficult to predict how environmental fluctuations will impact the hologenome.

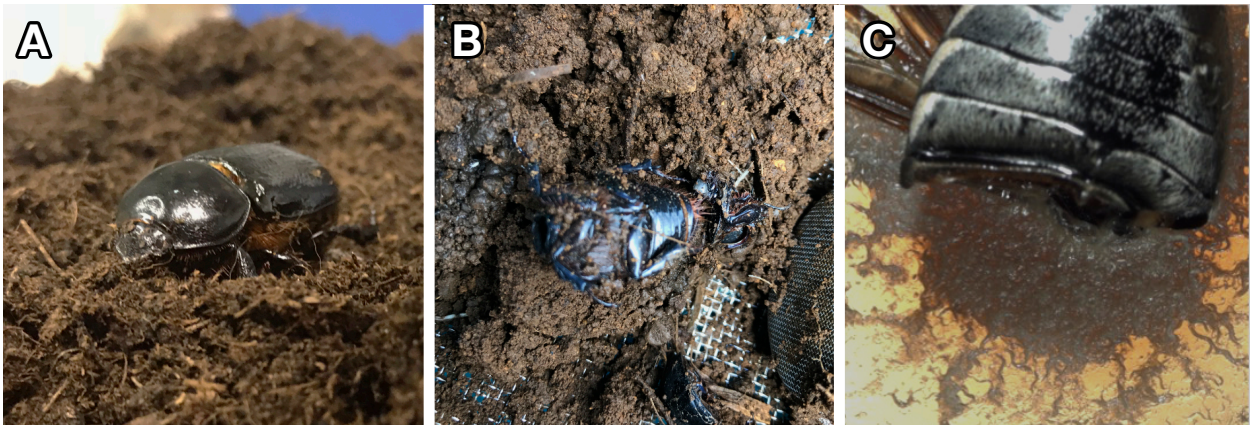

Figure 5. The bacteria-beetle blended battleground.
(A) *Oryctes borbonicus* dies in soil post-egg laying (image by Tess Renahan). **(B)** Bacterial blooms cover the carcass (image by Tess Renahan). **(C)** Ravenous nematodes emerge from the carcass, entering the beetle-based battleground bursting with bacteria and swarming with visiting voracious worms (image from Hong and Sommer, 2006).

The dynamics of microbes with *P. pacificus* are gradually being explored and detailed, with an emphasis on naturally occurring interactions in the wild (Figure 5). Recent studies identified the bacterial succession on the decaying *O. borbonicus* carcass and the effects various bacteria have on mouth form and predation (Meyer *et al.* 2017; Akduman *et al*. 2018; Akduman *et al*. 2020). Meyer and colleagues tracked nematode and bacteria successions on *O. borbonicus* for nearly two weeks, detailing the re-occurrence of dauers as early as one generation after exit and finding stable microbiomes (Meyer *et al.* 2017). Though providing first insights into the dynamics post-host death, this study was limited both spatially and temporally, as it observed only the carcass and spanned few generations. A new study also revealed involvement of bacteria-produced vitamin B_{12} on increased predatory behavior (Akduman *et al.* 2020); further exploration may indicate ecological relevance of beetle microbiome B_{12} production and its influence on

co-occurring nematodes. *Pristionchus pacificus* is exposed to various microbiomes that may differ depending on host species, location, and local soil properties. The microbes have the potential to influence characteristics of our favorite nematode, including two vital phenotypes subject to plasticity, dauer and mouth form. These polyphenisms especially come into play during competitive interactions among worms, wherein all species frustratingly shake their fist-like heads reproaching, “that wretched nematode!”

E. Thesis aims

"A BLANK, MY LORD. SHE NEVER TOLD HER LOVE,
BUT LET CONCEALMENT, LIKE A WORM I' THE BUD,
FEED ON HER DAMASK CHEEK. SHE PINED IN THOUGHT"

William Shakespeare, *Twelfth Night*

As I pined over my love for worms, I aimed to de-conceal the roles of two vital polyphenisms, dauer and mouth form, in intraguild predation, a befitting blend of competition and predation. I sought to expand on previous studies and bring ecological relevance to these plastic traits by studying them both in the wild and in lab, creating a natural setting to appropriately study characteristics influenced by the environment. To do so, I:

1. Developed a system to track both the dispersal of and plastic phenotypes of wild worms from their beetle hosts in large spatial and temporal scales, a first for nematodes. I did this both in the organisms' natural environments and in controlled mock-natural settings in the laboratory
2. Examined the potential cross-talk between the two polyphenisms using freshly isolated strains and domesticated strains, revealing carcass-environment specific effects on mouth form
3. Detailed the microbial succession on multiple developmental stages of two insect host species over months of nematode presence on the carcasses
4. Established a novel staining method to differentiate phenotypically similar nematode populations while determining the effects and circumstances of stage-specific pheromones on mouth form
5. Described a new, more intricate beetle-nematode system to further study complicated competitive interactions

The findings have resulted in several papers, often with collaborations. Effectively, we showed that in the face of competition and demand for food, predatory worms can kill two worms with one bite.

V Results

A. The genetics of phenotypic plasticity in nematode feeding structures

Sommer, R.J., Dardiry, M., Lenuzzi, M., Namdeo, S., **Renahan, T.**, Sieriebriennikov, B. and Werner, M.S. (2017). *Open Biology*, *7*(3), p.160332.

A.1 Synopsis

The intermingling of environment and genes create a bounty of phenotypes, often facilitating diversity and phenotypic novelty. This incredible ecological and evolutionary idea is referred to as phenotypic plasticity and has recently captured the attention of many biologists. Yet, due to the lack of established model organisms to study the phenomenon and dearth of specific genetic investigations there remains controversy. In an effort to ameliorate the situation, the current studies on nematode *Pristionchus pacificus* are summarized to illustrate the presence of a model system well-suited for phenotypic plasticity work. This roundworm maintains a mouth-form dimorphism that results in either strict bacterial-feeding adults or omnivorous adults that can predate on other worms. This plasticity is regulated by epigenetic-controlled developmental switch genes. Conclusions drawn from phylogeny along with comparative analyses substantiate the notion that phenotypic plasticity promotes diversity.

A.2 Own contribution

I participated in the discussions explored in the paper. My contribution is 10%.

B. Environmental influence on *Pristionchus pacificus* mouth form through different culture methods

Werner, M.S., Sieriebriennikov, B., Loschko, T., Namdeo, S., Lenuzzi, M., Dardiry, M., **Renahan, T**., Sharma, D.R. and Sommer, R.J. (2017). *Scientific Reports*, *7*(1), pp.1-12.

B. 1 Synopsis

The environmental factors perceived by juveniles can determine the phenotype of corresponding adults; thus, phenotypic plasticity can have substantial impacts on various fitness-related aspects of the organism, including behavior, morphology, and developmental trajectory. While the molecular mechanisms involved are gradually being examined, there remains a need for better, more amenable systems to really delve into details and set the foundation for future studies. The established model nematode *Pristionchus pacificus* meets the requirements for such demanding experimentation, and can be plied for unraveling the epigenetic, genetic, and genomic involvement in phenotypic plasticity utilizing its mouth-form dimorphism. Previously, there were no simple laboratory environmental settings that both affect the mouth forms and yield sizeable enough cultures necessary for molecular studies. Now, we have developed several assessable culture conditions that are inexpensive and non-exhaustive, are large-scale and reliably influence mouth-form ratios, plus the effects are reversible and occur via the pathways formerly described by forward genetic screens. In addition, several *Pristionchus* species were studied and found to react differently to the culture settings, opening more doors to investigate environmental effects on developmental plasticity at the macroevolutionary level.

B.2 Own contribution

I was involved in the RT-qPCR experiments. My contribution is 5%.

C. Adult influence on juvenile phenotypes by stage-specific pheromone production

Werner, M.S.*, Claaßen, M.H.*, **Renahan, T***., Dardiry, M. and Sommer, R.J. (2018). *iScience*, *10*, pp.123-134.

***co-first author**

C.1 Synopsis

Phenotypic plasticity is utilized by both animals and plants in reaction to population density. Curious to discover if either (or both) age class or cross-generational signaling affects plasticity dependent on density, we used mouth form-dimorphic *Pristionchus pacificus,* which plastically responds to elevated population density by developing a predatory morph, allowing for killing and consumption of nematode competitors. To do so, we established a novel dye-staining method to differentiate mixed populations of worms. We observed, strikingly, that only adult crowding resulted in the development of the predatory morph in juveniles. We tracked this finding to be caused by age/stage-specific pheromones, as determined using high-performance liquid chromatography-mass spectrometry of secreted metabolites and genetic mutants. Specifically, a complex ascarsoide dimer known to induce the predatory morph is only produced by late-stage juveniles and adults, though the mouth-form plasticity is restricted to early juveniles and is irreversible. Thus, age groups play a critical role in phenotypic plasticity, as cross-generational signaling from adults to juveniles signify a quickly growing population.

C. 2 Own contribution

I was involved in various aspects of project design, execution, and analysis for the staining assays, pheromone profiling, and supernatant experiments. I co-wrote the manuscript with one of the other first authors. My contribution is 30%.

D. Mechanism of murderous mushrooms paves path for parasitic helminth halt

Renahan, T. and Sommer, R.J. (2020). *Proceedings of the National Academy of Sciences*, *117*(13), pp.6974-6975.

D. 1 Synopsis

The vibrant interactions between fungi and nematodes epitomize the Red Queen hypothesis, as the two adapt and co-evolve throughout their antagonistic associations. In a journal-requested commentary on a recent research article exploring the susceptibility of nematode sensory cilia when struck by nematode-hungry carnivorous mushrooms (Lee et al., 2020), I reviewed the impressive findings and discussed relevance to application prospects and general evolutionary insights into predator-prey relationships. A newly identified killing mechanism of oyster mushroom *Pleurotus ostreatus* involves sparking a major calcium influx and hypercontraction of pharyngeal and head muscle cells, in turn causing quick necrosis of the whole nematode nervous system, plus muscle cells. Incredibly, this effect and mechanism is conserved across the various nematode species tested and developmental stages, and is unlike currently used anthelmintic drugs. Thus, Lee *et al*. uncovered both a beautiful illustration of predatory evolution and revealed a potential avenue for parasitic nematode treatment.

D. 2 Own contribution

I wrote this commentary with my advisor. My contribution is 50%.

E. *Rhabditophanes diutinus* a parthenogenetic clade IV nematode with dauer larvae

Dulovic, A., **Renahan, T.**, Röseler, W., Rödelsperger, C., Rose, A.M. and Streit, A. (2020). *PLoS Pathogens*, *16*(12), p.e1009113.

E.1 Synopsis

While *Caenorhabditis elegans* has proven to be an exceptional model system for numerous studies, it has failed as a standard to study the rudimentary biology and evolution of parasitic nematodes. This is in part due to the phylogenetic distance of *C. elegans* from parasitic nematode *Strongyloides* (nematode clades V and IV, respectively) and current lack of alternative models. *Strongyloides* spp. maintain distinctive biological characteristics and are of great medical and veterinary significance, thus capturing the interest of many researches and necessitating the establishment of an accessible, well-described, closely related free-living species. Enter *Rhabditophanes diutinus* (formerly KR3021), a free-living nematode belonging to a genus under the Strongyloidoidea nematode superfamily. A paucity of details had existed for this worm, yet it may prove valuable as a model for parasitism research. Now, the species is officially named, *R. diutinus*, and described, its life cycle determined, and stage-specific gene expression analyzed. Two developmental stages were classified that were not previously described for the species, dauer and arrested J2 larvae. Dauer morphology and dafachronic acid-treatment to prevent dauer induction in *R. diutinus* is the same as in *C. elegans* and similar to that of the infective larva (the analogous parasitic stage of dauer) in *Strongyloides*. In addition, the expression arrays of putative dauer/infective larva genes are similar. Therefore, *R. diutinus* can serve as an outgroup model system for parasitic nematode investigations and studying the evolution of parasitism.

E. 2 Own contribution

I was involved in the methodology and investigation of the two previously uncharacterized stages in this newly described species. My contribution is 20%.

F. Nematode biphasic 'boom and bust' dynamics are dependent on host bacterial load while linking dauer and mouth-form polyphenisms

Renahan, T., Lo, W.S., Werner, M.S., Rochat, J., Herrmann, M. and Sommer, R.J. (2021). *Environmental Microbiology*. *23*(9). pp. 5102-5113.

Highlighted by journal editors (Kanzaki, 2021) and cover image

F. 1 Synopsis

The multilayered interactions across the domains of life not only mold habitats but provide beautiful illustrations of co-evolution. The broad range of roundworm associations with various hosts and myriad microbes are the epitome of such illustrations. Yet, there is a dearth of studies investigating cross-kingdom dynamics in the soil after the naturally-occurring demise of insect hosts. La Réunion Island in the Indian Ocean is home to endemic rhinoceros beetle *Oryctes borbonicus*, which reliably harbors nematode *Pristionchus pacificus*, that displays two phenotypic plasticities. In adverse conditions, worms can enter an arrested, non-feeding dispersal stage called dauer. In addition, worms develop one of two mouth forms, becoming either a strict bacterial feeder or an omnivore that can predate on other worms. To investigate nematode dispersal patterns and link the two plastic characteristics, we collected beetles on the island, triggered death, and kept them in soil cages on Réunion and in an artificial-island setting the in the lab, maintaining great spatial and temporal resolutions. *Pristionchus pacificus* displays a biphasic "boom and bust" dispersal pattern that reflects carcass bacterial abundance but not bacterial species. Remarkably, all carcass dauers developed into predators, in contrast to non-carcass dauers maintained in lab, which exhibit standard mouth-form ratios. Altogether, we demonstrate that vital plastic characteristics are employed to ensure survival in the wild.

F. 2 Own contribution

I designed, conducted, and analyzed most experiments, along with writing the manuscript. My contribution is 90%.

G. Nematode interactions on beetle host indicate a role of mouth-form plasticity in resource competition

Renahan, T. and Sommer, R.J. (2021). *Frontiers in Ecology and Evolution. 9:752695*

G. 1 Synopsis

On the decaying insect carcass teeming with microbial life, competition comes alive in a beautiful display of this ubiquitous spectacle. Phenotypic plasticity allows organisms to dynamically respond to opponents, and is frequently employed in nematodes as a possible beneficial scheme. Indeed, insect-associated nematodes constantly face competition as they brawl to flourish on the dynamic and complex environment that is the decaying beetle host carcass. To investigate these interactions in more detail, we utilized formerly unstudied cockchafer *Gymnogaster bupthalma*, a beetle endemic to La Réunion Island and consistently co-infested with *Pristionchus mayeri* and *Acrostichus* spp., diplogastrids both capable of mouth-form and dauer plasticities. Worms develop either as solely bacterial eaters or as omnivores that can predate on fungi and nematodes. In adverse environmental conditions, worms may arrest development and become dauers, dispersal larvae equipped to endure unsuitable settings. We discovered a unique *Pristionchus* tactic while exploring this new beetle-nematode interaction in a natural setting, in which the species maintains a reproducing population on the carcass in parallel to steadily dispersing as dauer. In addition, *P. mayeri* becomes predaceous in the face of competitors, though the species is normally strictly bacterial-feeding.

G. 2 Own contribution

I was involved in the project conception and solely executed design and analysis. I wrote the manuscript. My contribution is 95%.

VI Discussion

"JUST UNDER THE SURFACE I SHALL BE, ALL TOGETHER AT FIRST,
THEN SEPARATE AND DRIFT, THROUGH ALL THE EARTH AND
PERHAPS IN THE END THROUGH A CLIFF INTO THE SEA,
SOMETHING OF ME. A TON OF WORMS IN AN ACRE, THAT IS A
WONDERFUL THOUGHT, A TON OF WORMS, I BELIEVE IT."

Samuel Beckett, *From An Abandoned Work*

Surrounded by a ton of worms is a delightful and stimulating thought, and one that has been a reality as I have plunged into the curious and exciting world of worms. This immersion has resulted in several new insights of both phenotypic plasticity's imperative role in competition for resources and the environmental conditions that influence development. The ecological significance of phenotypic plasticity is exceedingly well explored and described in several organisms, including plentiful plants, innumerable insects, and abundant amphibians.

The ecological significance of phenotypic plasticity in plants is evident. As plants compete for necessary sunlight and nutrients, they can plastically alter various aspects to deal with changing environments. Some modify flowering time while others elongate stems in efforts to compensate for decreased access to rays (Donohue 2003). Many adjust the formation and growth of roots depending on the density of nutrient availability (Hodge 2004). Prominent paradigms of the impact of plasticity have been continuously demonstrated in insects, namely the caste systems in ants, wasps, and bees, and the winged dispersal morph in aphids to escape poor conditions (Whitman and Ananthakrishnan 2009; Sloggett and Weisser 2002). Spadefoots have evolved several adaptations that enable living in extreme environments, with a few phenotypic plastic traits that have clear ecological implications. The tadpoles can plastically respond to altering water levels by accelerating or deaccelerating their developmental rates (Pfennig 1992). In addition, tadpoles can develop as either omnivores or carnivores, the latter enabling even cannibalism, allowing the predators to eliminate nonkin competitors (Pfennig and Murphy 2002). Tadpoles of the grey treefrog respond to

predators by lessening foraging activity (decreasing opportunity for detection) and by developing smaller bodies, thus increasing swimming agility (Van Buskirk and Relyea 1998; Van Buskirk 2000).

Yet, while the ecological relevance of the phenotypic plasticity phenomenon is well detailed in several distinguished phyla, in the emerging model organism *Pristionchus pacificus* it was previously poorly understood. The genetic and molecular mechanisms involved are thoroughly studied, but the lack of environmental significance left the system's story incomplete. Utilizing ubiquitous *P. pacificus* and its reliable association with sundry scarab beetles, we explored the dynamics of the worm's two crucial plastic traits, dauer and mouth form, in a natural setting both in the wild and in lab. In doing so, we were able to uncover and subsequently describe five major findings that help round out the roundworm's saga.

First, we developed a tracking system to determine nematode dispersal patterns from their beetle hosts in greatly expanded spatial and temporal scales from previous studies that were limited in these aspects. For over the course of three months (in addition, with a major gap, two years), we followed *P. pacificus* succession both on the beetle carcass and from it. Feeding stages and the arrested developmental stage, dauer, were inversely present, displaying a biphasic "boom and bust" dispersal dynamic: populations reproduced while bacterial food abundance was relatively high, and then entered dauer as resources decreased, preceding a major migration of dauers from the carcass, thus allowing a repopulation of the bacteria and a subsequent increase of growing worms, until the devouring microbes finished demolishing the carcass, leaving only the exoskeleton. Though, after two years, single-digit number of dauers remain on the chitin structure, perhaps waiting for a new host to emerge. The surprising finding resulting from this experiment is the speed at which these worms wiggle away and the distance covered in a seemingly short timespan; future studies can pinpoint the exact rate at which nematodes move.

Second, we revealed carcass-specific factors that influence mouth form by examining the potential cross-talk between the two plastic traits utilizing both wild

strains freshly isolated from the beetle hosts and domesticated strains. All worms exiting dauer on the carcass developed as predators, even though successive generations from these worms displayed various mouth-form ratios. Yet, this exclusively predatory formation is restricted to carcass-derived dauers; post-dauer worms of domesticated strains maintain the strains' normal mouth-form preferences. In addition, non-dauer worms on the carcass developed both forms, as did dauers in the surrounding soil. Thus, there are factors specific to the insect carcass that influence only dauers to become predatory. Current studies are exploring how the natural environment may impact mouth forms, and single worm RNA-seq of dauer and post-dauer carcass worms and domesticated strains will provide insight into potential biological differences.

Third, we detailed the carcasses' bacterial succession over the course of three months, covering the most active worm period and shortly after population growth has ceased. Surprisingly, the bacterial composition was relatively stable, and at least to the resolution of 16S classification, there was no obvious array corresponding to the biphasic boom and bust dispersal dynamic seen in the nematodes. Though, at ten weeks, after most worms have left the carcass and only dauers remain, there is a slight increase of Bacteroidetes, which is known to be associated with dauer frequency in *C. elegans* (Samuel *et al.* 2016). In addition, certain natural bacteria can influence *P. pacificus* survival (Akduman *et al.* 2018). Thus, there may be undetected impacts from certain microbes, necessitating ongoing studies investigating bacterial influences on *P. pacificus* in a soil setting.

Fourth, we established a novel dye-staining method to differentiate similar nematode populations in mixed cultures. By doing do, we were able to explore age-class effects on mouth form and determined certain pheromones to be stage-specific, including one that induces the predatory morph. Adults produce a predator-inducing pheromone that signals to juveniles impending population increase, as the developmental plasticity is density-dependent. While this could be indicative of kin selection, perhaps juveniles evolved to recognize adult pheromones. Regardless, this demonstrates that adults are a critical age group and provides a setting for further

investigation of metabolite production. Specifically, further studies revealed that metabolite production in earlier stages are needed for formation of this late-stage pheromone, and RNA-seq analysis pinpointed several genes potentially involved in small molecule production pathways, based on *C. elegans* homologs involved in ascaroside synthesis. Ongoing studies include CRISPR/Cas9 mutagenesis of these genes, which will illuminate on this molecular mechanism that has an ecological impact via influence on an imperative plastic phenotype.

Fifth, we have described and established a previously unexplored beetle-nematode system that enables expansion of experiment types. While most field studies in our lab focused on abundant scarab beetle *Oryctes borbonicus* and *Pristionchus pacificus,* we now have another system: cockchafer *Gymnogaster bupthalma* that hosts both *Pristionchus mayeri* and *Acrostichus* spp., and often co-infestation of both genera. Strikingly, carcass-derived *P. mayeri* develops as predators in conditions dense with competitors, in stark contrast to domesticated *P. mayeri*, which stubbornly remains strictly bacterial-feeding, even under conventional predatory-inducing conditions. This exciting finding has set the stage for investigating the potential canalization of the bacterial morph in our domesticated strain. Single worm RNA-seq of carcass-derived and domesticated worms of various developmental stages and mouth forms, along with predatory assays, will provide major insights into the extraordinary phenomenon that is phenotypic plasticity.

A forthcoming manuscript will set the stage for future studies utilizing the two beetle systems by detailing the microbial successions of *G. bupthalma* adults and larvae and *O. borbonicus* larvae. With the establishment of the microbial classification of certain beetle developmental stages and at specific times of decay, experiments to pinpoint potential effects of particular bacteria on phenotypic plasticity can be employed. Thus, the model organism *P. pacificus* now not only maintains detailed molecular insights, but also ecological significance that further cements the worm as an imperative and useful system to explore phenotypic plasticity at various levels. Many more wonderful thoughts will emerge and inspire from acres of tons of worms.

VII References

Akduman, N., Lightfoot, J. W., Röseler, W., Witte, H., Lo, W. S., Rödelsperger, C., & Sommer, R. J. (2020). Bacterial vitamin B 12 production enhances nematode predatory behavior. *The ISME journal*, *14*(6), 1494-1507.

Akduman, N., Rödelsperger, C., & Sommer, R. J. (2018). Culture-based analysis of *Pristionchus*-associated microbiota from beetles and figs for studying nematode-bacterial interactions. *PLoS One*, *13*(6), e0198018.

Alatorre-Rosas, R., & Kaya, H. K. (1990). Interspecific competition between entomopathogenic nematodes in the genera *Heterorhabditis* and *Steinernema* for an insect host in sand. *Journal of Invertebrate Pathology*, *55*(2), 179-188.

Bardgett, R. D., & Van Der Putten, W. H. (2014). Belowground biodiversity and ecosystem functioning. *Nature*, *515*(7528), 505-511.

Bedding, R. A., Molyneux, A. S., & Akhurst, R. J. (1983). *Heterorhabditis spp*., *Neoaplectana spp*., and *Steinernema kraussei*: interspecific and intraspecific differences in infectivity for insects. *Experimental Parasitology*, *55*(2), 249-257.

Bento, G., Ogawa, A., & Sommer, R. J. (2010). Co-option of the hormone-signalling module dafachronic acid–DAF-12 in nematode evolution. *Nature*, *466*(7305), 494-497.

Berg, M., Stenuit, B., Ho, J., Wang, A., Parke, C., Knight, M., Alvarez-Cohen, L. and Shapira, M. (2016). Assembly of the *Caenorhabditis elegans* gut microbiota from diverse soil microbial environments. *The ISME journal*, *10*(8), 1998-2009.

Blanco-Pérez, R., Bueno-Pallero, F. Á., Neto, L., & Campos-Herrera, R. (2017). Reproductive efficiency of entomopathogenic nematodes as scavengers. Are they able to fight for insect's cadavers? *Journal of Invertebrate Pathology*, *148*, 1-9.

Blaxter, M. (2011). Nematodes: the worm and its relatives. *PLoS Biology*, *9*(4), e1001050.

Bordenstein, S. R., & Theis, K. R. (2015). Host biology in light of the microbiome: ten principles of holobionts and hologenomes. *PLoS Biology*, *13*(8), e1002226.

Bose, N., Meyer, J.M., Yim, J.J., Mayer, M.G., Markov, G.V., Ogawa, A., Schroeder, F.C. and Sommer, R.J. (2014). Natural variation in dauer pheromone production and sensing supports intraspecific competition in nematodes. *Current Biology*, *24*(13), 1536-1541.

Bose, N., Ogawa, A., von Reuss, S. H., Yim, J. J., Ragsdale, E. J., Sommer, R. J., & Schroeder, F. C. (2012). Complex small-molecule architectures regulate phenotypic plasticity in a nematode. *Angewandte Chemie*, *124*(50), 12606-12611.

Bouchard, P., Grebennikov, V. V., Smith, A. B., & Douglas, H. (2009). Biodiversity of coleoptera. In: Foottit, R. G., Adler, P. D., editors. *Insect Biodiversity: Science and Society*. Oxford: Wiley-Blackwell. 265-301.

Bovien, P. (1937). Some types of association between nematodes and insects. *Videnskabelige Meddelelser fra Dansk Naturhistorisk Forening*, *101*, 1-114.

Brown, F. D., D'Anna, I., & Sommer, R. J. (2011). Host-finding behaviour in the nematode *Pristionchus pacificus*. *Proceedings of the Royal Society B: Biological Sciences*, *278*(1722), 3260-3269.

Buffington, S. A., Di Prisco, G. V., Auchtung, T. A., Ajami, N. J., Petrosino, J. F., & Costa-Mattioli, M. (2016). Microbial reconstitution reverses maternal diet-induced social and synaptic deficits in offspring. *Cell*, *165*(7), 1762-1775.

Burnell, A., & Stock, S. P. (2000). *Heterorhabditis*, *Steinernema* and their bacterial symbionts—lethal pathogens of insects. *Nematology*, *2*(1), 31-42.

Burrows, R. B. (1962). Comparative morphology of *Ancylostoma tubaeforme* (Zeder, 1800) and *Ancylostoma caninum* (Ercolani, 1859). *The Journal of Parasitology*, 715-718.

Buskirk, J., & Relyea, R. A. (1998). Selection for phenotypic plasticity in *Rana sylvatica* tadpoles. *Biological Journal of the Linnean Society*, *65*(3), 301-328.

Buskirk, V. (2000). Functional mechanisms of an inducible defence in tadpoles: morphology and behaviour influence mortality risk from predation. *Journal of Evolutionary Biology*, *13*(2), 336-347.

Butcher, R. A. (2017). Small-molecule pheromones and hormones controlling nematode development. *Nature Chemical Biology*, *13*(6), 577-586.

Campbell, J. F., & Gaugler, R. (1993). Nictation behaviour and its ecological implications in the host search strategies of entomopathogenic nematodes (Heterorhabditidae and Steinernematidae). *Behaviour*, 155-169.

Campos-Herrera, R., Půža, V., Jaffuel, G., Blanco-Pérez, R., Čepulytė-Rakauskienė, R., & Turlings, T. C. (2015). Unraveling the intraguild competition between *Oscheius spp*. nematodes and entomopathogenic nematodes: implications for their natural distribution in Swiss agricultural soils. *Journal of Invertebrate Pathology*, *132*, 216-227.

Carrier, T. J., & Reitzel, A. M. (2018). Convergent shifts in host-associated microbial communities across environmentally elicited phenotypes. *Nature Communications*, *9*(1), 1-9.

Cassada, R. C., & Russell, R. L. (1975). The dauerlarva, a post-embryonic developmental variant of the nematode *Caenorhabditis elegans*. *Developmental Biology*, *46*(2), 326-342.

Chang, T., Jung, B.K., Sohn, W.M., Hong, S., Shin, H., Ryoo, S., Lee, J., Lee, K.H., Khieu, V., Huy, R. and Chai, J.Y. (2020). Morphological and Molecular Diagnosis of *Necator americanus* and *Ancylostoma ceylanicum* Recovered from Villagers in Northern Cambodia. *The Korean Journal of Parasitology*, *58*(6), 619.

Cinkornpumin, J.K., Wisidagama, D.R., Rapoport, V., Go, J.L., Dieterich, C., Wang, X., Sommer, R.J. and Hong, R.L. (2014). A host beetle pheromone regulates development and behavior in the nematode *Pristionchus pacificus*. *eLife*, *3*, e03229.

Darwin, C. (1859). *On the origin of species by means of natural selection*. London: Murray.

DeWitt, T. J., & Scheiner, S. M. (Eds.). (2004). *Phenotypic plasticity: functional and conceptual approaches*. Oxford: Oxford University Press.

Dillman, A. R., & Sternberg, P. W. (2012). Entomopathogenic nematodes. *Current Biology*, *22*(11), R430.

Dirksen, P., Marsh, S.A., Braker, I., Heitland, N., Wagner, S., Nakad, R., Mader, S., Petersen, C., Kowallik, V., Rosenstiel, P. and Félix, M.A. (2016). The native microbiome of the nematode *Caenorhabditis elegans*: gateway to a new host-microbiome model. *BMC Biology*, *14*(1), 1-16.

Dong, C., Weadick, C. J., Truffault, V., & Sommer, R. J. (2020). Convergent evolution of small molecule pheromones in *Pristionchus* nematodes. *eLife*, *9*, e55687.

Donohue, K. (2003). Setting the stage: phenotypic plasticity as habitat selection. *International Journal of Plant Sciences*, *164*(S3), S79-S92.

Downes, M. J., & Griffin, C. T. (1996). Dispersal behaviour and transmission strategies of the entomopathogenic nematodes *Heterorhabditis* and *Steinernema*. *Biocontrol Science and Technology*, *6*(3), 347-356.

Duncan, L. W., Dunn, D. C., Bague, G., & Nguyen, K. (2003). Competition between entomopathogenic and free-living bactivorous nematodes in larvae of the weevil *Diaprepes abbreviatus*. *Journal of Nematology*, *35*(2), 187.

Eng, M. S., Preisser, E. L., & Strong, D. R. (2005). Phoresy of the entomopathogenic nematode *Heterorhabditis marelatus* by a non-host organism, the isopod *Porcellio scaber*. *Journal of Invertebrate Pathology*, *88*(2), 173-176.

Fedyaeva, M. A., Neretina, T. V., Konovalova, O. P., & Tchesunov, A. V. (2016). Two known and one new species of Draconematidae and Epsilonematida (Nematoda, Desmodorida) from the White Sea, North Russia. *Zootaxa*, *4121*(4), 383-411.

Félix, M. A., & Duveau, F. (2012). Population dynamics and habitat sharing of natural populations of *Caenorhabditis elegans* and *C. briggsae*. *BMC Biology*, *10*(1), 1-19.

Ferris, H. (2010). Contribution of nematodes to the structure and function of the soil food web. *Journal of Nematology*, *42*(1), 63.

Frézal, L., & Félix, M. A. (2015). The natural history of model organisms: *C. elegans* outside the Petri dish. *eLife*, *4*, e05849.

Fürst von Lieven, A. (2002). Functional morphology, origin and phylogenetic implications of the feeding mechanism of *Tylopharynx foetida* (Nematoda: Diplogastrina). *Russian Journal of Nematology*, *10*, 11-23.

Gerisch, B., Weitzel, C., Kober-Eisermann, C., Rottiers, V., & Antebi, A. (2001). A hormonal signaling pathway influencing *C. elegans* metabolism, reproductive development, and life span. *Developmental Cell*, *1*(6), 841-851.

Gibbs, K. A., Urbanowski, M. L., & Greenberg, E. P. (2008). Genetic determinants of self identity and social recognition in bacteria. *Science*, *321*(5886), 256-259.

Giblin-Davis, R. M., Williams, D. S., Wergin, W. P., Dickson, D. W., Hewlett, T. E., Bekal, S., & Becker, J. (2001). Ultrastructure and development of *Pasteuria sp*.(S-1 strain), an obligate endoparasite of *Belonolaimus longicaudatus* (Nemata: Tylenchida). *Journal of Nematology*, *33*(4), 227.

Glass, N. L., & Kaneko, I. (2003). Fatal attraction: nonself recognition and heterokaryon incompatibility in filamentous fungi. *Eukaryotic Cell*, *2*(1), 1-8.

Golden, J. W., & Riddle, D. L. (1982). A pheromone influences larval development in the nematode *Caenorhabditis elegans*. *Science*, *218*(4572), 578-580.

Golden, J. W., & Riddle, D. L. (1984a). A *Caenorhabditis elegans* dauer-inducing pheromone and an antagonistic component of the food supply. *Journal of Chemical Ecology*, *10*(8), 1265-1280.

Golden, J. W., & Riddle, D. L. (1984b). The *Caenorhabditis elegans* dauer larva: developmental effects of pheromone, food, and temperature. *Developmental Biology*, *102*(2), 368-378.

Gould, A.L., Zhang, V., Lamberti, L., Jones, E.W., Obadia, B., Korasidis, N., Gavryushkin, A., Carlson, J.M., Beerenwinkel, N. and Ludington, W.B. (2018). Microbiome interactions shape host fitness. *Proceedings of the National Academy of Sciences*, *115*(51), E11951-E11960.

Hartley, C. J., Lillis, P. E., Owens, R. A., & Griffin, C. T. (2019). Infective juveniles of entomopathogenic nematodes (*Steinernema* and *Heterorhabditis*) secrete ascarosides and respond to interspecific dispersal signals. *Journal of Invertebrate Pathology*, *168*, 107257.

Hedrich, R., & Fukushima, K. (2021). On the origin of carnivory: Molecular physiology and evolution of plants on an animal diet. *Annual Review of Plant Biology*, *72*, 133-153.

Herrmann, M., Kienle, S., Rochat, J., Mayer, W. E., & Sommer, R. J. (2010). Haplotype diversity of the nematode *Pristionchus pacificus* on Réunion in the Indian Ocean suggests multiple independent invasions. *Biological Journal of the Linnean Society*, *100*(1), 170-179.

Herrmann, M., Mayer, W. E., Hong, R. L., Kienle, S., Minasaki, R., & Sommer, R. J. (2007). The nematode *Pristionchus pacificus* (Nematoda: Diplogastridae) is associated with the oriental beetle *Exomala orientalis* (Coleoptera: Scarabaeidae) in Japan. *Zoological Science*, *24*(9), 883-889.

Herrmann, M., Mayer, W. E., & Sommer, R. J. (2006). Nematodes of the genus *Pristionchus* are closely associated with scarab beetles and the Colorado potato beetle in Western Europe. *Zoology*, *109*(2), 96-108.

Hodge, A. (2004). The plastic plant: root responses to heterogeneous supplies of nutrients. *New Phytologist*, *162*(1), 9-24.

Hong, R. L., & Sommer, R. J. (2006). *Pristionchus pacificus*: a well-rounded nematode. *BioEssays*, *28*(6), 651-659.

Hussey, R. S. (1989). Disease-inducing secretions of plant-parasitic nematodes. *Annual Review of Phytopathology*, *27*(1), 123-141.

Inoda, T. (2012). Predaceous diving beetle, *Dytiscus sharpi sharpi* (Coleoptera: Dytiscidae) larvae avoid cannibalism by recognizing prey. *Zoological Science*, *29*(9), 547-552.

Ishibashi, N., & Kondo, E. (1990). Behavior of infective juveniles. In: Gaugler, R., Kaya, H. K., editors. *Entomopathogenic Nematodes in Biological Control*. Boca Raton: CRC Press.139-150.

Jeong, P.Y., Jung, M., Yim, Y.H., Kim, H., Park, M., Hong, E., Lee, W., Kim, Y.H., Kim, K. and Paik, Y.K. (2005). Chemical structure and biological activity of the *Caenorhabditis elegans* dauer-inducing pheromone. *Nature*, *433*(7025), 541-545.

Kaplan, F., Alborn, H.T., von Reuss, S.H., Ajredini, R., Ali, J.G., Akyazi, F., Stelinski, L.L., Edison, A.S., Schroeder, F.C. and Teal, P.E. (2012). Interspecific nematode signals regulate dispersal behavior. *PloS One*, *7*(6), e38735.

Kaya, H. K., Bedding, R. A., & Akhurst, R. J. (1993). An overview of insect-parasitic and entomopathogenic nematodes. In: Bedding, R. A., Akhurst, R. J., & Kaya, H. K., editors. *Nematodes and the biological control of insect pests*. 1-10.

Kaya, H., & Campbell, J. (2000). Influence of insect associated cues on the jumping behavior of entomopathogenic nematodes (Steinernema spp.). *Behaviour*, *137*(5), 591-609.

Kaya, H. K., & Gaugler, R. (1993). Entomopathogenic nematodes. *Annual Review of Entomology*, *38*(1), 181-206.

Kaya, H. K., & Koppenhöfer, A. M. (1996). Effects of microbial and other antagonistic organism and competition on entomopathogenic nematodes. *Biocontrol Science and Technology*, *6*(3), 357-372.

Kolodny, O., & Schulenburg, H. (2020). Microbiome-mediated plasticity directs host evolution along several distinct time scales. *Philosophical Transactions of the Royal Society B*, *375*(1808), 20190589.

Koppenhöfer, A. M., & Fuzy, E. M. (2006). Effect of soil type on infectivity and persistence of the entomopathogenic nematodes *Steinernema scarabaei*, *Steinernema glaseri, Heterorhabditis zealandica*, and *Heterorhabditis bacteriophora*. *Journal of Invertebrate Pathology*, *92*(1), 11-22.

Koppenhöfer, A. M., & Kaya, H. K. (1996). Coexistence of two steinernematid nematode species (Rhabditida: Steinernematidae) in the presence of two host species. *Applied Soil Ecology*, *4*(3), 221-230.

Koppenhöfer, A. M., Kaya, H. K., Shanmugam, S., & Wood, G. L. (1995). Interspecific competition between steinernematid nematodes within an insect host. *Journal of Invertebrate Pathology*, *66*(2), 99-103.

Lambshead, P. J. D., & Boucher, G. (2003). Marine nematode deep-sea biodiversity–hyperdiverse or hype? *Journal of Biogeography*, *30*(4), 475-485.

Lambshead, P. J. D., Chen, Z. X., Chen, W. Y., Chen, S. Y., & Dickson, S. W. (2004). Marine nematode biodiversity. In: Chen Z. X., Chen S. Y., Dickson D. W., editors. *Nematology: Advances and Perspectives Volume 1: Nematode Morphology, Physiology and Ecology*. London: CABI Publishing. 436-467.

Ledón-Rettig, C. C., Moczek, A. P., & Ragsdale, E. J. (2018). *Diplogastrellus* nematodes are sexually transmitted mutualists that alter the bacterial and fungal communities of their beetle host. *Proceedings of the National Academy of Sciences*, *115*(42), 10696-10701.

Lee, D., Lee, H., Kim, N., Lim, D. S., & Lee, J. (2017). Regulation of a hitchhiking behavior by neuronal insulin and TGF-β signaling in the nematode *Caenorhabditis elegans*. *Biochemical and Biophysical Research Communications*, *484*(2), 323-330.

Lightfoot, J. W., Wilecki, M., Rödelsperger, C., Moreno, E., Susoy, V., Witte, H., & Sommer, R. J. (2019). Small peptide–mediated self-recognition prevents cannibalism in predatory nematodes. *Science*, *364*(6435), 86-89.

Lorenzen, S. (1994). *The phylogenetic systematics of free living nematodes*. London: The Ray Society.

Markov, G.V., Meyer, J.M., Panda, O., Artyukhin, A.B., Claaßen, M., Witte, H., Schroeder, F.C. and Sommer, R.J. (2016). Functional conservation and divergence of daf-22 paralogs in *Pristionchus pacificus* dauer development. *Molecular Biology and Evolution*, *33*(10), 2506-2514.

Martin, M. O. (2002). Predatory prokaryotes: an emerging research opportunity. *Journal of Molecular Microbiology and Biotechnology*, *4*(5), 467-478.

McGaughran, A., & Morgan, K. (2015). Population genetics and the La Réunion case study. In: Sommer, R. J., editor. *Pristionchus pacificus: A Nematode Model for Comparative and Evolutionary Biology.* Leiden-Boston: Brill. 197-219.

McGaughran, A., Rödelsperger, C., Grimm, D.G., Meyer, J.M., Moreno, E., Morgan, K., Leaver, M., Serobyan, V., Rakitsch, B., Borgwardt, K.M. and Sommer, R.J. (2016). Genomic profiles of diversification and genotype–phenotype association in island nematode lineages. *Molecular Biology and Evolution*, *33*(9), 2257-2272.

Mehlhorn, H. (2008). *Encyclopedia of parasitology: Volume 1, AM.*, (Edn. 3). Berlin Heidelberg: Springer.

Meyer, J. M., Baskaran, P., Quast, C., Susoy, V., Rödelsperger, C., Glöckner, F. O., & Sommer, R. J. (2017). Succession and dynamics of *Pristionchus* nematodes and their microbiome during decomposition of *Oryctes borbonicus* on La Réunion Island. *Environmental Microbiology*, *19*(4), 1476-1489.

Molyneux, A. S. (1986). *Heterorhabditis spp*. and *Steinernema* (= Neoaplectana) spp.: temperature, and aspects of behavior and infectivity. *Experimental Parasitology*, *62*(2), 169-180.

Montalvo-Katz, S., Huang, H., Appel, M. D., Berg, M., & Shapira, M. (2013). Association with soil bacteria enhances p38-dependent infection resistance in *Caenorhabditis elegans*. *Infection and Immunity*, *81*(2), 514-520.

Moreno, E., McGaughran, A., Rödelsperger, C., Zimmer, M., & Sommer, R. J. (2016). Oxygen-induced social behaviours in *Pristionchus pacificus* have a distinct evolutionary history and genetic regulation from *Caenorhabditis elegans*. *Proceedings of the Royal Society B: Biological Sciences*, *283*(1825), 20152263.

Morgan, K., McGaughran, A., Ganeshan, S., Herrmann, M., & Sommer, R. J. (2014). Landscape and oceanic barriers shape dispersal and population structure in the island nematode *Pristionchus pacificus*. *Biological Journal of the Linnean Society*, *112*(1), 1-15.

Morgan, K., McGaughran, A., Villate, L., Herrmann, M., Witte, H., Bartelmes, G., Rochat, J. and Sommer, R.J. (2012). Multi locus analysis of *Pristionchus pacificus* on La Réunion Island reveals an evolutionary history shaped by multiple introductions, constrained dispersal events and rare out-crossing. *Molecular Ecology*, *21*(2), 250-266.

Noguez, J. H., Conner, E. S., Zhou, Y., Ciche, T. A., Ragains, J. R., & Butcher, R. A. (2012). A novel ascaroside controls the parasitic life cycle of the entomopathogenic nematode *Heterorhabditis bacteriophora*. *ACS Chemical Biology*, *7*(6), 961-966.

O'Callaghan, K. M., Zenner, A. N., Hartley, C. J., & Griffin, C. T. (2014). Interference competition in entomopathogenic nematodes: male *Steinernema* kill members of their own and other species. *International Journal for Parasitology*, *44*(13), 1009-1017.

Ogawa, A., & Sommer, R. J. (2009). Strategies to get arrested. *Science*, *326*(5955), 944-945.

Osche, G. (1955). The pre-adaptation of free-living nematodes to parasitism. *Verhandlungen der Deutschen Zoologischen Gesellschaft. (Zoologischer Anzeiger)*, (Suppl. 19), 391-397.

Panda, O., Akagi, A.E., Artyukhin, A.B., Judkins, J.C., Le, H.H., Mahanti, P., Cohen, S.M., Sternberg, P.W. and Schroeder, F.C. (2017). Biosynthesis of Modular Ascarosides in *C. elegans*. *Angewandte Chemie*, *129*(17), 4807-4811.

Penkov, S., Ogawa, A., Schmidt, U., Tate, D., Zagoriy, V., Boland, S., Gruner, M., Vorkel, D., Verbavatz, J.M., Sommer, R.J. and Knölker, H.J. (2014). A wax ester promotes collective host finding in the nematode *Pristionchus pacificus*. *Nature Chemical Biology*, *10*(4), 281-285.

Peters, A. (1996). The natural host range of *Steinernema* and *Heterorhabditis spp*. and their impact on insect populations. *Biocontrol Science and Technology*, *6*(3), 389-402.

Pfennig, D. W. (1992). Polyphenism in spadefoot toad tadpoles as a locally adjusted evolutionarily stable strategy. *Evolution*, *46*(5), 1408-1420.

Pfennig, D. W. (Ed.). (2021). *Phenotypic Plasticity & Evolution: Causes, Consequences, Controversies*. Boca Raton: Taylor & Francis.

Pfennig, D. W., & Murphy, P. J. (2002). How fluctuating competition and phenotypic plasticity mediate species divergence. *Evolution*, *56*(6), 1217-1228.

Phillips, G., Moulton, J. K., & Bernard, E. C. (2020). *Heth pivari n. sp.* (Nematoda: Ransomnematoidea: Hethidae) from the indigenous North American millipede *Narceus gordanus* (Spirobolida: Spirobolidae), with keys for worldwide *Heth spp. Zootaxa*, *4861*(4), 486-514.

Platt, H. M., Shaw, K. M., & Lambshead, P. J. D. (1984). Nematode species abundance patterns and their use in the detection of environmental perturbations. *Hydrobiologia*, *118*(1), 59-66.

Poinar Jr, G. O. (1983). *The natural history of nematodes.* Englewood Cliffs: Prentice Hall, Inc.

Poinar Jr, G.O. (1990). Taxonomy and biology of Steinernematidae and Heterorhabditidae. In: Gaugler, R., Kaya, H. K., editors. *Entomopathogenic Nematodes in Biological Control*. Boca Raton: CRC Press. *54.*

Procter, D. L. (1990). Global overview of the functional roles of soil-living nematodes in terrestrial communities and ecosystems. *Journal of Nematology*, *22*(1), 1.

Ragsdale, E. J., Müller, M. R., Rödelsperger, C., & Sommer, R. J. (2013). A developmental switch coupled to the evolution of plasticity acts through a sulfatase. *Cell*, *155*(4), 922-933.

Renahan, T., & Hong, R. L. (2017). A species-specific nematocide that results in terminal embryogenesis. *Journal of Experimental Biology*, *220*(18), 3238-3247.

Riddle, D. L., Swanson, M. M., & Albert, P. S. (1981). Interacting genes in nematode dauer larva formation. *Nature*, *290*(5808), 668-671.

Rota-Stabelli, O., Daley, A. C., & Pisani, D. (2013). Molecular timetrees reveal a Cambrian colonization of land and a new scenario for ecdysozoan evolution. *Current Biology*, *23*(5), 392-398.

Samuel, B. S., Rowedder, H., Braendle, C., Félix, M. A., & Ruvkun, G. (2016). *Caenorhabditis elegans* responses to bacteria from its natural habitats. *Proceedings of the National Academy of Sciences*, *113*(27), E3941-E3949.

Schlichting, C. D., & Pigliucci, M. (1998). *Phenotypic evolution: a reaction norm perspective*. Sunderland: Sinauer Associates, Inc.

Shin, S.C., Kim, S.H., You, H., Kim, B., Kim, A.C., Lee, K.A., Yoon, J.H., Ryu, J.H. and Lee, W.J. (2011). *Drosophila* microbiome modulates host developmental and metabolic homeostasis via insulin signaling. *Science*, *334*(6056), 670-674.

Sloggett, J. J., & Weisser, W. W. (2002). Parasitoids induce production of the dispersal morph of the pea aphid, *Acyrthosiphon pisum*. *Oikos*, *98*(2), 323-333.

Smith, P., & Tchernov, E. (1992). *Structure, Function, and Evolution of Teeth*. Tel Aviv: Freund Publishing House Ltd.

Sommer, R. J. (Ed.). (2015). *Pristionchus pacificus: A Nematode Model for Comparative and Evolutionary Biology*. Leiden-Boston: Brill.

Sommer, R., Carta, L. K., Kim, S. Y., & Sternberg, P. W. (1996). Morphological, genetic and molecular description of *Pristionchus pacificus sp. n.*(Nematoda: Neodiplogasteridae). *Fundamental and Applied Nematology*, *19*, 511-522.

Sonnenburg, J. L., & Bäckhed, F. (2016). Diet–microbiota interactions as moderators of human metabolism. *Nature*, *535*(7610), 56-64.

Stork, N. E., McBroom, J., Gely, C., & Hamilton, A. J. (2015). New approaches narrow global species estimates for beetles, insects, and terrestrial arthropods. *Proceedings of the National Academy of Sciences*, *112*(24), 7519-7523.

Sudhaus, W. (2008). Evolution of insect parasitism in rhabditid and diplogastrid nematodes. *Advances in Arachnology and Developmental Biology*, *12*, 143-161.

Suez, J., Korem, T., Zeevi, D., Zilberman-Schapira, G., Thaiss, C.A., Maza, O., Israeli, D., Zmora, N., Gilad, S., Weinberger, A. and Kuperman, Y. (2014). Artificial sweeteners induce glucose intolerance by altering the gut microbiota. *Nature*, *514*(7521), 181-186.

Susoy, V., Herrmann, M., Kanzaki, N., Kruger, M., Nguyen, C.N., Rödelsperger, C., Röseler, W., Weiler, C., Giblin-Davis, R.M., Ragsdale, E.J. and Sommer, R.J. (2016). Large-scale diversification without genetic isolation in nematode symbionts of figs. *Science Advances*, *2*(1), e1501031.

Susoy, V., Ragsdale, E. J., Kanzaki, N., & Sommer, R. J. (2015). Rapid diversification associated with a macroevolutionary pulse of developmental plasticity. *eLife*, *4*, e05463.

Susoy, V., & Sommer, R. J. (2016). Stochastic and conditional regulation of nematode mouth-form dimorphisms. *Frontiers in Ecology and Evolution*, *4*, 23.

Thomas, J. H., Birnby, D. A., & Vowels, J. J. (1993). Evidence for parallel processing of sensory information controlling dauer formation in *Caenorhabditis elegans*. *Genetics*, *134*(4), 1105-1117.

Van Den Hoogen, J., Geisen, S., Routh, D., Ferris, H., Traunspurger, W., Wardle, D.A., De Goede, R.G., Adams, B.J., Ahmad, W., Andriuzzi, W.S. and Bardgett, R.D. (2019). Soil nematode abundance and functional group composition at a global scale. *Nature*, *572*(7768), 194-198.

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., & Dufresne, A. (2015). The importance of the microbiome of the plant holobiont. *New Phytologist*, *206*(4), 1196-1206.

Vieira, P., & Gleason, C. (2019). Plant-parasitic nematode effectors—insights into their diversity and new tools for their identification. *Current Opinion in Plant Biology*, *50*, 37-43.

Von Lieven, A. F., & Sudhaus, W. (2000). Comparative and functional morphology of the buccal cavity of Diplogastrina (Nematoda) and a first outline of the phylogeny of this taxon. *Journal of Zoological Systematics and Evolutionary Research*, *38*(1), 37-63.

Wallace, A.R. (1880). *Island Life; Or, The Phenomena and Causes of Insular Faunas and Floras.* London: Macmillan and Co..

West-Eberhard, M. J. (1989). Phenotypic plasticity and the origins of diversity. *Annual review of Ecology and Systematics*, *20*(1), 249-278.

West-Eberhard MJ. (2003). *Developmental plasticity and evolution*. Oxford: Oxford University Press.

Whitman, D. W., & Ananthakrishnan, T. N. (2009). *Phenotypic plasticity of insects: mechanisms and consequences*. Boca Raton: Science Publishers, Inc..

Wilecki, M., Lightfoot, J. W., Susoy, V., & Sommer, R. J. (2015). Predatory feeding behaviour in *Pristionchus* nematodes is dependent on phenotypic plasticity and induced by serotonin. *The Journal of Experimental Biology*, *218*(9), 1306-1313.

Williamson, V. M., & Gleason, C. A. (2003). Plant–nematode interactions. *Current Opinion in Plant Biology*, *6*(4), 327-333.

Zilber-Rosenberg, I., & Rosenberg, E. (2008). Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. *FEMS Microbiology Reviews*, *32*(5), 723-735.

VIII Appendix

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Review

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THE ROYAL SOCIETY PUBLISHING

The genetics of phenotypic plasticity in nematode feeding structures

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Phenotypic plasticity has been proposed as an ecological and evolutionary concept. Ecologically, it can help study how genes and the environment interact to produce robust phenotypes. Evolutionarily, as a facilitator it might contribute to phenotypic novelty and diversification. However, the discussion of phenotypic plasticity remains contentious in parts due to the absence of model systems and rigorous genetic studies. Here, we summarize recent work on the nematode *Pristionchus pacificus*, which exhibits a feeding plasticity allowing predatory or bacteriovorous feeding. We show feeding plasticity to be controlled by developmental switch genes that are themselves under epigenetic control. Phylogenetic and comparative studies support phenotypic plasticity and its role as a facilitator of morphological novelty and diversity.

1. Introduction

All organisms have to adapt to the environment and to environmental variation. Often, alternative conditions result in different expressions and values of traits, a phenomenon referred to as 'phenotypic plasticity'. Generally, phenotypic (or developmental) plasticity is defined as the property of a given genotype to produce different phenotypes depending on distinct environmental conditions [1,2]. In addition to being an ecological concept that allows studying how organisms respond to environmental variation, phenotypic plasticity also represents an integral part of the evolutionary process. Given these ecological and evolutionary implications, it is not surprising that the concept of phenotypic plasticity has been contentious ever since its introduction at the beginning of the 20th century. For some, plasticity is the major driver and facilitator of phenotypic diversification, and, as such, of greatest importance for understanding evolution and its underlying mechanisms [1–3]. For others, phenotypic plasticity represents environmental noise and is sometimes considered to even hinder evolution because environmentally induced variation may slow down the rate of adaptive processes [4,5]. This controversy largely depends on two limitations. First, there is confusion over the different types of plasticity found in nature. Plasticity can be adaptive or non-adaptive, reversible or irreversible, conditional or stochastic, and continuous or discrete, all of which require careful evaluations of examples of plasticity for their potential evolutionary significance. Second, the absence of plasticity model systems has long hampered the elucidation of potential molecular and genetic mechanisms, the identification of which would provide a framework for theoretical considerations.

In 1965, Bradshaw made one of the most important contributions to the concept of phenotypic plasticity when he proposed that plasticity must have a genetic basis. This idea grew out of the observation that the plasticity of a trait is independent of the phenotype of the plastic trait itself [6]. However, little progress was made to identify underlying mechanisms, largely due to the absence of laboratory model systems of plasticity. Here, we summarize recent studies on phenotypic plasticity of feeding structures in the nematode *Pristionchus pacificus*. The

Table 1. History of phenotypic plasticity.

Date	Scientist(s)	Theory
1909	Woltereck	reaction norm
1913	Johannsen	genotype–phenotype distinction
1940–1950	Waddington Schmalhausen	canalization/assimilation
1965	Bradshaw	genetic basis of plasticity
1998–2003	Schlichting/Pigliucci West-Eberhard	facilitator hypothesis

advantages of this system have allowed unbiased genetic approaches that provide detailed insight into the genetic control of plasticity and a molecular framework for studying the mechanisms of plasticity and genetic–environmental interactions. A model system approach in nematodes might therefore help clarify the role of plasticity in evolution by shedding light on its molecular mechanisms and macro-evolutionary potentials. We will start with a brief historical account of phenotypic plasticity and its role for the evolution of novelty.

2. A historical account

The history of phenotypic plasticity begins at the beginning of the 20th century (table 1) [7]. In 1909, Richard Woltereck carried out the first experiments on plastic characters using the water flea *Daphnia*. He coined the term 'reaction norm' (or norm of reaction) to describe the relationship between the expressions of phenotypes across a range of different environments [3]. However, it was Johannsen (1911) who first distinguished between genotype and phenotype, and thereby introduced the concept of genotype–environment interaction [8]. This concept was only developed further three decades later by the Russian biologist Schmalhausen and the British developmental biologist Waddington. In particular, Waddington, using environmental perturbation of development, provided important conceptual contributions [9]. For example, he introduced the concept of genetic assimilation based on his work with the bithorax and crossveinless phenotypes in *Drosophila*. When fly pupae were exposed to heat shock, some of them developed a crossveinless phenotype. Upon artificial selection for multiple generations, this trait became fixed in some animals even without heat shock. Similarly, when flies were treated with ether vapour, some exhibited a homeotic bithorax phenotype, which again could be fixed even without ether induction after artificial selection for approximately 20 generations. Waddington argued that genetic assimilation allows the environmental response of an organism to be incorporated into the developmental programme of the organism. While it is now known that the fixation of the bithorax phenotype was based on the selection of standing genetic variation at a homeotic gene [10], at the time these findings were controversially discussed and often referred to as Lamarckian mechanisms. Given the missing genetic foundation of development and plasticity in the 1940s, it is not surprising that Waddington's claim for an extended evolutionary synthesis found little support among neo-Darwinists [11].

The major conceptual advancement for plasticity research was in 1965 when Anthony Bradshaw proposed that phenotypic plasticity and the ability to express alternative phenotypes must be genetically controlled [6]. Some plants develop alternative phenotypes in response to extreme environmental conditions. Using a comparative approach, Bradshaw realized that the plasticity of a trait could differ between close relatives of the same genus, independent of the trait itself. From this observation he concluded that the genetic control of a character is independent of the character's plasticity. This remarkable conclusion represents one of the most important testimonies of the power of comparative approaches and the key foundation for modern studies of plasticity.

It is not surprising that botanists have paid detailed attention to reaction norm and plasticity for breeding purposes, and the first modern monographs that advertised the significance of phenotypic plasticity for development and evolution were written by active practitioners in this field [3]. Many examples of plasticity from animals are known as well, often in insects. The migratory locust *Schistocerca gregaria* can form two alternative phenotypes in relation to food availability. Adult *Schistocerca* are dark with large wings when food is abundant, whereas they are green with small wings when food is limited [12]. Similarly, many butterflies are known to form distinct wing patterns in the dry and rainy season in the tropics or in spring and summer in more temperate climates [13]. Perhaps the most spectacular examples of plasticity are those found in hymenopterans forming the basis for eusociality in insects and resulting in the most extreme forms of morphological and behavioural novelties. Mary-Jane West-Eberhard, after a long and active career studying social behaviours in Hymenoptera, proposed an extended evolutionary theory that links development and plasticity to evolution. Her monograph *Developmental plasticity and evolution* provides an exhaustive overview on alternative phenotypes in nature [2]. Building on the now available genetic understanding of developmental processes, she proposed plasticity to represent a major facilitator and driver for the evolution of novelty and the morphological and behavioural diversification in animals and plants.

This long path from Johannsen, Waddington and Bradshaw to current plasticity research has resulted in a strong conceptual framework for the potential significance of plasticity for evolution (table 1). However, scepticism remains, largely due to the near absence of associated genetic and molecular mechanisms of plasticity [14]. To overcome these limitations, plasticity research requires model systems that tie developmental plasticity in response to environmental perturbations to laboratory approaches. Before summarizing the recent inroads obtained in one laboratory model for phenotypic plasticity, the next paragraph will briefly summarize the different forms of plasticity.

3. Some important terminology: the different forms of plasticity

By definition, the concept of phenotypic plasticity incorporates many unrelated phenomena, which has resulted in enormous confusion and debate about its potential for evolutionary adaptations [15]. Three major distinctions are necessary to properly evaluate the potential significance of plasticity for evolution. First, phenotypic plasticity can be adaptive or non-adaptive, and only the former can contribute to adaptive evolution when organisms are faced with a new or altered environment.

In contrast, non-adaptive plasticity in response to extreme and often stressful environments is likely to result in maladaptive traits that are without evolutionary significance [15].

Second, plasticity can be continuous or discrete, the latter resulting in alternative phenotypes often referred to as polyphenisms. Such alternative phenotypes have several advantages for experimental analysis and evaluation in the field. Most importantly, alternative phenotypes can more readily be distinguished from genetic polymorphisms that can also result in phenotypic divergence. Multiple examples of polyphenisms from aerial and subterranean stem and leaf formation in water plants, insect wing and body form dimorphisms and the casts of social insects have been studied in detail to analyse the interaction between the genotype and the environment in the specification of plastic traits [2]. The binary readout of alternative phenotypes provides a major advantage of such experimental analyses.

Third, plasticity might be regulated by conditional and stochastic factors [16]. While the former is more common, additional stochastic elements of regulation are known in some examples of plasticity and such cases have several experimental advantages. Most examples of plasticity have environment *a* inducing phenotype **A** and environment *b* inducing phenotype **B**. However, organisms might form alternative phenotypes **A** and **B** in part due to stochastic factors that are independent of environmental alterations. The potential role of stochastic factors has been largely overlooked in plant and animal systems, but is well known in microbes. Phenotypic heterogeneity or bistability is known in many bacteria to result in phenotypically distinct subpopulations of cells [17,18]. Persister cell formation in *Staphylococcus aureus* and spore formation in *Bacillus subtilis* represent just a few examples of phenotypic heterogeneity that occur to a certain extent in a stochastic manner. Antibiotic resistance seen by persister cells resulted in detailed molecular and mechanistic insight into the stochastic regulation of phenotypic heterogeneity [19].

Adaptive versus non-adaptive, continuous versus discrete, and conditional versus stochastic regulation of plasticity represent important distinctions for the evaluation and significance of plastic traits in development and evolution. However, one additional factor that often complicates a proper evaluation of plasticity is the inherent difficulty to distinguish between genetic polymorphisms and polyphenisms. Genetic polymorphisms are a cornerstone of mainstream evolutionary theory for the generation of phenotypic divergence. Therefore, empirical studies on plasticity would profit from a proper distinction between polymorphisms and polyphenisms. Besides inbred lines in outbreeding species, self-fertilization in hermaphroditic organisms results in isogenic lines. Such isogenic lines can rule out contributions of genetic polymorphisms. Some plants, nematodes and other animals with a hermaphroditic mode of reproduction are therefore ideal for studies of plasticity, mimicking the isogenic advantages of bacteria with phenotypic heterogeneity.

In the following, we summarize recent insight into the genetic regulation of a mouth-form feeding plasticity in the nematode *P. pacificus*. This example of plasticity is adaptive, represents a dimorphic trait with two alternative phenotypes, and contains conditional and stochastic elements of regulation. *Pristionchus pacificus* is a hermaphroditic species with isogenic propagation, and is amenable to forward and reverse genetic analysis [20,21]. We begin with a brief summary of mouth-form polyphenism in this nematode species.

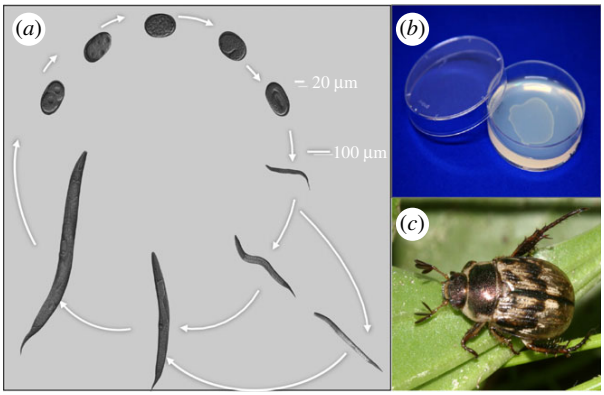

Figure 1. *Pristionchus pacificus* and growth. (*a*) Adult hermaphrodites lay eggs that develop through four larval stages to become adult. The first juvenile stage remains in the eggshell in *P. pacificus*. Under harsh and unfavourable conditions, worms develop into an arrested and long-lived dauer stage. (*b*) In the laboratory, worms are grown on agar plates with *Escherichia coli* as food source. Under these conditions, worms complete their direct life cycle in 4 days (20°). (*c*) The oriental beetle *Exomala orientalis* from Japan and the United States is one of the scarab beetle hosts on which *P. pacificus* is found in the dauer larval stage.

4. Mouth-form polyphenism as a case study

The genus *Pristionchus* belongs to the nematode family Diplogastridae, which shows entomophilic associations (figure 1) and omnivorous feeding strategies, including predation on other nematodes [22]. Usually, nematodes stay in the arrested dauer stage—a nematode-specific form of dormancy—in or on the insect vector (figure 1*a*). Nematode–insect associations represent a continuum between two most extreme forms, with dauer larvae of some species jumping on and off their carriers (phoresy), whereas others wait for the insect to die in order to resume development on the insect carcass (necromeny). Insect carcasses represent heterogeneous environments full of a variety of microbes. Such insect carcasses are best characterized by a boom and bust strategy of many of its inhabitants. While many nematodes, yeasts, protists and bacteria are known to proliferate on insect cadavers, few, if any, of these systems have been fully characterized, in particular with regard to species succession during decomposition.

Pristionchus pacificus and related nematodes live preferentially on scarab beetles (i.e. cockchafers, dung beetles and stag beetles; figure 1*c*) [23]. On living beetles, *P. pacificus* is found exclusively in the arrested dauer stage and decomposition experiments indicate that adult worms are found on the cadaver only 7 days after the beetle's death [24]. *Pristionchus* and other nematodes live on and wait for the beetle to die, resulting in enormous competition for food and survival on the carcass. It was long known that *Pristionchus* and other diplogastrid nematodes form teeth-like denticles in their mouths, which allow predatory feeding (figure 2*a*) [25]. Also, it was long known that many species form two alternative mouth-forms. In the case of *P. pacificus*, animals decide during larval development in an irreversible manner to adopt a eurystomatous (Eu) or a stenostomatous (St) mouth-form (figure 2*a*) [25]. Eu animals form two teeth with a wide buccal cavity, representing predators. In contrast, St animals have a single tooth with a narrow buccal cavity and are strict

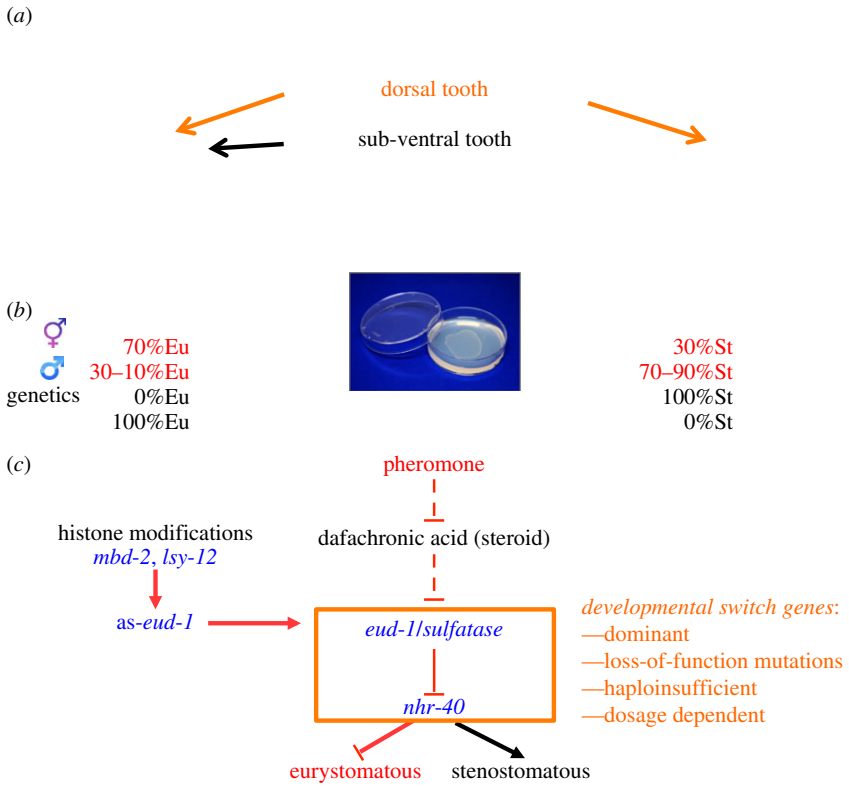

Figure 2. Genetic regulation of phenotypic plasticity of *P. pacificus* feeding structures. (*a*) Mouth dimorphism. During larval development, *P. pacificus* individuals make an irreversible decision to develop a eurystomatous morph with two teeth (orange and black arrows) and a broad buccal cavity (white arrow), or alternatively, a stenostomatous morph with a single dorsal tooth (orange arrow) and a narrow buccal cavity (white arrow). (*b*) Under fixed laboratory conditions, mouth-form plasticity shows stochastic regulation resulting in hermaphrodites having approximately 70% eurystomatous mouth-forms, whereas males have been 10–30% eurystomatous animals. In genetic screens, monomorphic mutants can be isolated that are either 100% stenostomatous or 100% eurystomatous. (*c*) Partial genetic network regulating mouth-form plasticity. The sulfatase-encoding *eud-1* gene and the nuclear hormone receptor are developmental switch mutations, which are dominant, *loss-of-function* and dosage dependent, resulting in all-stenostomatous or all-eurystomatous phenotypes, respectively. Small molecule signalling acts upstream of *eud-1* and involves pheromones and steroid hormone signalling, which are not a subject of this review. Histone modifications are crucial for mouth-form regulation and act through an antisense message at the *eud-1* locus (as-*eud-1*).

microbial feeders. Selection experiments have shown that the mouth-form dimorphism represents an example of phenotypic plasticity because isogenic animals can form both mouth-forms [25]. The dimorphism is discrete and adaptive with strong fitness effects preferring St and Eu animals under bacterial and predatory conditions, respectively [26,27]. Most importantly, mouth-form plasticity is regulated by conditional factors such as starvation and crowding [25], but also contains stochastic elements of regulation. Specifically, a nearly constant ratio of 70–90% Eu : 30–10% St animals is formed under fixed environmental conditions (figure 2*b*). It is this aspect of stochastic regulation resulting in the occurrence of both mouth-forms under standard laboratory conditions that allows manipulation of plasticity by genetic, molecular and chemical tools [16].

5. Genetics of nematode feeding plasticity

Pristionchus pacificus has been developed as a model system in evolutionary biology [20,21]. While only distantly related to *Caenorhabditis elegans*, it shares a number of features: self-fertilization, a short generation time of 4 days and monoxenic growth on *E. coli*. Adopting the functional toolkit of *C. elegans*, forward and reverse genetic tools are available in *P. pacificus*, including CRISPR-Cas9 genetic engineering and genetic transformation [28,29]. In addition, the known beetle association allowed a vast collection of *P. pacificus* strains and genomes to be catalogued [30,31].

Given the stochastic mouth-form dimorphism of wild-type *P. pacificus* animals when grown on bacteria, mutagenesis screens for monomorphic mutants can be performed to isolate strains deficient in the formation of one particular mouth-form (figure 2*b*). The first such unbiased genetic screen resulted in a *eu*rystomatous-form *d*efective mutant, *eud-1*, which turned out to be dominant and represents a developmental switch gene (figure 2*c*) [32]. Mutant *eud-1* animals are all-St, resulting in the complete absence of Eu animals. In contrast, overexpression of *eud-1* in wild-type or *eud-1* mutant animals reverts this phenotype to all-Eu. These and other experiments showed that *eud-1* is haploinsufficient and dosage dependent. *eud-1* alleles are dominant, and their all-St phenotype results from reduction-of-function, but not gain-of-function mutations. Consistently, *eud-1* mutant alleles were rescued with a wild-type copy of *eud-1*, whereas overexpression of a mutant copy of the gene did not result in any phenotype, as would usually be the case for gain-of-function mutations (figure 2*c*) [32].

A suppressor screen for Eu animals in an all-St *eud-1* mutant background resulted in the identification of the nuclear hormone receptor *nhr-40* (figure 2*c*) [33]. Interestingly, *nhr-40* is also part of the developmental switch constituting similar

genetic features but with an opposite phenotype to *eud-1*: *nhr-40* mutants are all-Eu, while overexpression results in all-St lines. *nhr-40* mutants are again dominant as loss-of-function mutants and haplo-insufficient. Thus, two genes regulating mouth-form plasticity show a dominant null or reduction-of-function phenotype. This is in strong contrast to the overall pattern in nematodes. Screens for dominant mutations in *C. elegans* resulted in many gain-of-function alleles, whereas *unc-108* represents the only gene that when mutated results in a dominant null phenotype, indicating haplo-insufficient genes to be rare [34].

Together, the experiments summarized above allow four major conclusions. First, unbiased genetic analysis of *P. pacificus* feeding plasticity indicates that plasticity is indeed under genetic control. *eud-1* and *nhr-40* mutants are monomorphic, being either all-St or all-Eu. Thus, genes affect mouth-form plasticity without affecting the character state itself; in *eud-1* mutants the St mouth-form is properly formed, similar to the Eu form in *nhr-40* mutant animals. Second, both genes are part of a developmental switch with loss-of-function and overexpression, resulting in complete but opposite phenotypes. Developmental switches had long been predicted to play an important role in plasticity regulation [2], but due to the previous absence of genetic models of plasticity, little genetic evidence was obtained. Third, *eud-1* and *nhr-40* are both located on the X chromosome. *Pristionchus pacificus* has an XO karyotype in males, similar to *C. elegans* [35]. Interestingly, males have predominantly a St mouth-form [25] and *eud-1* and *nhr-40* mutant males are all-St and all-Eu, respectively. Thus, *eud-1* and *nhr-40* escape male dosage compensation, a process that is just beginning to be investigated in *P. pacificus* [36]. Finally, it is interesting to note that *eud-1* resulted from a recent duplication [32]. While *C. elegans* contains one *eud-1*/sulfatase copy located on an autosome, *P. pacificus* contains three copies, with the two recently evolved genes being located on the X chromosome. However, CRISPR/Cas9-induced mutations in the two other *eud-1*-like genes in *P. pacificus* suggest that there are no specific phenotypes associated with the knockout of both genes [37].

6. Epigenetic control of switch genes

Two common aspects of *eud-1* and *nhr-40* mutants resulting in monomorphic, plasticity-defective phenotypes are that they show no other obvious phenotypes. In contrast, an unbiased search for mouth-form defects in a collection of mutants previously isolated for their egg-laying- or vulva-defective phenotypes identified *mbd-2* and *lsy-12* mutants to resemble an all-St *eud-1*-like phenotype [38]. *mbd-2* is egg-laying-defective and encodes a member of the methyl-binding protein family that is strongly reduced in *C. elegans* but not in *P. pacificus* [39,40]. *lsy-12* encodes a conserved histone-acetyltransferase, and *mbd-2* and *lsy-12* mutants were shown to result in massive histone modification defects involving multiple gene activation marks, such as H3K4me3, H3K9ac and H3K27ac [38]. Given that *mbd-2*, *lsy-12* and *eud-1* mutants have nearly identical mouth-form monomorphism, *eud-1* was itself a potential target for histone modification, and indeed *eud-1* expression is downregulated in *mbd-2* and *lsy-12* mutants. Interestingly, however, histone modification defects affect an antisense message at the *eud-1* locus, and overexpression experiments with this as-*eud-1* transcript suggest that as-*eud-1* positively regulates *eud-1* expression [38]. Together, these findings strongly suggest that the developmental switch is under epigenetic control. In principle, the epigenetic regulation of a switch mechanism is ideally suited to incorporate environmental information and environmental variation. However, information about associated mechanisms in *P. pacificus* awaits future studies, whereas several studies in insects recently already indicated the involvement of epigenetic mechanisms in gene-environmental interactions [41–43]. In conclusion, the use of forward genetic approaches in a laboratory model system provide strong evidence for the regulation of nematode feeding plasticity by developmental switch genes. Furthermore, epigenetic mechanisms including histone modifications and antisense RNA-mediated regulation might be crucial for gene–environment interactions.

7. Macro-evolutionary potentials

The genetic and epigenetic control of feeding plasticity in *P. pacificus* provides a basis to study how organisms sense and respond to the environment and to environmental variation. But is plasticity also important for evolution? Answering this question requires comparative studies that when performed in a phylogenetic context might provide insight into the significance of plasticity for evolutionary processes. Micro-evolutionary studies, by comparing many different wild isolates of *P. pacificus*, indicated strong differences in Eu : St ratios between isolates that correlated with *eud-1* expression [32]. Two recent studies have moved this analysis to the macro-evolutionary level, suggesting that phenotypic plasticity indeed facilitates rapid diversification. Susoy and co-workers studied the evolution of feeding structures in more than 90 nematode species using geometric morphometrics [44]. These species included dimorphic taxa, such as *P. pacificus*, but also monomorphic species that never evolved feeding plasticity, such as *C. elegans* (primary monomorphic), and those that had secondarily lost it (secondary monomorphic). This study found that feeding dimorphism was indeed associated with a strong increase in complexity of mouth-form structures [44]. At the same time, the subsequent assimilation of a single mouth-form phenotype (secondary monomorphism) coincided with a decrease in morphological complexity, but an increase in evolutionary rates. Thus, the gain and loss of feeding plasticity have led to increased diversity in these nematodes [8].

A second case of mouth-form plasticity increasing morphological diversification came from a striking example of fig-associated *Pristionchus* nematodes. Besides the worldwide branch of the genus that is associated with scarab beetles (currently more than 30 species), a recent study identified *Pristionchus* species, such as *P. borbonicus*, that live in association with fig wasps and figs [16]. These nematodes are extraordinarily diverse in their mouth morphology for two reasons. First, *P. borbonicus* and others form five distinct mouth-forms that occur in succession in developing fig synconia, thereby increasing the polyphenism from two to five distinct morphs. Second, the morphological diversity of these five morphs exceeds that of several higher taxa, although all five morphs are formed by the same species [16]. These findings strongly support the facilitator hypothesis, and they also indicate that ecological diversity can be maintained in the absence of genetic variation as all this diversity is seen within a single species and without associated speciation and radiation events [45].

8. Perspective

Phenotypic plasticity represents a striking phenomenon observed in organisms of all domains of life. It has been a contentious concept and was partially dismissed by mainstream evolutionary theory because many unrelated phenomena have been inappropriately mixed under the same heading. Following and extending previous attempts by Ghalambor *et al*. [15], we have tried to clarify terminology to provide necessary distinctions that will help study and evaluate plasticity, and establish its significance for evolution. Second, the use of a laboratory model system approach has provided strong evidence for the genetic control of feeding plasticity in *P. pacificus*. This genetic framework can serve as a paradigm to study in detail how the same genotype interacts with the environment to control this plastic trait. Besides nematodes, insects and diverse plants are very important multicellular organisms for the study of phenotypic plasticity. In particular, work on butterfly wing patterns and the coloration of caterpillars, but also horn size in different beetles, provide powerful inroads in the proper evaluation of plasticity [46,47]. Together, these studies on plants, insects and nematodes will provide mechanistic insight into this fascinating biological principle and will help provide an extended framework for evolution.

Competing interests. We declare we have no competing interests.

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References

1. Pigliucci M. 2001 *Phenotypic plasticity: beyond nature and nurture: syntheses in ecology and evolution*. Baltimore, MD: Johns Hopkins University Press.
2. West-Eberhard MJ. 2003 *Developmental plasticity and evolution*. Oxford, UK: Oxford University Press.
3. Schlichting CD, Pigliucci M. 1998 *Phenotypic evolution.* Sunderland, MA: Sinauer Associates.
4. de Jong G. 2005 Evolution of phenotypic plasticity: patterns of plasticity and the emergence of ecotypes. *New Phytol.* **166**, 101–117. (doi:10.1111/j.1469-8137.2005.01322.x)
5. Wund MA. 2012 Assessing the impacts of phenotypic plasticity on evolution. *Integr. Comp. Biol.* **52**, 5–15. (doi:10.1093/icb/ics050)
6. Bradshaw AD. 1965 Evolutionary significance of phenotypic plasticity in plants. *Adv. Genet.* **13**, 115–155. (doi:10.1016/S0065-2660(08)60048-6)
7. Nicoglou A. 2015 Phenotypic plasticity: from microevolution to macroevolution. In *Handbook of evolutionary thinking in the sciences* (eds T Heams, P Huneman, G Lecointre, M Silberstein), pp. 285–318. Heidelberg, Germany: Springer.
8. Nijhout HF. 2015 To plasticity and back again. *Elife* **4**, e06995. (doi:10.7554/eLife.06995)
9. Waddington CH. 1959 Canalisation of development and genetic assimilation of acquired characters. *Nature* **183**, 1654–1655. (doi:10.1038/1831654a0)
10. Gibson G, Hogness DS. 1996 Effect of polymorphism in the *Drosophila* regulatory gene *Ultrabithorax* on homeotic stability. *Science* **271**, 200–203. (doi:10.1126/science.271.5246.200)
11. Amundson R. 2005 *The changing role of the embryo in evolutionary thought*. Cambridge, UK: Cambridge University Press.
12. Whitman DW, Ananthakrishnan TN. 2009 *Phenotypic plasticity in insects*. Plymouth, NH: Science Publishers.
13. Nijhout HF. 1991 *The development and evolution of butterfly wing patterns*. Washington, DC: Smithsonian Institution Press.
14. Laland K *et al.* 2014 Does evolutionary theory need a rethink? *Nature* **514**, 161–164. (doi:10.1038/514161a)
15. Ghalambor CK, McKay JK, Carroll SP, Reznick DN. 2007 Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. *Funct. Ecol.* **21**, 394–407. (doi:10.1111/j.1365-2435.2007.01283.x)
16. Susoy V, Sommer RJ. 2016 Stochastic and conditional regulation of nematode mouth-form dimorphisms. *Front. Ecol. Evol.* **4**, 23. (doi:10.3389/fevo.2016.00023)
17. Dubnau D, Losick R. 2006 Bistability in bacteria. *Mol. Microbiol.* **61**, 564–572. (doi:10.1111/j.1365-2958.2006.05249.x)
18. de Jong IG, Haccou P, Kuipers OP. 2011 Bet hedging or not? A guide to proper classification of microbial survival strategies. *Bioessays* **33**, 215–223. (doi:10.1002/bies.201000127)
19. Smits WK, Kuipers OP, Veening JW. 2006 Phenotypic variation in bacteria: the role of feedback regulation. *Nat. Rev. Microbiol.* **4**, 259–271. (doi:10.1038/nrmicro1381)
20. Sommer RJ, McGaughran A. 2013 The nematode Pristionchus pacificus as a model system for integrative studies in evolutionary biology. *Mol. Ecol.* **22**, 2380–2393. (doi:10.1111/mec.12286)
21. Sommer RJ. 2015 Pristionchus pacificus*: a nematode model for comparative and evolutionary biology*. Leiden, Netherlands: Brill.
22. Kanzaki N, Giblin-Davis RM. 2015 Diplogastrid systematics and phylogeny. In Pristionchus pacificus*: a nematode model for comparative and evolutionary biology* (eds RJ Sommer), pp. 43–76. Leiden, Netherlands: Brill.
23. Ragsdale EJ. 2015 Mouth dimorphis and the evolution of novelty and diversity. In Pristionchus pacificus*: a nematode model for comparative and evolutionary biology* (ed. RJ Sommer), pp. 301–329. Leiden, Netherlands: Brill.
24. Meyer JM, Baskaran P, Quast C, Susoy V, Rödelsperger C, Glöckner FO, Sommer RJ. In press. Succession and dynamics of *Pristionchus* nematodes and their microbiome during decomposition of *Oryctes borbonicus* on La Réunion Islan. *Environ. Microbiol*. (doi:10.1111/1462-2920.13697)
25. Bento G, Ogawa A, Sommer RJ. 2010 Co-option of the hormone-signalling module dafachronic acid-DAF-12 in nematode evolution. *Nature* **466**, 494–497. (doi:10.1038/nature09164)
26. Serobyan V, Ragsdale EJ, Muller MR, Sommer RJ. 2013 Feeding plasticity in the nematode *Pristionchus pacificus* is influenced by sex and social context and is linked to developmental speed. *Evol. Dev.* **15**, 161–170. (10.1111/ede.12030)
27. Serobyan V, Ragsdale EJ, Sommer RJ. 2014 Adaptive value of a predatory mouth-form in a dimorphic nematode. *Proc. R. Soc. B* **281**, 20141334. (doi:10.1098/rspb.2014.1334)
28. Schlager B, Wang X, Braach G, Sommer RJ. 2009 Molecular cloning of a dominant roller mutant and establishment of DNA-mediated transformation in the nematode *Pristionchus pacificus*. *Genesis* **47**, 300–304. (doi:10.1002/dvg.20499)
29. Witte H, Moreno E, Rodelsperger C, Kim J, Kim JS, Streit A, Sommer RJ. 2015 Gene inactivation using the CRISPR/Cas9 system in the nematode *Pristionchus pacificus*. *Dev. Genes Evol.* **225**, 55–62. (doi:10.1007/s00427-014-0486-8)
30. Morgan K, McGaughran A, Villate L, Herrmann M, Witte H, Bartelmes G, Rochat J, Sommer RJ. 2012 Multi locus analysis of *Pristionchus pacificus* on La Reunion Island reveals an evolutionary history shaped by multiple introductions, constrained dispersal events and rare out-crossing. *Mol. Ecol.* **21**, 250–266. (doi:10.1111/j.1365-294X.2011.05382.x)
31. Rodelsperger C, Neher RA, Weller AM, Eberhardt G, Witte H, Mayer WE, Dieterich C, Sommer RJ. 2014 Characterization of genetic diversity in the nematode *Pristionchus pacificus* from population-scale resequencing data. *Genetics* **196**, 1153–1165. (doi:10.1534/genetics.113.159855)
32. Ragsdale EJ, Muller MR, Rodelsperger C, Sommer RJ. 2013 A developmental switch coupled to the evolution of plasticity acts through a sulfatase. *Cell* **155**, 922–933. (doi:10.1016/j.cell.2013.09.054)

33. Kieninger MR, Ivers NA, Rodelsperger C, Markov GV, Sommer RJ, Ragsdale EJ. 2016 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. (doi:10.1016/j.cub.2016.06.018)
34. Park EC, Horvitz HR. 1986 Mutations with dominant effects on the behavior and morphology of the nematode *Caenorhabditis elegans*. *Genetics* **113**, 821–852.
35. Pires-daSilva A, Sommer RJ. 2004 Conservation of the global sex determination gene tra-1 in distantly related nematodes. *Genes Dev.* **18**, 1198–1208. (doi:10.1101/gad.293504)
36. Lo TW, Pickle CS, Lin S, Ralston EJ, Gurling M, Schartner CM, Bian Q, Doudna JA, Meyer BJ. 2013 Precise and heritable genome editing in evolutionarily diverse nematodes using TALENs and CRISPR/Cas9 to engineer insertions and deletions. *Genetics* **195**, 331–348. (doi:10.1534/genetics.113.155382)
37. Ragsdale EJ, Ivers NA. 2016 Specialization of a polyphenism switch gene following serial duplications in *Pristionchus* nematodes. *Evolution* **70**, 2155–2166. (doi:10.1111/evo.13011)
38. Serobyan V, Xiao H, Namdeo S, Rodelsperger C, Sieriebriennikov B, Witte H, Roseler W, Sommer RJ. 2016 Chromatin remodelling and antisense-mediated up-regulation of the developmental switch gene *eud-1* control predatory feeding plasticity. *Nat. Commun.* **7**, 12337. (doi:10.1038/ncomms12337)
39. Gutierrez A, Sommer RJ. 2004 Evolution of *dnmt-2* and *mbd-2*-like genes in the free-living nematodes *Pristionchus pacificus, Caenorhabditis elegans* and *Caenorhabditis briggsae*. *Nucleic Acids Res.* **32**, 6388–6396. (doi:10.1093/nar/gkh982)
40. Gutierrez A, Sommer RJ. 2007 Functional diversification of the nematode *mbd2/3* gene between *Pristionchus pacificus* and *Caenorhabditis elegans*. *BMC Genet.* **8**, 57. (doi:10.1186/1471-2156-8-57)
41. Simola DF *et al.* 2016 Epigenetic re(programming) of caste-specific behavior in the ant *Camponotus floridanus*. *Science* **351**, 37–39. (doi:10.1126/science.aac6633)
42. Gibert JM, Mouchel-Vielh E, De Castro S, Peronnet F. 2016 Phenotypic plasticity through transcriptional regulation of the evolutionary hotspot gene tan in *Drosophila melanogaster*. *PLoS Genet.* **12**, e1006218. (doi:10.1371/journal.pgen.1006218)
43. Kucharski R, Maleszka J, Foret S, Maleszka R. 2008 Nutritional control of reproductive status in honeybees via DNA methylation. *Science* **319**, 1827–1830. (doi:10.1126/science.1153069)
44. Susoy V, Ragsdale EJ, Kanzaki N, Sommer RJ. 2015 Rapid diversification associated with a macroevolutionary pulse of developmental plasticity. *Elife* **4**, e05463. (doi:10.7554/eLife.05463)
45. Phillips PC. 2016 Evolution: five heads are better than one. *Curr. Biol.* **26**, R283–R285. (doi:10.1016/j.cub.2016.02.048)
46. Emlen DJ, Hunt J, Simmons LW. 2005 Evolution of sexual dimorphism and male dimorphism in the expression of beetle horns: phylogenetic evidence for modularity, evolutionary lability, and constraint. *Am. Nat.* **166**(Suppl. 4), S42–S68. (doi:10.1086/444599)
47. Moczek AP, Sultan S, Foster S, Ledon-Rettig C, Dworkin I, Nijhout HF, Abouheif E, Pfennig DW. 2011 The role of developmental plasticity in evolutionary innovation. *Proc. R. Soc. B* **278**, 2705–2713. (doi:10.1098/rspb.2011.0971)

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Environmental influence on *Pristionchus pacificus* mouth form through different culture methods

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Environmental cues can impact development to elicit distinct phenotypes in the adult. The consequences of phenotypic plasticity can have profound effects on morphology, life cycle, and behavior to increase the fitness of the organism. The molecular mechanisms governing these interactions are beginning to be elucidated in a few cases, such as social insects. Nevertheless, there is a paucity of systems that are amenable to rigorous experimentation, preventing both detailed mechanistic insight and the establishment of a generalizable conceptual framework. The mouth dimorphism of the model nematode *Pristionchus pacificus* offers the rare opportunity to examine the genetics, genomics, and epigenetics of environmental influence on developmental plasticity. Yet there are currently no easily tunable environmental factors that affect mouth-form ratios and are scalable to large cultures required for molecular biology. Here we present a suite of culture conditions to toggle the mouth-form phenotype of *P. pacificus*. The effects are reversible, do not require the costly or labor-intensive synthesis of chemicals, and proceed through the same pathways previously examined from forward genetic screens. Different species of *Pristionchus* exhibit different responses to culture conditions, demonstrating unique gene-environment interactions, and providing an opportunity to study environmental influence on a macroevolutionary scale.

Phenotypes can be dramatically influenced by environmental conditions experienced during development, a phenomenon referred to as developmental plasticity[1–3]. Examples of plastic phenotypes have been studied for nearly a century, including differences in morphology[4], sex and caste determination[5–7], and innate immunity[8]. However, despite long-held interest in the field, and decade's worth of progress linking genotype to phenotype, relatively little is known about the mechanisms connecting environment to phenotype. To study the mechanisms of environmental influence on phenotype, easily tunable methods to induce phenotypic changes and model organisms amenable to molecular biology techniques are required. For example, temperature and diet have been utilized to explore plasticity in insects and nematodes[9–14], some of which have revealed fundamental principles of dynamic gene regulation. In particular, investigating life cycle plasticity in *C. elegans* contributed to our understanding of nutrition and endocrine signaling[15–18], and the discovery of regulatory RNAs[19]. However, the number of case studies remains small, and heuristic insight of ecologically relevant phenotypes within an evolutionary framework is still lacking.

The model organism *P. pacificus* exhibits an environmentally sensitive developmental switch of its feeding structures[20]. In the wild *P. pacificus* exists in a dormant state (dauer) on beetles. When beetles die *Pristionchus* exits the dauer state to feed on decomposition bacteria, and proceeds to reproductive maturity[21,22] (Fig. 1A). While developing under crowded conditions a "wide-mouthed" eurystomatous (Eu) morph with two teeth is built, which allows adults to prey on other nematodes (Fig. 1B). Alternatively, a "narrow-mouthed" stenostomatous (St) morph with one tooth relegates diet exclusively to microorganisms (Fig. 1C). While Eu animals can exploit additional food sources[23] and attack and kill competitors[24], St animals mature slightly faster[25], creating a tradeoff of strategies depending on the environment perceived during development. Under monoxenic growth conditions in the laboratory using *Escherichia coli* OP50 bacteria as a food source on NGM-agar plates, 70–90% of the reference *P. pacificus* strain PS312 develop the Eu morph. Metabolic studies have elucidated compounds that affect this mouth-form decision. For example, the steroid hormone dafachronic acid shifts mouth-form frequencies to St[20]. Conversely, the pheromone dasc#1 shifts the frequency to Eu[26]. Recent mutant screens

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Figure 1. Life cycle and phenotypic plasticity of *Pristionchus pacificus*. (**A**) *P. pacificus* exist in a necromenic relationship with host beetles (i.e. shown here *Lucanus cervus*), and upon decomposition of the beetles the worms exit the dormant (dauer) state. Photo taken by M Herrmann and R Sommer. Depending on environmental conditions experienced during this period, adults develop either (**B**) a wide-mouth "eurystomatous" (Eu) morph with an additional tooth allowing them to prey on other nematodes, or (**C**) a microbivorous narrow mouth "stenostomatous" (St) morph. (**D**) Diagram integrating the environment into known gene-phenotype interactions of the *P. pacificus* mouth-form pathway.

have established several genes in the mouth-form regulatory pathway[27–29]. The sulfatase *eud-1* (*eu*rystomatous *d*efective) is a dosage-dependent "switch" gene[30]: *eud-1* mutants are 100% St, while overexpression of a *eud-1*

transgene confers 100% Eu[27]. The nuclear-hormone-receptor *Ppa-nhr-40* was identified as a suppressor of *eud-1*, and regulates downstream genes[28]. *C. elegans* homologs of the epigenetic enzymes acetyltransferase *lsy-12* and methyl-binding protein *mbd-2* have also been identified to control mouth-form plasticity, and are attractive factors for channeling environmental cues to changes in gene regulation. Both mutants led to global losses of activating-histone modifications, and decreased expression of *eud-1*[29].

Identification of these switch genes affords the opportunity to track regulatory mechanisms that respond to environmental cues[31,32]. Unfortunately, the application of small molecules to affect mouth-form ratios in large enough quantities for biochemical fractionation or epigenetic profiling (e.g. ChIP) is impractical given the labor and expense of chemical synthesis or purification. Moreover, it is difficult to obtain consistent mouth-form ratios with pharmacological compounds as they are in constant competition with endogenous hormones and pheromones[20]. Finally, while crowding/starvation can also induce the Eu morph, it is technically challenging to compare different population densities, or to synchronize starved vs. un-starved larvae. To adequately study environmental effects on phenotypic plasticity, cheap, consistent, and simple methods are needed that can tune mouth-form ratios in synchronized populations. Here, we establish a set of culture conditions to affect environmental influence on mouth form. These methods are fast, reproducible, and only require the differential application of buffer, and culturing state (solid vs. liquid). Intriguingly, different species of *Pristionchus* exhibit different response regimes, suggesting evolutionary divergence of gene-environment interactions.

Results

Liquid culture affects *Pristionchus pacificus* mouth-form. In order to accumulate large amounts of biological material for molecular and biochemical experiments we grew the laboratory California strain (PS312) of *P. pacificus* in liquid culture. To our surprise, this culture condition reversed the mouth-form phenotype from preferentially Eu to preferentially St. To better examine this observation we screened mouth-forms of adults representing a parental generation (P), and obtained[33] and split eggs evenly to either agar plates or liquid culture, and screened adults of the next generation (G1) (Fig. 2A). Reproducibly, this simple difference in culturing method led to a dramatic shift in mouth-form ratio ($>95\%$ Eu on agar compared to ~10% Eu in liquid culture, $p < 0.001$, *paired t-test*) (Fig. 2B). Importantly, *P. pacificus* developed at similar rates in agar and liquid culture, allowing facile comparisons between conditions (Fig. 2C), and arguing against nutritional deprivation inducing the mouth-form shift. St animals have a slightly faster development than Eu animals when grown on agar[25], however we found developmental speed to be indistinguishable between morphs in liquid culture (Supplementary Fig. 1). The different environmental conditions present distinct energy requirements (eg. swimming and feeding on motile bacteria in 3-dimensional liquid culture) that might offset potential tradeoffs in resource allocation.

Next, we investigated whether the change in mouth-form ratio induced by liquid culture was capable of being inherited. The mouth-from ratio of adults was consistent with the culture method they developed in regardless of the culture method of the parental generation, suggesting the effect is not transgenerational (Fig. 2D). These results also demonstrate the immediate and robust nature of this plasticity, and similar experiments coupled to mutagenesis may be useful for identifying genes involved in the ability to sense and respond to changing environments.

Buffer components and culture state affect mouth form. To investigate the potential influence of culture conditions on mouth form we examined differences in buffer composition, and solid vs. liquid culturing state. In our previous experiments we had used standard liquid culture protocols for *C. elegans*[33], which utilize S media (S), whereas we normally grow *P. pacificus* on Nematode Growth Media (NGM) agar plates[33]. To assess the contribution of the chemical composition of the medium, as opposed to solid vs. liquid environments (hereafter referred to as 'culture state'), we performed reciprocal culture experiments. Nematodes that were grown on either S-agar or NGM-liquid exhibited intermediate mouth-form ratios ($51 \pm 5\%$ Eu and $38 \pm 13\%$ Eu, respectively, $p < 0.001$ relative to solid or liquid states of the same medium, *paired t-test*) (Table 1d,h,i,p), revealing a growth-medium composition effect. However, as these mouth-form ratios were in-between the extremes of NGM-agar and S-liquid, it also suggests other environmental factors are operating.

S medium contains phosphate (50 mM) and sulfate (14 mM) - both of which have previously been shown to affect mouth-form ratios at 120 mM[27]. To test whether this concentration of phosphate was causing the S-medium effect we made alternative formulations by replacing phosphate with 50 mM Tris ("T-Medium") or Hepes ("H-Medium"), pH 7.5. Liquid culture in T- and H-medium yielded reproducibly higher Eu ratios ($35 \pm 8\%$ and $28 \pm 10\%$, respectively, $p < 0.05$, *paired t-test*) (Table 1d–f), demonstrating a specific, albeit subtle contribution from phosphate. Furthermore, *P. pacificus* grown in axenic (without bacteria)[34], M9[33], or PBS (which does not contain sulfate) -based liquid cultures were all highly St (Table 1a–c). Although nematode survival rate was poor in PBS, and development was slowed in axenic culture (9–10 days for sexual maturation, rather than 3–4).

Rotation speed of liquid culture affects mouth form. Further exploration of liquid culture methodology revealed that decreasing the rotation per minute (rpm) also affected mouth-form ratios. Previous experiments that led to high St ratios had been performed at 180 rpm, but when shifted to "slow" speeds of 70 or 50 rpm, the mouth-form ratio shifted to an intermediate Eu bias ($55 \pm 11\%$ and $66 \pm 9\%$, respectively, $p < 0.05$, *t-test*) (Table 1j,l). The simplicity of changing rpm shaking-speed to affect mouth-form ratios is an intriguing environmental perturbation as other factors like food source, buffer, and culturing state are identical. When examined without bacteria, it became evident that at slow speeds (<90 rpm) nematodes aggregated in the center of the liquid column, whereas at higher speeds they were dispersed. When combined with conditions that exhibited intermediate St ratios the effects were additive, yielding up to $87 \pm 3\%$ Eu with NGM-liquid culture (Table 1k,m,n). The higher density of nematodes at slow speeds suggests that pheromones may be responsible. Consistent with this hypothesis, we passed multiple *P. pacificus* generations from one liquid culture to another,

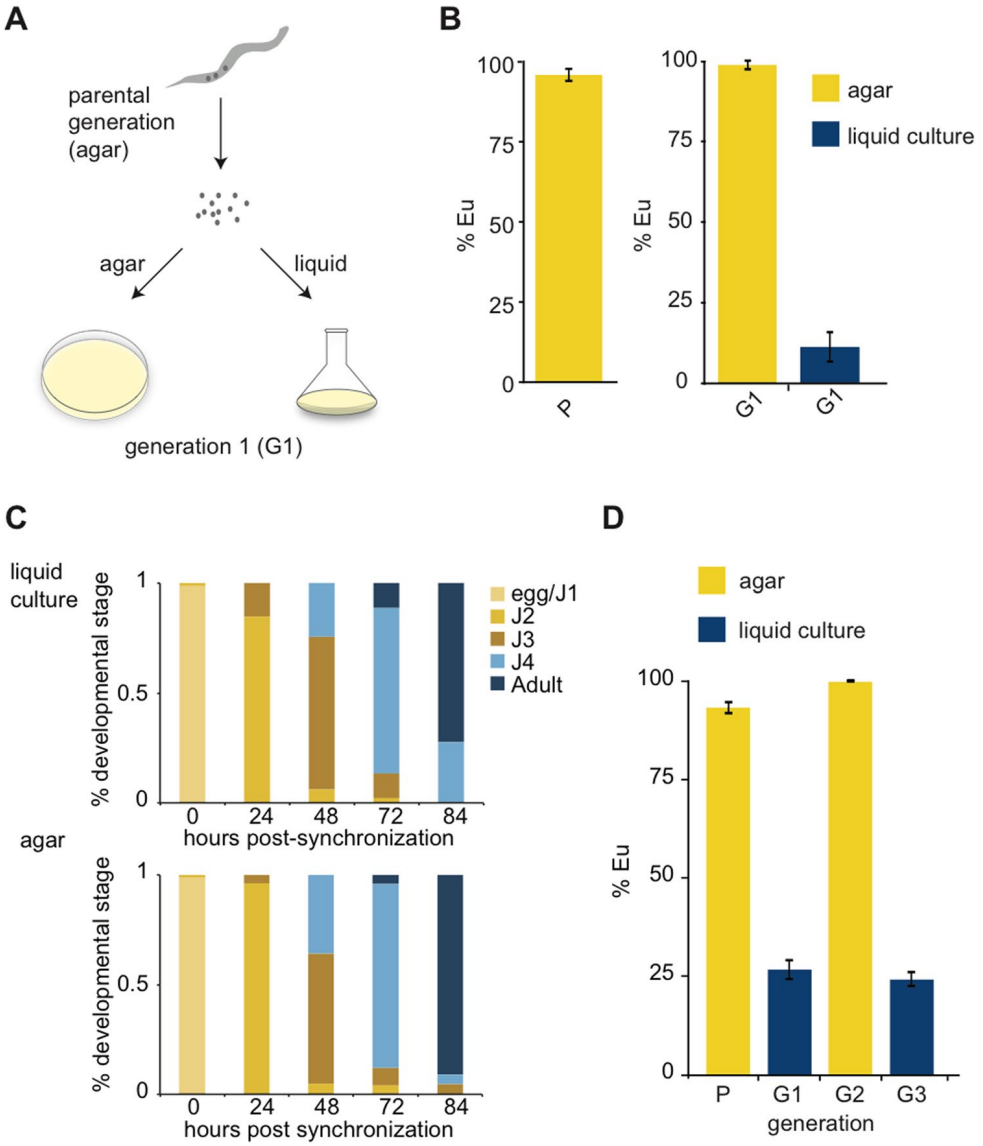

Figure 2. Different culture methods affect mouth-form phenotypic plasticity. (**A**) Diagram of experimental design to compare culture conditions from the same population after bleach synchronization. (**B**) Mouth-form ratios presented as percent eurystomatous (% Eu) from the parental generation (P) and the next generation (G1) grown in either liquid culture or agar plates, $n = 18$ biological replicates, $p < 0.05$, *students two-tailed t-test*, error bars represent SEM. (**C**) Developmental stages of bleach-synchronized *P. pacificus* in either agar plates or liquid culture. Bar graphs represent a typical experiment measuring >30 animals at the indicated time-points. (**D**) Mouth-form ratios of switching experiments between agar and liquid cultures. Nematodes were bleached between generations (P, G1, G2), and eggs-J1 larvae were passed to the next condition, $n = 3$, error bars represent SEM.

either by a 1:10 dilution, or by bleaching and washing. When passed by bleaching the next generation remained highly St (8 ± 4%). However when passed by dilution the next generation of worms exhibited intermediate Eu ratios (51 ± 16%, $p < 0.05$, *unpaired t-test*), perhaps because pheromones from the first generation were passed on to the second.

Liquid culture affects body morphology. We also observed morphological differences of body length and width between agar and liquid culture, demonstrating an additional plastic response (Supplementary Figure 2). Worms that develop in liquid culture exhibit longer, narrower bodies compared to worms that develop in agar, a phenomenon that has also been observed in *C. elegans*[33]. To disentangle whether the effect on mouth form is discrete or connected to the change in body shape we grew worms in NGG culture, which is intermediate between liquid and solid states[35]. Similar to liquid culture, adult worms grown in NGG exhibited a more slender body morphology than on agar plates ($p < 0.05$, Mann-Whitney), but they exhibited the highly Eu mouth-form ratio of worms grown in agar culture (Supplementary Fig. 2, Table 1d,o,p). While it is difficult to completely exclude the possibility that they are connected, there is no obvious correlation between the St mouth form and

	Condition	% Eu	S.E.M.
A	LC PBS, 180 rpm	7	3.2
B	LC Axenic Culture, 180 rpm	8.8	8.8
C	LC M9, 180 rpm	11.5	6.2
D	LC S-medium, 180 rpm	12.8	3.2
E	LC H-medium, 180 rpm	28	10.1
F	LC T-medium, 180 rpm	35	7.6
G	LC S-medium, 100 rpm	35.2	3
H	LC NGM, 180 rpm	37.8	12.9
I	AG S-medium	51.4	5.4
J	LC S-medium, 70 rpm	55.1	10.9
K	LC T-medium, 50 rpm	61.5	16.9
L	LC S-medium, 50 rpm	65.9	9
M	LC H-medium, 50 rpm	70.6	15
N	LC NGM, 50 rpm	87.3	3.3
O	NGG	97.1	2.5
P	AG NGM	98.7	0.7

Table 1. Buffer/ions and physical culture state affect mouth-form phenotype. A panel of culturing methods covers phenotypic ratios from ~10–99% Eu. LC = liquid culture, AG = agar, T and H medium = S-medium with phosphate replaced with 50 mM Tris or HEPES, pH 7.5, respectively, NGG = NGM with agar replaced with Gelrite/Gelzan CM (Sigma)[35]. $N \geq 3$ biological replicates per condition, and standard error mean (SEM) is presented in the last column. Mouth-form phenotypes were assessed 4–5 days after bleach-synchronization (see Methods).

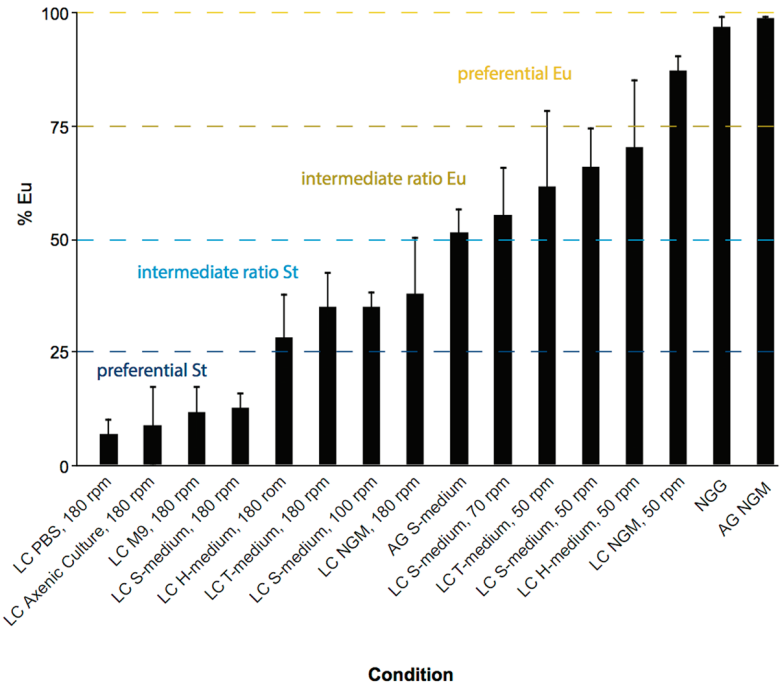

Figure 3. Comprehensive evaluation of culture method on mouth-form ratio in *P. pacificus*. Same data as in Table 1, but presented according to gradation of effect on mouth-form phenotype, from low to high % eurystomatous. LC = liquid culture, AG = agar, T and H medium = S-medium with phosphate replaced with 50 mM Tris or HEPES, pH 7.5, respectively, NGG = NGM with agar replaced with Gelrite/Gelzan CM (Sigma)[35]. Error bars represent standard error mean (SEM) for different biological replicates ($n \geq 3$, Methods).

slender morphology observed in liquid culture. Therefore, it seems these two instances of phenotypic plasticity are under distinct regulation.

Collectively, we have established a broad range of culturing methods that allow the acquisition of almost any mouth-form ratio from an isogenic strain (Fig. 3). A variety of liquid culture conditions, including buffers without phosphates or sulfates, exhibited an effect on mouth form, suggesting an unknown environmental effect that is perhaps specific to solid or liquid states.

Liquid culture acts upstream of known switch genes. Next, we sought to place the environmental effects of liquid culture relative to known genetic and environmental factors. First, we examined whether liquid culture had an effect on mutants that are 100% Eu on agar plates[27,28]. Animals from a *eud-1* overexpression line and *Ppa-nhr-40* mutant line remained 100% Eu in liquid culture, arguing that these genes act downstream of the environmental effect of liquid culture (Fig. 4A). Next, we assessed whether the dasc#1 pheromone was capable of inducing the Eu mouth-form in liquid culture, as it does on agar. dasc#1 experiments demonstrate a large variability in phenotypic ratio (Fig. 4B), however they typically exhibited a higher Eu proportion than control worms without dasc#1 treatment ($p = 0.068$, *paired t-test*). This intermediate and variable effect suggests that liquid culture and the dasc#1 pheromone act in parallel and antagonistically to each other. Finally, we also compared the expression of four genes in different culturing conditions that are up-or down-regulated in *eud-1* mutants (100% St) vs. wild-type (70–100% Eu)[27]. There was a strong correspondence between *eud-1* vs. wild-type RNA-seq data, and liquid vs. agar culture RT-qPCR (Fig. 4C,D). These results provide further evidence that the environmental effect of liquid culture is upstream of *eud-1*, and that this method is suitable for studying genetic pathways that have been determined through mutational experiments[27–29].

Liquid culture effect is dependent on genetic background. Finally, we explored whether there was a macro-evolutionary difference in responses to culture conditions. We chose four *Pristionchus* species that flank *P. pacificus* phylogenetically; three are highly Eu on agar (>95%), and one is highly St (>95%) (Fig. 5A,B). Remarkably, each species exhibited distinct phenotypic responses to liquid culture. For example, *P. maupasi* was highly Eu in both conditions, while *P. entomophagus* shifted to almost 100% St (Fig. 5C) in liquid culture. Meanwhile *P. mayeri* was St in both culture conditions. Taken together, these data show a genetic basis to environmental effects on phenotypic plasticity, which can be exploited for evolutionary, genetic, and molecular exploration of plasticity mechanisms. Whether these differences in response reflect adaptive changes to different environments, or are a result of drift remains to be seen in future investigations.

Discussion

We describe multiple methods for the culture of preferentially St (<25% Eu), intermediate St (25–50% Eu), intermediate Eu (50–75 Eu%), and preferentially Eu (>75% Eu) *P. pacificus* (Fig. 3, Methods). Growth rates are similar between conditions, allowing the generation of developmentally synchronized populations. The effects are immediate, and immediately reversible when switching between liquid and agar, suggesting they are not transgenerational. Importantly, the genetic pathways towards building each respective mouth form are consistent with pathways established from prior forward genetics[27,28]. Finally, the environmental response is unique in four species of *Pristionchus* tested, arguing that evolution has acted, passively or actively, on gene-environment interactions. The ability to toggle between mouth forms with simple culturing conditions provides powerful new tools to study the genetic and molecular mechanisms of phenotypic plasticity.

Perturbation of environmental factors such as salt concentration[15,36–38], pathogen[8,39–42], temperature[7,10,13,43–45], and diet[46,47] have been exploited for decades to study adaptive responses. More recent genome-wide profiling of epigenetic information carriers has revealed potential mechanisms for communicating stimuli to changes in gene expression. So called 'poised' or 'permissive' chromatin states can respond to external signals, leading to changes in transcription that ultimately affect tissue differentiation[48–55]. The time is now ripe to test whether similar processes affect phenotypic plasticity, a critical link between ecology and molecular mechanism that has just begun to be explored[56–60].

Our panel of *P. pacificus* culture conditions saturates the mouth-form frequency space (Fig. 3). The ability to shift ratios by rpm shaking-speed provides perhaps the cleanest method because of its simplicity. In shaking speeds greater than 90 rpm nematodes are dispersed, while below 90 rpm they are concentrated in the center of the liquid vortex. Since different buffer formulations also affected mouth-form ratios, and the combination with slow rpm yielded an additive effect, it seems that alterations in the abundance, diffusion, and local concentration of pheromones and ions (i.e. phosphate and sulfate) contribute to the observed differences between liquid and agar culture conditions. However, we note that densely packed nematodes at slow rpm (much denser than on a plate) in NGM-liquid media are still insufficient to recapitulate the >95% Eu phenotype seen on NGM-agar plates. While it remains possible that these are the only contributing factors, we speculate an additional unknown factor is extant related to bacterial density, metabolism, or the liquid environment itself.

Whether liquid culture is a direct stimulator of the St mouth form is currently unknown. Field observations and competition experiments are required to (1) assess if *Pristionchus* experiences wet-enough conditions in the wild to mimic liquid culture conditions as with other lotic, lentic or marine nematodes[61–63], and (2) determine whether the St mouth form provides an advantage in this environment. Both *C. elegans* and *P. pacificus* exhibit a slender morphology in liquid culture, suggesting a conserved plastic response to this environment. It is conceivable that a liquid culture-dependent signaling pathway related to mouth form also exists, although it could be mediated indirectly through other factors. Seemingly unrelated stimuli are capable of inducing the same developmental pathway by eventually descending on a downstream switch or "evocator"[64–66]. Regardless of the ultimate environmental factor, our analysis of gene expression in liquid culture reflects patterns observed in constitutive St mutants, suggesting that similar downstream pathways are utilized (Fig. 1D). Importantly however, we did not observe faster St development in liquid culture as has been observed on agar, and which is predicted to be the tradeoff advantage of the St morph[25]. It is formally possible that we did not have enough temporal resolution to identify the small but significant differences previously observed (55 hours for St and 61 hours for Eu). It is also worth noting that laboratory culture conditions are highly artificial, and it is perhaps not surprising that they could affect ecological strategies. Nevertheless, our results suggest that caution should be taken when studying *P. pacificus* ecology across different environments, as it may be context dependent. Going forward, it will be

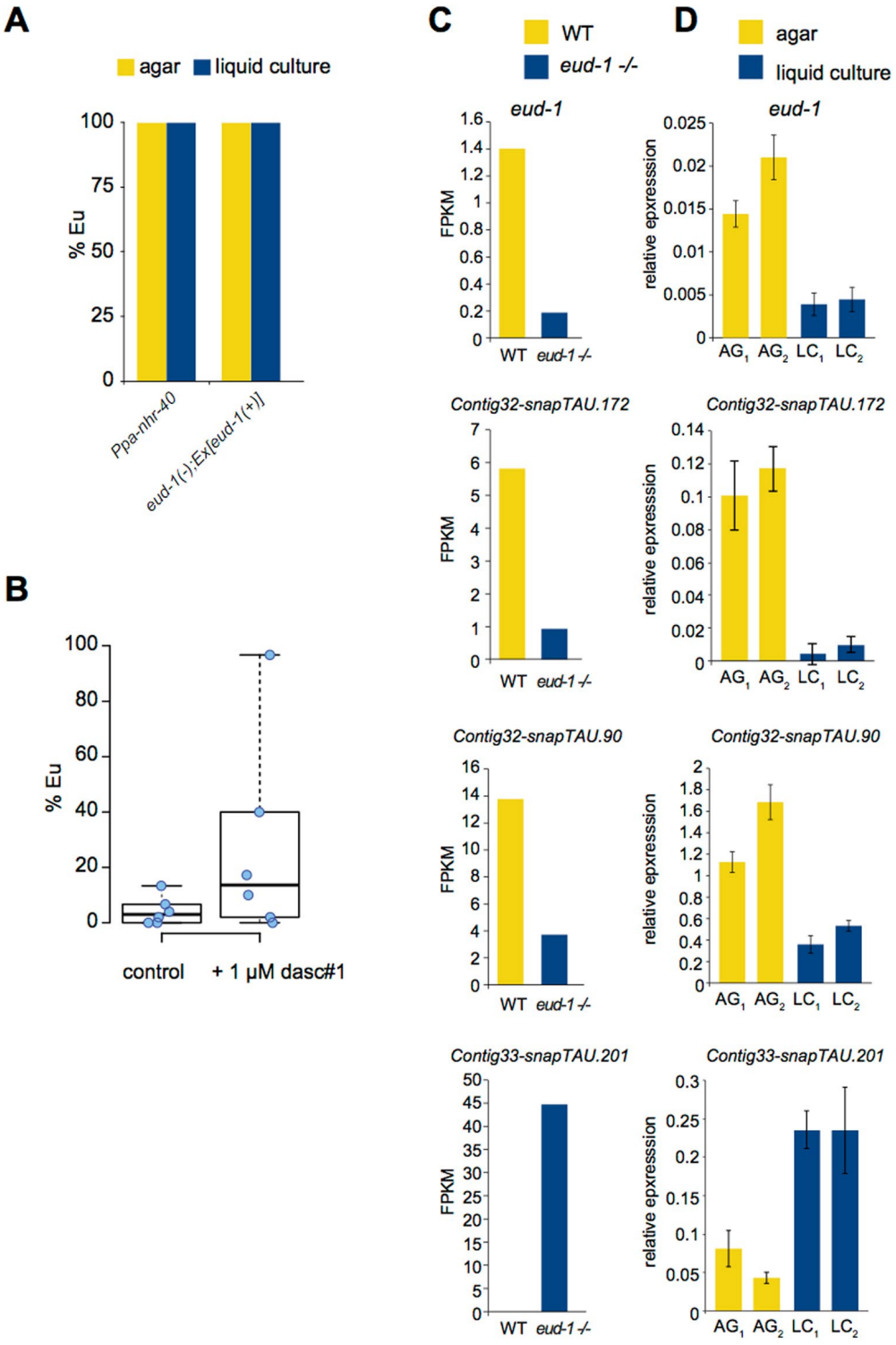

Figure 4. The environmental effect of liquid culture is upstream of known genetic components and induces similar pathways. (**A**) Mouth-form ratios of *eud-1* overexpression[27] and a *Ppa-nhr-40*[28] mutant in liquid culture reveals no effects, suggesting these genes are downstream, $n = 3$ biological replicates. (**B**) Addition of 1 µM dasc#1 exhibits a variable response that appears to induce Eu, although it is not statistically significant ($p = 0.068$). (**C**) Expression analysis of four genes by RNA-seq from *eud-1* mutants (the average of 4 homozygous mutant alleles is represented)[27] (100% St) compared to the RS2333 California strain (70–100% Eu), y-axis = fpkm (relative expression). (**D**) Reverse transcription-quantitative PCR (RT-qPCR) of *P. pacificus* PS312 grown in liquid culture/S-medium (LC) vs. NGM-agar plates (AG) for the two biological replicates displayed, with four technical replicates each. The y-axis represents $2^{\Delta Ct}$ (relative expression) compared to the housekeeping gene *Ppa-Y45F10D.4* (iron binding protein)[69], error bars represent standard deviation of n = 4 technical replicates.

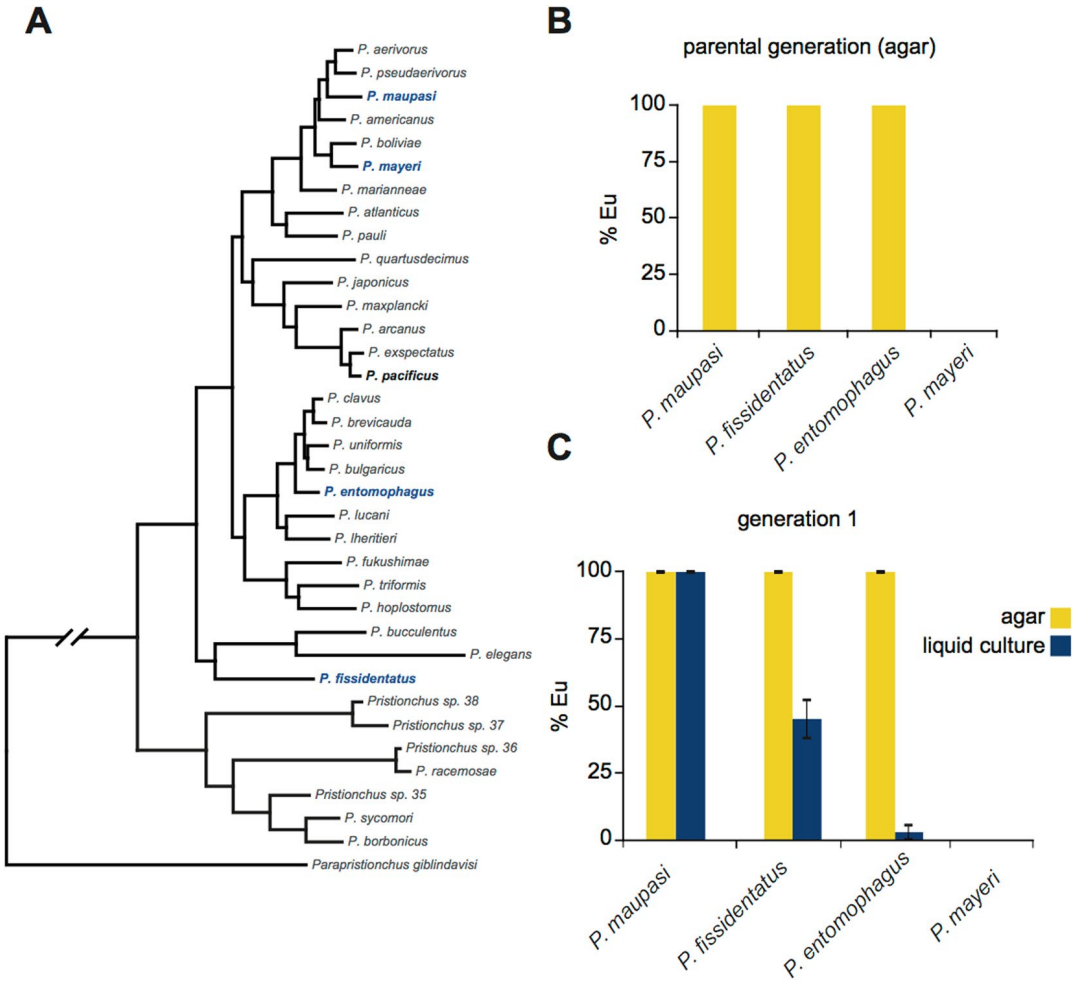

Figure 5. Macro-evolutionary view of liquid culture environmental influence. (**A**) Phylogeny of *Pristionchus* species[70] highlighting *P. pacificus* (bold), *P. fissidentatus*, *P. mayeri*, *and P. entomophagus* (blue). (**B**) Mouth-form ratio of parental generations ($n = 3$) of indicated species on NGM-agar after three consecutive healthy generations on OP50. (**C**) Mouth-form ratios of indicated species in either NGM-agar or liquid culture/S-medium ($n = 3$), error bars represent SEM.

informative to assess if the developmental speed of different species correlates with their response to liquid culture, and the aqueous content in which they are found in nature.

Which culture method is utilized will depend on the purpose of the experiment. Exploiting intermediate ratio conditions may be useful to study genes or other environmental factors predicated to effect mouth form but in an unknown direction (Eu or St). For experiments that require the greatest separation in mouth-form frequencies we recommend S-medium at 180 rpm (St) vs. NGM agar plates (Eu). We also frequently observed a modest degree of variation, which is expected for a stochastic phenotypic trait[67]. As such, every measurement utilizing these culturing methods should be performed side-by-side with control samples. It is our hope that these methods will be a contribution to the study of environmental effects on *P. pacificus* mouth form, and phenotypic plasticity in general.

Methods

Strains and species. For all *P. pacificus* experiments the California strain PS312 was used, except comparisons to RNA-seq data, which used a more grown-out version of the same strain (RS2333). For experiments with different species (Fig. 5) *P. maupasi*, *P. fissidenatus*, and *P. mayeri* were compared to *P. pacificus*. Epistasis experiments (Fig. 4A) were performed with *Ppa-nhr-40*(*tu505*) and *eud-1*(*tu445*);*tuEx[eud-1*(+)*]*.

Culture methods. Five young adult *Pristionchus* nematodes were passed every 4–6 days on 10 ml NGM-agar, 60 mm plates at 20 °C seeded with 300 µl of overnight cultures of *Escherichia coli* OP50 (grown in LB at 37 °C) and covered with parafilm to avoid experiencing starvation for three consecutive generations[33]. The mouth-form phenotype of 4th generation adults represents the parental (P) generation (Fig. 2A,C, and D). Prior to all subsequent phenotyping experiments adults were synchronized by washing off of plates with M9 using plastic Pasteur pipettes into 15 ml conical tubes, and adding 30% final volume NaOH/bleach (0.5 ml NaOH, 1 ml bleach/3.5 ml washed worms) for 9 minutes with gentle agitation every few minutes. Carcasses were filtered through a 120 µm

nylon net (Millipore) fixed between two rubber gaskets in a plastic funnel, washed by applying 3 ml M9 drop-wise on the filter, then pelleted 500 × g, 1 minute, room temperature. Eggs-J1 were washed by gentle re-suspension in 3 ml M9, and re-centrifuged 500 × g, 1 minute, room temperature. It is important not to wash worms with S-medium before or directly after bleach because it will start to precipitate. M9 wash was removed by pipette, and then eggs-J1s were ready for re-suspension in the appropriate buffer depending on the experiment.

For the majority of experiments, eggs-J1 larvae were re-suspended in 100 µl M9 × the number of test conditions (i.e. 200 µl for comparing one agar vs. one liquid culture condition). For re-culturing on agar, eggs-J1 were pipetted in the center of the OP50 lawn on 60 mm agar plates (NGM or S-medium), then the plate was tilted in 360° to spread and dry the eggs. Afterwards the plates were stored at 20° and adults were phenotyped 4–5 days later (see below for details of phenotyping). For culturing in liquid formats, 100 µl of eggs-J1 were pipetted into 10 ml of medium in 50 ml-volume autoclaved Erlenmeyer flasks. To prepare monoxenic liquid cultures the amount of OP50 *E. coli* was empirically determined. For all liquid cultures described (except axenic culture) 100 ml of overnight OP50 *E. coli* (grown in LB) to an optical density (OD_{600}) of 0.5, was pelleted 30 minutes, 4 °C at 3,000 × g in an SLA-3000 rotor and re-suspended in 10 ml filter-sterilized (0.22 µm, Millipore) S-medium[33] unless otherwise noted (e.g. M9 or PBS, Fig. 2). The concentration of bacteria is a critical parameter. The procedure described above led to healthy cultures of *P. pacificus* at the normal developmental rate observed on agar plates (3–4 days[21]), while adding less (50 ml or 10 ml) OP50 led to slower rates, or even the inability to develop beyond the J2 larval stage when significantly less was added. Liquid cultures were incubated 180 rpm, 20–22 °C unless otherwise noted for "slow" rpm experiments (50 and 70 rpm).

For experiments with "H" or "T" medium, S-medium was prepared as before[33] except that phosphates were replaced with 50 mM of HEPES or Tris, pH 7.5, respectively. Axenic culture was prepared according to Samuel *et al.*[34] with the exception that flavin-mononucleotide was replaced with riboflavin (Sigma) at the same amount, and cultures were shaken at 180 rpm instead of 70. As previously noted[34] with *C. elegans*, *P. pacificus* also develops slower in axenic culture, reaching maturity (adults) at 9–10 days after adding eggs. Culture in NGG was performed similar to Muschiol and Traunspurger 2007[35]. In short, 3 ml of NGM was prepared with agar replaced with Gelrite/Gelzan CM (Sigma) at 0.75 g/L and seeded with 300 µl of OP50 and bleached eggs, then incubated at 20 °C.

To collect nematodes from liquid cultures for tracking developmental stages or mouth-form phenotyping we developed a filtering method using removable 5 µm filters (Millipore) combined with the Sterifil aseptic system (47 mm, Millipore). Filters are applied to the Sterifil apparatus and a small amount of M9 is added and vacuumed through to ensure a tight and continuous seal. Then liquid cultures are decanted into the funnel and slowly vacuumed. All *P. pacificus* developmental stages are large enough to be blocked by the 5 µm filter, while bacteria pass through. However when attempting to isolate J2s we recommend applying 2 × 5 µm filters. After all liquid has passed through the filter, nematodes were washed with ~25 ml of M9 by decanting directly on to the filter and applying vacuum pressure. Then the funnel was removed, and forceps were used to transfer the filter to an open 50 ml conical tube in a curved shape to fit into the opening. Nematodes were then washed from the filter by repeatedly applying the same 1 ml of M9 over the filter. Then this 1 ml was transferred to 1.5 ml microcentrifuge tube, and incubated at room temperature for 5 minutes to allow adults to swim to the bottom. Adults were pelleted by a quick (2–3 seconds) centrifugation, and the supernatant was removed. If juveniles are desired, the tube, now free of bacteria after filtering, can also be centrifuged at max speed >5 minutes to pellet. Nematode pellets were then phenotyped, or flash-frozen in liquid N2 and stored −80 °C for subsequent processing (e.g. RT-qPCR).

Developmental rate determination. Worms were grown in liquid culture after bleach synchronization then filtered through a 20 µM filter 2 hours post bleach to isolate synchronous J2 animals, and then returned to liquid culture. Individual aliquots from the same flasks were monitored at regular intervals, and mouth-forms of adults were recorded at the J4-adult transition ($n = 2$). Flasks were rotated at 50 rpm to obtain large quantities of both St and Eu animals. Although not shown, several J4 were present at the earlier time points of 59 and 62 hours, which verified that we were observing the J4-adult transition.

Mouth-form phenotyping. For phenotyping nematodes grown on agar plates or NGG[35], adults were selected with a wire pick and transferred to 3–5 µl of M9 spotted on 4% agar pads (containing 10 mM sodium azide) on a standard microscope slide, then covered with a cover slip. For nematodes grown in liquid culture, after gently pelleting adults, they were re-suspended in the remaining M9 and 3–5 µl were directly pipetted onto the agar pad. When comparing mouth-forms of different conditions, we often performed 'blind' comparisons by writing the identity of the sample (i.e. "agar" or "liquid") on the slide, and then using laboratory tape to cover the identity, and blindly selecting a slide before placing it in the microscope holder. After counting, the identity of the sample was revealed by removing the tape. Phenotyping was performed at 40–100x/1.4 oil objective on a Differential Interference Contrast (DIC) microscope (Zeiss) according to buccal landmarks previously described[20]. In short, Eu were determined by the presence of a wide-mouth, a hooked dorsal tooth, and an additional subventral tooth. Conversely St animals were determined by a narrow-mouth, flint-like dorsal tooth, and absence of a subventral tooth (Fig. 1B,C). The number of biological replicates (n) was ≥3 for all conditions, and as high as 18 for liquid culture/S-medium, with each replicate including ≥50 animals with the exception that PBS and NGM-liquid cultures yielded significantly fewer animals, and included ≥20 animals per replicate. Mouth-forms were assessed 4–5 days after bleach-synchronization. Error bars represent standard error means (SEM), and statistical significance was assessed by *paired 2-tailed t-tests* unless otherwise indicated in the text.

dasc#1 experiments. dasc#1 was added at 1 µM final concentration according to previous methods[26] to eggs-J1 larvae in liquid culture. Mouth-forms were phenotyped as described above after 4 days and compared to

control liquid cultures without dasc#1. The p-value was determined by a 1-tailed, paired *t-test* for $n = 6$ biological replicates.

Morphology measurements. Length and width measurements were performed on synchronized adult animals four days after bleaching. Measurements were made of 12 animals grown on agar, 13 grown on NGG, and 10 in liquid culture using the ImageJ plug-in WormSizer[68]. Box plots in Supplementary Figure 2 show quartile edges (25% and 75%) of the distribution and medians (black bars), made in R {boxplot(shape~Condition, data = worm_sizes, horizontal = TRUE, notch = FALSE)}.

Expression analysis. RNA-seq data was obtained from Ragsdale, Müller *et al.*[27], and average fpkms from 4 mutant alleles of *eud-1* vs. one wild-type California RS2333 were plotted. For RT-qPCR, RNA was first extracted from either 1 agar plate or 1 liquid culture of synchronized young adults (4 days post-bleaching) of the California strain PS312 (same as RS2333 but an earlier frozen stock) by Trizole extraction followed by purification with Zymo RNA-Clean & Concentrator-25 columns following manufacturers instructions from Zymo. 500–1,000 ng of purified RNA was converted to cDNA using SuperScript II (Invitrogen) for 1 hour with Oligo$(dT)_{18}$ primer in 20 µl reactions, and then heat-inactivated with 40 µl of 150 mM KOH/20 mM Tris-base for 10 minutes at 99 °C followed by 40 µl of 150 mM HCl, and 100 µl of TE. 4 µl of cDNA was used for each technical replicate in 10 µl qPCR reactions with 1x LightCycler® 480 SYBR Green I Master Mix (Roche) and 0.25 µM of each primer on a Light-Cycler 480, 384 well format. All primer sets were validated for single amplicon production with Tm melt-curve analysis, and efficiency with a 5-log titration of cDNA. Relative expression ($2^{\Delta Ct}$) was measured relative to *Ppa-Y45F10D.4* (iron binding protein)[69] for each gene.

Data availability. All data generated or analyzed during this study are included in this article and its Supplementary Information files.

References

1. West-Eberhard, M. J. Developmental Plasticity and Evolution. (2003).
2. Bradshaw, A. D. Evolutionary Significance of Phenotypic Plasticity in Plants. *Adv. in Genetics* **13**, 115–155 (1965).
3. Gauze, G. F. *Problems of evolution*. **37**, part 2 (1947).
4. Huxley, J. Problems of relative growth. (1932).
5. Weaver, N. Rearing Honeybee Larvae on Royal Jelly in the Laboratory. *Bee World* (1955).
6. Charnov, E. L. & Bull, J. When is sex environmentally determined? *Nature* **266**, 828–830 (1977).
7. Ferguson, M. W. & Joanen, T. Temperature of egg incubation determines sex in Alligator mississippiensis. *Nature* **296**, 850–853 (1982).
8. Palacios, M. G., Sparkman, A. M. & Bronikowski, A. M. Developmental plasticity of immune defence in two life-history ecotypes of the garter snake, Thamnophis elegans - a common-environment experiment. *J Anim Ecol* **80**, 431–437 (2011).
9. Waddington, C. H. Genetic Assimilation of an Acquired Character. *Evolution* **7**, 118 (1953).
10. Gibert, J.-M., Peronnet, F. & Schlötterer, C. Phenotypic Plasticity in Drosophila Pigmentation Caused by Temperature Sensitivity of a Chromatin Regulator Network. *PLoS Genet* **3**, e30 (2007).
11. Golden, J. W. & Riddle, D. L. The Caenorhabditis elegans dauer larva: Developmental effects of pheromone, food, and temperature. *Developmental Biology* **102**, 368–378 (1984).
12. Sikkink, K. L., Reynolds, R. M. & Ituarte, C. M. Rapid evolution of phenotypic plasticity and shifting thresholds of genetic assimilation in the nematode Caenorhabditis remanei. *G3:* (2014).
13. Powsner, L. The effects of temperature on the durations of the developmental stages of Drosophila melanogaster. *Physiological Zoology* (1935).
14. Brakefield, P. M., Gates, J., Keys, D. & Kesbeke, F. Development, plasticity and evolution of butterfly eyespot patterns. *Nature* (1996).
15. Fielenbach, N. & Antebi, A. C. elegans dauer formation and the molecular basis of plasticity. *Genes & Development* **22**, 2149–2165 (2008).
16. Riddle, D. L., Swanson, M. M. & Albert, P. S. Interacting genes in nematode dauer larva formation. *Nature* **290**, 668–671 (1981).
17. Albert, P. S. & Riddle, D. L. Mutants of Caenorhabditis elegans that form dauer-like larvae. *Developmental Biology* **126**, 270–293 (1988).
18. Gottlieb, S. & Ruvkun, G. daf-2, daf-16 and daf-23: genetically interacting genes controlling Dauer formation in Caenorhabditis elegans. *Genetics* **137**, 107–120 (1994).
19. Lee, R. C., Feinbaum, R. L. & Ambros, V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. **75**, 843–854 (1993).
20. 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).
21. Sommer, R. J. & McGaughran, A. The nematode Pristionchus pacificus as a model system for integrative studies in evolutionary biology. *Molecular Ecology* **22**, 2380–2393 (2013).
22. Meyer, J. M. *et al*. Succession and dynamics of Pristionchus nematodes and their microbiome during decomposition of Oryctes borbonicus on La Réunion Island. *Environmental Microbiology* **19**, 1476–1489 (2017).
23. Serobyan, V., Ragsdale, E. J. & Sommer, R. J. Adaptive value of a predatory mouth-form in a dimorphic nematode. *Proceedings of the Royal Society of London B: Biological Sciences* **281**, 20141334–989 (2014).
24. 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. *J. Exp. Biol.* **218**, 1306–1313 (2015).
25. Serobyan, V., Ragsdale, E. J., Müller, M. R. & Sommer, R. J. Feeding plasticity in the nematode Pristionchus pacificus is influenced by sex and social context and is linked to developmental speed. *Evolution & Development* **15**, 161–170 (2013).
26. Bose, N. *et al*. Complex Small-Molecule Architectures Regulate Phenotypic Plasticity in a Nematode. *Angewandte Chemie International Edition* **51**, 12438–12443 (2012).
27. Ragsdale, E. J., Müller, M. R., Rödelsperger, C. & Sommer, R. J. A Developmental Switch Coupled to the Evolution of Plasticity Acts through a Sulfatase. **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. *Curr. Biol.* (2016).
29. Serobyan, V. *et al*. Chromatin remodelling and antisense-mediated up-regulation of the developmental switch gene eud-1 control predatory feeding plasticity. *Nat Commun* **7**, 12337 (2016).
30. Mather, K. & De Winton, D. Adaptation and counter-adaptation of the breeding system in Primula. *Annals of Botany* (1941).
31. Sommer, R. J. *et al*. The genetics of phenotypic plasticity in nematode feeding structures. *Open Biology* **7**, 160332–118 (2017).

32. Serobyan, V. & Sommer, R. J. Developmental systems of plasticity and trans-generational epigenetic inheritance in nematodes. *Current Opinion in Genetics & Development* **45**, 51–57 (2017).
33. Stiernagle, T. *Maintenance of C. elegans*, *WormBook*, *ed. The C. elegans Research Community*. (WormBook, 2006).
34. Samuel, T. K., Sinclair, J. W., Pinter, K. L. & Hamza, I. Culturing Caenorhabditis elegans in Axenic Liquid Media and Creation of Transgenic Worms by Microparticle Bombardment. *Journal of Visualized Experiments: JoVE* e51796 (2014).
35. Muschiol, D. & Traunspurger, W. Life cycle and calculation of the intrinsic rate of natural increase of two bacterivorous nematodes from chemoautotrophic Movile Cave, Romania. *Nematology* **9**, 271–284 (2007).
36. Barberon, M. *et al*. Adaptation of Root Function by Nutrient-Induced Plasticity of Endodermal Differentiation. *Cell* **164**, 447–459 (2016).
37. Abbruzzese, G. *et al*. Leaf morphological plasticity and stomatal conductance in three Populus alba L. genotypes subjected to salt stress. *Environmental and Experimental Botany* **66**, 381–388 (2009).
38. Wang, Y. *et al*. Salt-induced plasticity of root hair development is caused by ion disequilibrium in Arabidopsis thaliana. *J Plant Res* **121**, 87–96 (2008).
39. Touchon, J. C. T. C., Gomez-Mestre, I. G.-M. & Warkentin, K. M. W. M. Hatching plasticity in two temperate anurans: responses to a pathogen and predation cues. *Canadian Journal of Zoology* **84**, 556–563 (2006).
40. Hong, J. K. & Hwang, B. K. Induction by pathogen, salt and drought of a basic class II chitinase mRNA and its *in situ* localization in pepper (Capsicum annuum). *Physiologia Plantarum* **114**, 549–558 (2002).
41. Pulendran, B., Palucka, K. & Banchereau, J. Sensing Pathogens and Tuning Immune Responses. *Science* **293**, 253–256 (2001).
42. Huang, Q. *et al*. The Plasticity of Dendritic Cell Responses to Pathogens and Their Components. *Science* **294**, 870–875 (2001).
43. Plunkett, C. R. Temperature as a tool of research in phenogenetics: methods and results (1932).
44. Woodward, D. E. & Murray, J. D. On the Effect of Temperature-Dependent Sex Determination on Sex Ratio and Survivorship in Crocodilians. *Proceedings of the Royal Society of London B: Biological Sciences* **252**, 149–155 (1993).
45. Manenti, T., Loeschcke, V., Moghadam, N. N. & Sørensen, J. G. Phenotypic plasticity is not affected by experimental evolution in constant, predictable or unpredictable fluctuating thermal environments. *Journal of Evolutionary Biology* **28**, 2078–2087 (2015).
46. Brzek, P., Kohl, K., Caviedes-Vidal, E. & Karasov, W. H. Developmental adjustments of house sparrow (Passer domesticus) nestlings to diet composition. *J. Exp. Biol.* **212**, 1284–1293 (2009).
47. Watson, E., MacNeil, L. T., Arda, H. E., Zhu, L. J. & Walhout, A. J. M. Integration of Metabolic and Gene Regulatory Networks Modulates the C. elegans Dietary Response. *Cell* **153**, 253–266 (2013).
48. Bernstein, B. E. *et al*. A Bivalent Chromatin Structure Marks Key Developmental Genes in Embryonic. *Stem Cells.* **125**, 315–326 (2006).
49. Rougvie, A. E. & Lis, J. T. Postinitiation transcriptional control in Drosophila melanogaster. *Mol. Cell. Biol.* **10**, 6041–6045 (1990).
50. Rada-Iglesias, A. *et al*. A unique chromatin signature uncovers early developmental enhancers in humans. *Nature* **470**, 279–283 (2011).
51. Zentner, G. E., Tesar, P. J. & Scacheri, P. C. Epigenetic signatures distinguish multiple classes of enhancers with distinct cellular functions. *Genome Res.* **21**, 1273–1283 (2011).
52. Maxwell, C. S. *et al*. Pol II Docking and Pausing at Growth and Stress Genes in C. elegans. *Cell Rep* **6**, 455–466 (2014).
53. Gaertner, B. *et al*. Poised RNA Polymerase II Changes over Developmental Time and Prepares Genes for Future Expression. *Cell Rep* **2**, 1670–1683 (2012).
54. Ernst, J. *et al*. Mapping and analysis of chromatin state dynamics in nine human cell types. *Nature* **473**, 43–49 (2011).
55. Hsu, H. T. *et al*. Recruitment of RNA polymerase II by the pioneer transcription factor PHA-4. *Science* **384**, 1372–1376 (2015).
56. Ozawa, T. *et al*. Histone deacetylases control module-specific phenotypic plasticity in beetle weapons. *Proc. Natl. Acad. Sci. USA* **113**, 15042–15047 (2016).
57. Kucharski, R., Maleszka, J., Foret, S. & Maleszka, R. Nutritional Control of Reproductive Status in Honeybees via DNA Methylation. *Science* **319**, 1827–1830 (2008).
58. Simola, D. F. *et al*. Epigenetic (re)programming of caste-specific behavior in the ant Camponotus floridanus. *Science* **351**, aac6633–aac6633 (2016).
59. Kooke, R. *et al*. Epigenetic basis of morphological variation and phenotypic plasticity in Arabidopsis thaliana. *Plant Cell* **27**, 337–348 (2015).
60. Zhang, Y. Y., Fischer, M., Colot, V. & Bossdorf, O. Epigenetic variation creates potential for evolution of plant phenotypic plasticity. *New Phytologist* **197**, 314–322 (2013).
61. Traunspurger, W. The biology and ecology of lotic nematodes. *Freshwater Biology* **44**, 29–45 (2000).
62. Tietjen, J. H. & Lee, J. J. Life history and feeding habits of the marine nematode, Chromadora macrolaimoides steiner. *Oecologia* **12**, 303–314 (1973).
63. Herman, P. M. J. & Vranken, G. Studies of the life-history and energetics of marine and brackish-water nematodes. *Oecologia* **77**, 457–463 (1988).
64. Waddington, C. H. *Organisers and Genes by C. H. Waddington*. (The University Press, 1940).
65. Waddington, C. H. Canalization of development and the inheritance of acquired characters. *Nature* (1942).
66. Baeuerle, P. A. & Baltimore, D. IkappaB: a specific inhibitor of the NF-kappaB transcription factor. *Science* (1988).
67. Susoy, V. & Sommer, R. J. Stochastic and Conditional Regulation of Nematode Mouth-Form Dimorphisms. *Front. Ecol. Evol.* **4**, 6706 (2016).
68. Moore, B. T., Jordan, J. M. & Baugh, L. R. WormSizer: high-throughput analysis of nematode size and shape. *PLoS ONE* **8**, e57142 (2013).
69. Schuster, L. N. & Sommer, R. J. Expressional and functional variation of horizontally acquired cellulases in the nematode Pristionchus pacificus. *Gene* **506**, 274–282 (2012).
70. Susoy, V. *et al*. Large-scale diversification without genetic isolation in nematode symbionts of figs. *Science Advances* **2**, e1501031–e1501031 (2016).

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Author Contributions

M.S.W., B.S. and R.J.S. conceived and designed the experiments. M.S.W. and T.L. performed mouth-form experiments with help from B.S. in making axenic culture, RNA-seq analysis (also with assistance from S.N.), and rpm experiments. M.S.W., B.S., S.N., M.L., M.D., T.R. and D.R.S. all contributed to RT-qPCR experiments. M.S.W. wrote the manuscript with edits and assistance from R.J.S., and with contribution and approval from all other authors.

Additional Information

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Supplementary Information

Environmental influence on *Pristionchus pacificus* mouth form through different culture methods

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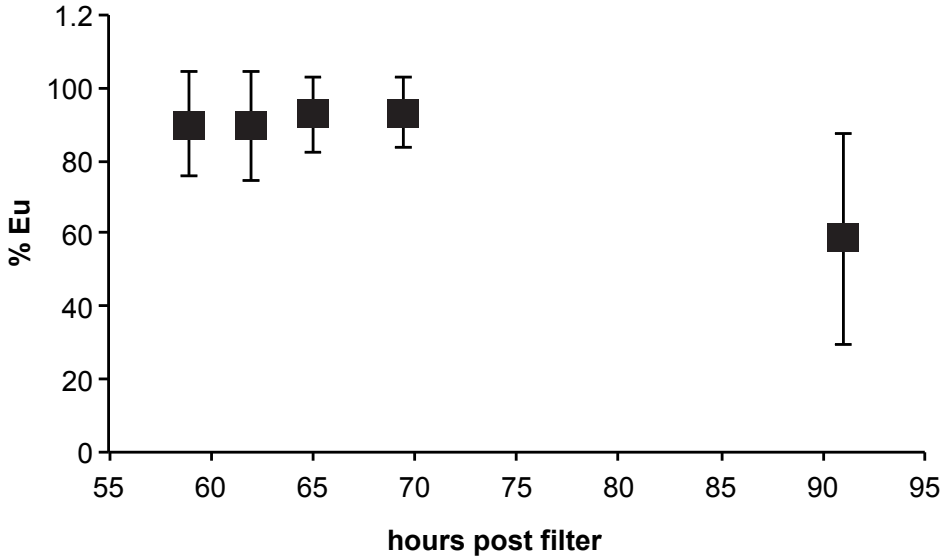
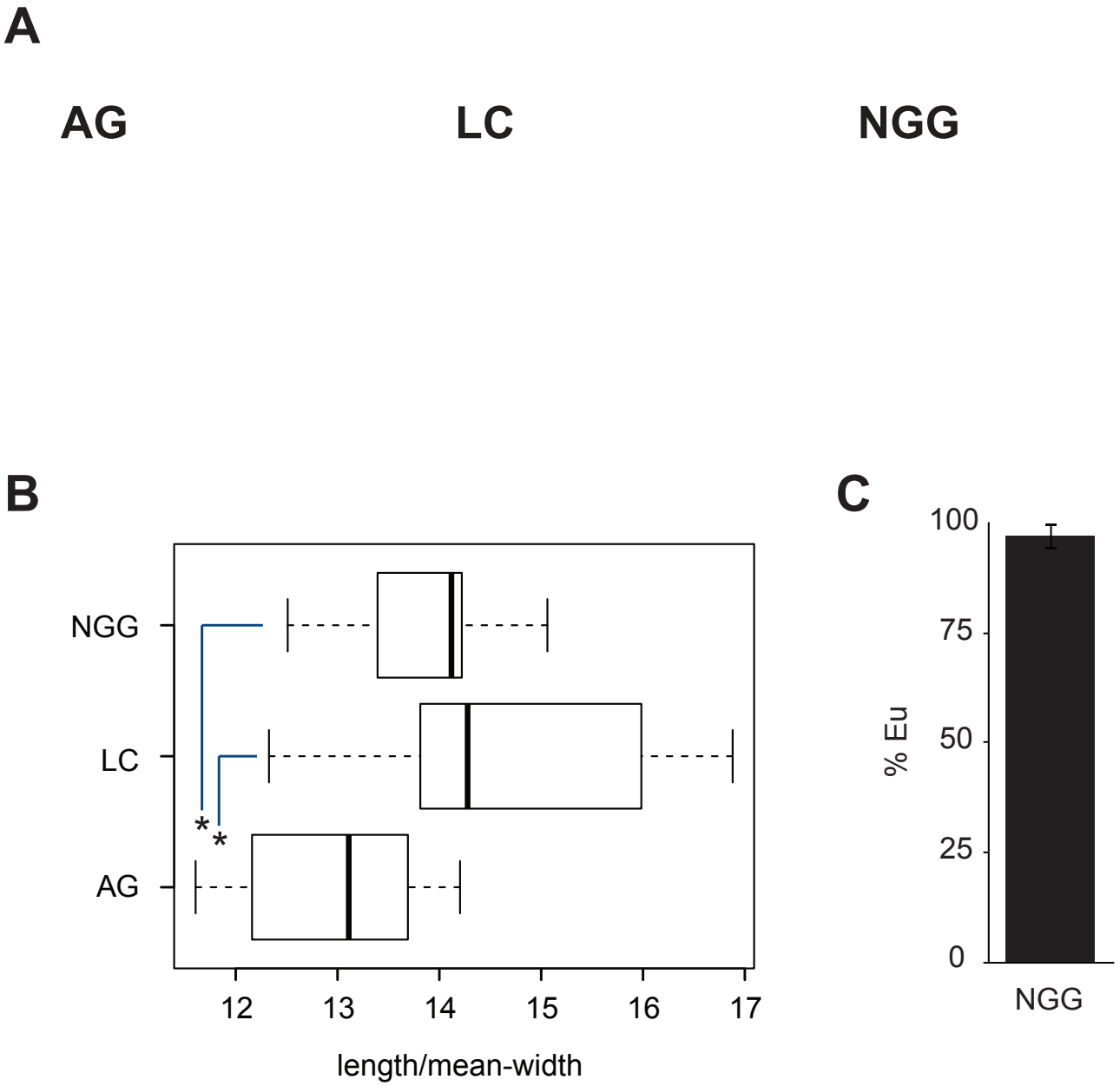


Supplementary Figure 1. Growth rate of morphs in liquid culture. Percent Eu of adult hermaphrodites grown in liquid at the J4-adult transition (59-70 hours post filter), and at 91 hours, a time point at which we normally collect and phenotype animals (n = 2). Worms were incubated at 22° C in S-Medium with 50 rpm shaking to induce sufficient numbers of both, St and Eu animals, allowing statistical significance testing ($p > 0.05$ between any two time-points arguing against slower development of Eu animals, two-tailed t-test).



Supplementary Figure 2. Slender morphology does not correlate with mouth-form. (A) Images of *P. pacificus* grown in liquid culture and NGG display more slender morphology than on agar plates, quantified in (**B**). Measurements of adults from the same synchronized population were made with Wormsizer[68], n = 12 (agar), 13 (NGG), and 10 (liquid culture = 'LC'). Statistical significance was measured with a nonparametric Mann-Whitney U test in R. (**C**) Same as in Figure 2, mouth-form ratio of adult PS312 grown in NGG, n = 3.

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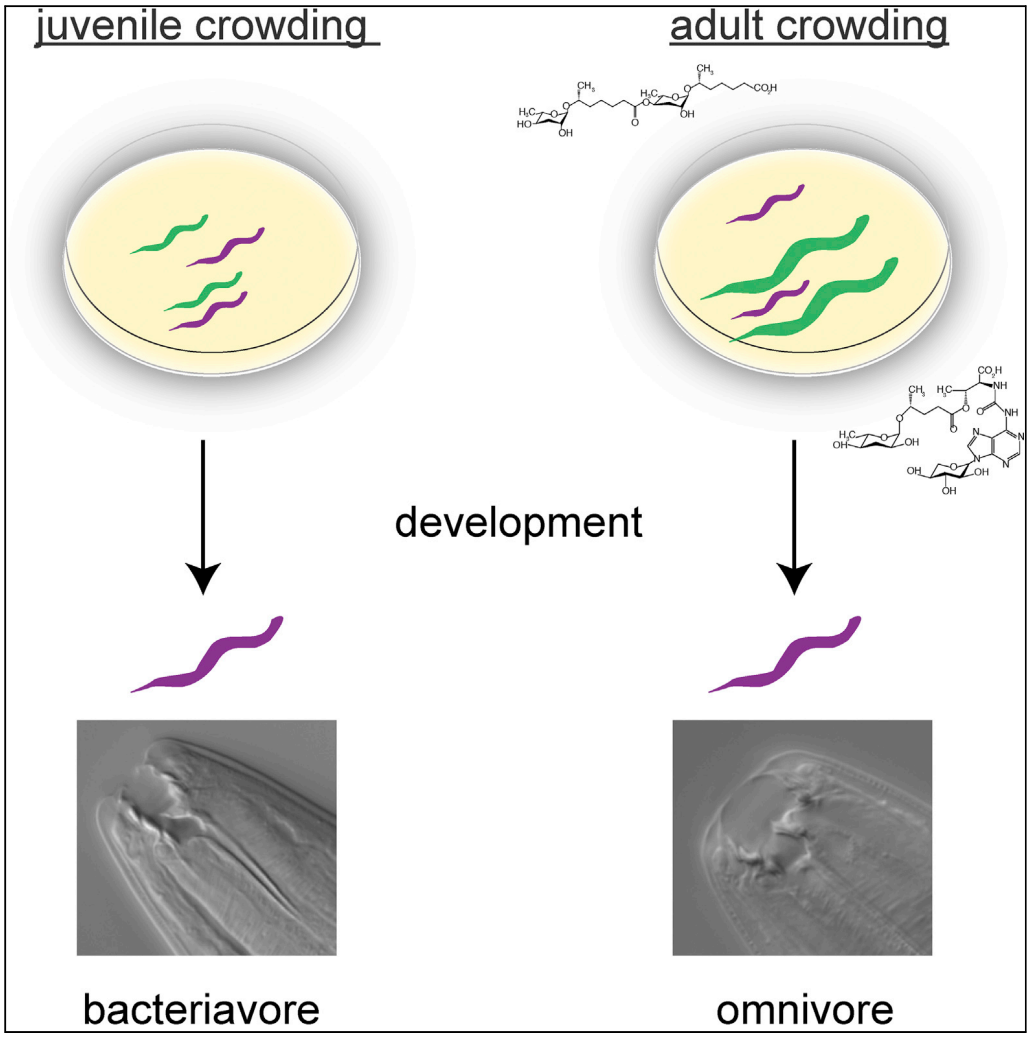

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HIGHLIGHTS

Novel vital dye method for tracking mixed nematode populations

Adult, but not juvenile, crowding induces the omnivorous morph in *P. pacificus*

Omnivorous morph-inducing pheromones are produced late in development

Age class is an important component of density-dependent phenotypic plasticity

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Adult Influence on Juvenile Phenotypes by Stage-Specific Pheromone Production

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SUMMARY

Many animal and plant species respond to population density by phenotypic plasticity. To investigate if specific age classes and/or cross-generational signaling affect density-dependent plasticity, we developed a dye-based method to differentiate co-existing nematode populations. We applied this method to *Pristionchus pacificus*, which develops a predatory mouth form to exploit alternative resources and kill competitors in response to high population densities. Remarkably, adult, but not juvenile, crowding induces the predatory morph in other juveniles. High-performance liquid chromatography-mass spectrometry of secreted metabolites combined with genetic mutants traced this result to the production of stage-specific pheromones. In particular, the *P. pacificus*-specific di-ascaroside#1 that induces the predatory morph is induced in the last juvenile stage and young adults, even though mouth forms are no longer plastic in adults. Cross-generational signaling between adults and juveniles may serve as an indication of rapidly increasing population size, arguing that age classes are an important component of phenotypic plasticity.

INTRODUCTION

Population density is an important ecological parameter, with higher densities corresponding to increased competition for resources (Hastings, 2013). In addition to density-dependent selection (MacArthur, 1962; Travis et al., 2013), which operates on evolutionary timescales, some organisms can respond dynamically to population density through phenotypic plasticity. For example, plants can sense crowding by detecting the ratio of red (chlorophyll absorbing) to far red (non-absorbing) light, and respond by producing higher shoots (Dudley and Schmitt, 2015). Locusts undergo solitary to swarm (i.e., gregarious) transition as a result of increased physical contact (Pener and Simpson, 2009; Simpson et al., 2001). Intriguingly, population density can also have cross-generational effects, defined here as the density of one age group affecting the phenotypes of another. For example, adult crowding of the desert locust *Schistocerca gregaria* (Maeno and Tanaka, 2008; Simpson and Miller, 2007) and migratory locust *Locusta migratoria* (Chen et al., 2015; Ben Hamouda et al., 2011) also influences the egg size, number, and morphology of their progeny, high population densities of red squirrels elicit hormonal regulation in mothers to influence faster-developing offspring (Dantzer et al., 2013), and crowding in aphids can induce winged progeny from flightless parents (Slogget and Weisser, 2002, Sutherland, 1969). In many species, population density and cross-generational signaling are communicated by pheromones; however, the precise nature, mechanisms of induction, age specificity, and exact ecological role are not well understood.

Nematodes are a powerful model system to investigate the mechanisms of density-dependent plasticity because many small molecule pheromones that affect plastic phenotypes have been characterized (Butcher, 2017; Butcher et al., 2007; von Reuss et al., 2012). For example, in the model organism *Caenorhabditis elegans,* high population densities induce entry into a stress-resistant dormant "dauer" stage (Fielenbach and Antebi, 2008). The decision to enter dauer was revealed to be regulated by a family of small molecule nematode-derived modular metabolites (NDMMs) called ascarosides that act as pheromones (Butcher et al., 2007, 2008; Jeong et al., 2005). Ascarosides consist of an ascarylose sugar with a fatty acid side chain and modular head and terminal groups (Figure 1A). The level and composition of ascarosides were later shown to be dependent on sex (Chasnov et al., 2007; Izrayelit et al., 2012) and development (Kaplan et al., 2011), although it is thought that early larval development into dauer can be induced by pheromones from all developmental stages (Golden and Riddle, 1982). Subsequent studies revealed that specific NDMMs also regulate other life history traits, such as mating (Chasnov et al., 2007; Izrayelit et al., 2012), social behavior (Srinivasan et al., 2012), and developmental speed (Ludewig et al., 2017). Although NDMMs are broadly conserved (Choe et al., 2012; Dong et al., 2018; Markov et al., 2016), inter- and intraspecific competition have driven the evolution of distinct response regimes (different levels of sensitivity to the

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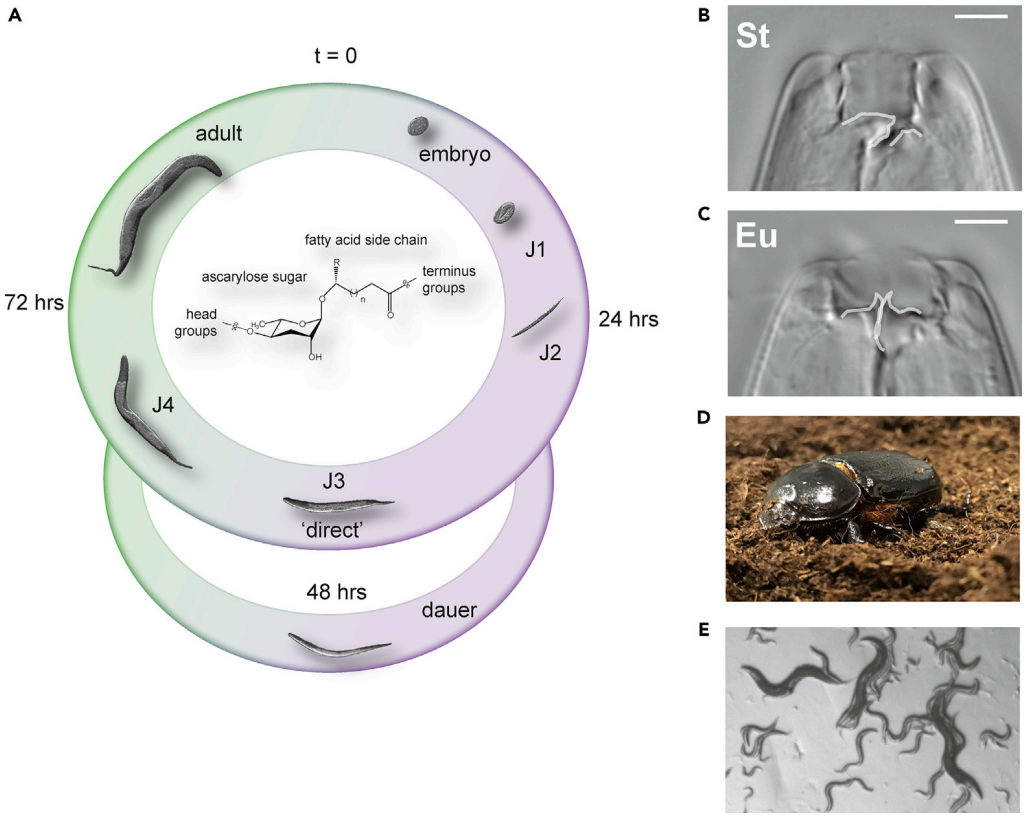

Figure 1. Life Cycle and Developmental Plasticity of the Model Nematode *Pristionchus pacificus*

(A) The life cycle of *P. pacificus* consists of four juvenile stages (J1–J4) until sexual maturation (adult hermaphrodites). Like many nematodes *P. pacificus* can enter a long-living "dormant" dauer state that is resistant to harsh environmental conditions. The decision to continue through the direct life cycle or enter dauer is regulated by small-molecule-excreted ascarosides (chemical structure adapted from Butcher, 2017).

(B and C) (B) *P. pacificus* can also adopt one of two possible feeding structures; either a microbivorous narrow mouth (stenostomatous, St) or (C) an omnivorous wide mouth (eurystomatous, Eu) with an extra tooth that can be utilized to kill and eat other nematodes or fungi. White lines indicate the presence of an extra tooth (right side) in the Eu morph or its absence in the St morph, and the dorsal tooth (left side), which is narrow and elongated (flint-like) in St and hook like in Eu. Scale bar, 5 μM.

(D) *P. pacificus* is often found in a necromenic association with beetles (e.g., shown here *Oryctes borbonicus*, photo taken by Tess Renahan) in the dauer state and resumes the free-living life cycle upon beetle death to feed on the ensuing microbial bloom.

(E) RSC017 mixed-staged worms on agar plates.

same pheromone, or sensitivity to different pheromones) for the same phenotypes (Bose et al., 2014; Choe et al., 2012; Diaz et al., 2014; Falcke et al., 2018; Greene et al., 2016). In addition, distinct plastic phenotypes have evolved that are regulated by more complex ascaroside structures (Bose et al., 2012).

In *Pristionchus pacificus,* a soil-associated nematode that is reliably found on scarab beetles (Figure 1A) (Herrmann et al., 2006, 2007; Sommer and McGaughran, 2013), an ascaroside dimer (dasc#1) that is not found in *C. elegans* regulates the development of a predatory mouth form (Bento et al., 2010; Bose et al., 2012; Sommer et al., 2017). Mouth-form plasticity represents an example of a morphological novelty that results in predatory behavior to exploit additional resources and kill competitors. Specifically, adult *P. pacificus* exhibit either a narrow stenostomatous (St) mouth (Figure 1B), which is restricted to bacterial feeding, or a wide eurystomatous (Eu) mouth with an extra denticle (Figure 1C), which allows for feeding on bacteria and fungi (Sanghvi et al., 2016), and predation on other nematodes (Wilecki et al., 2015). This type of phenotypic plasticity is distinct between direct, non-arrested development and indirect (dauer) development because the mouth form decision results in two alternative life history strategies in the adult (for review, see Sommer & Mayer, 2015). Recent studies in *P. pacificus* have begun to investigate the dynamics and succession of nematodes on decomposing beetle carcasses to better understand the ecological significance of mouth-form plasticity (Meyer et al., 2017). These studies revealed that on a carcass

(Figure 1D), *P. pacificus* exits the dauer diapause to feed on microbes, and then re-enters dauer after food sources have been exhausted, displaying a "boom-and-bust" ecology (Meyer et al., 2017; Sommer and McGaughran, 2013). Presumably different stages of this succession comprise different ratios of juveniles and adults, and recognizing the age structure of a population as a juvenile could provide predictive value for adulthood. However, it is unknown whether the mouth-form decision is sensitive to crowding by different age classes (example of crowding by different age groups, Figure 1E). More broadly, whereas age classes are known to be important for population growth and density-dependent selection (Hastings, 2013; Charlesworth, 1994; 1972), their role in phenotypic plasticity has thus far been largely unexplored.

Although nematodes have many experimental advantages, including easy laboratory culture and advanced genetic, genomic, and the aforementioned chemical tools, their small size has made investigations at the organismal level and in experimental ecology challenging. For example, no *in vivo* methodologies are currently available to label distinct populations without the need for transgenics, which is only available in select model organisms such as *C. elegans*, *P. pacificus*, and some of their relatives. Here, we combine a novel dye-staining method with the first developmental pheromone profiling in *P. pacificus* to study the potential effects of age on density-dependent plasticity. This vital dye method allows tracking adults with juveniles, or juveniles with juveniles, and can be applied to any nematode system that can be cultured under laboratory conditions. In contrast to dauer, we found that mouth form is strongly affected by cross-generational signaling. Specifically, only adult crowding induces the predatory morph, which is controlled by stage-specific pheromones.

RESULTS

A Vital Dye Method for Labeling Nematode Populations

To directly test if different age groups of *P. pacificus* influence mouth form, we required two synchronized populations to co-habit the same space, yet still be able to identify worms from different age groups. To do so, we developed a dye-staining methodology to robustly differentiate between nematode populations. After trying several vital dyes, we identified that neutral red (Thomas and Lana, 2008) and CellTracker Green BODIPY (Thermo) stain nematode intestines brightly and specifically to their respective channels (Figures 2A–2E and S1, Transparent Methods). These dyes stained all nematodes tested including *C. elegans* (Figure S2) and dauer larvae (Figures S3A and S3B). Both dyes lasted more than 3 days and neutral red >5 days (Figures S3C–S3G), allowing long-term tracking of mixed nematode populations. Importantly, neither neutral red nor CellTracker Green staining affected viability, developmental rate, or the formation of specific morphological structures, such as *P. pacificus* mouth form (Figure S4). Thus, neutral red and CellTracker Green allow specific labeling of worm populations to study age-dependent effects on phenotypes.

Adult but Not Juvenile Crowding Induces the Predatory Mouth Form in *P. pacificus*

To assess potential intra- or inter-generational influence on *P. pacificus* mouth form, we stained 200 juveniles stage 2 (J2s) of the highly St strain RSC017 (Figure 3A) with neutral red and added an increasing number of CellTracker Green-stained RSC017 adults or juveniles (J2s or J3/4s) (Figure 2F). After 3 days, we phenotyped red animals that had developed into adults. Almost half (48%) of the population developed an Eu mouth form with 500 adult animals, compared with less than 4% with 500 J2 or J3/4 juveniles ($n > 100$ from 2–5 independent biological replicates; for display, summed percentages are shown in Figures 3B–3D). We performed a direct statistical comparison between crowded plates and controls (no added crowding animals) for every number and stage of crowding. After multiple testing corrections, only 200 and 500 adult-crowded plates yielded significant differences compared with control (un-crowded) plates (*Bonferroni-corrected* $p = 6.9 \times 10^{-3}$ and $<2.2 \times 10^{-16}$, respectively, Fisher's exact test on Eu counts). To ascertain if there is a general difference between juvenile or adult crowding, we performed a binomial regression on replicate Eu count data, with stage (J2–J4 versus adults) and number of crowding animals included as fixed effects (Transparent Methods, Table S1). Indeed, we observed a significant difference between adult and juvenile crowding and the incidence of Eu morphs ($p = 1.32 \times 10^{-2}$).

We were also curious if dauers, which have a thickened cuticle and represent a distinct stage in the boom-and-bust life cycle of nematodes, could still respond to adults. Indeed, the same trend that was observed with juveniles was seen with dauers; only 200 and 500 adults significantly induced the Eu mouth form, albeit to a more muted extent (Figures 3E and 3F) (*Bonferroni-corrected* $p = 2.4 \times 10^{-2}$ and 7.3×10^{-5}, respectively; Fisher's exact test; and binomial regression between dauer and adult crowding $p = 2.96 \times 10^{-3}$).

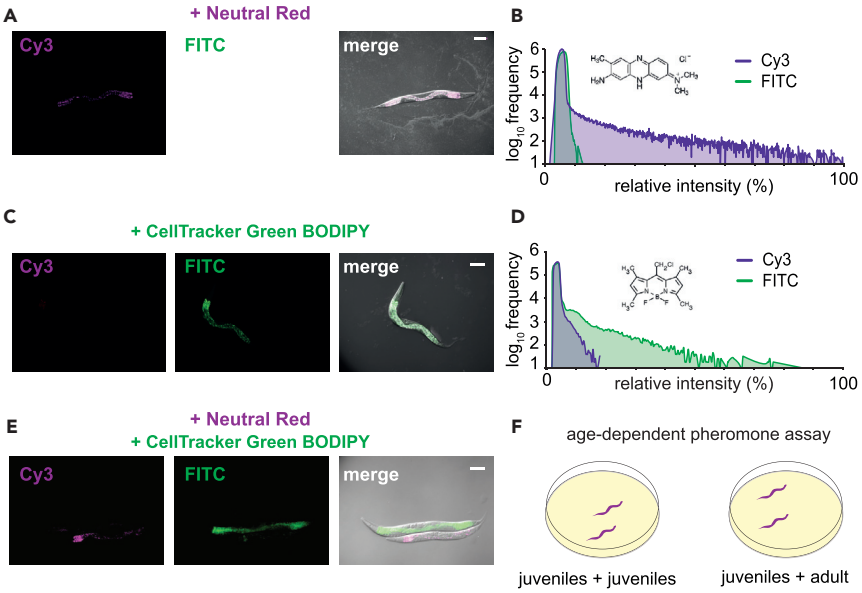

Figure 2. Vital Dye Method in Nematodes Allows Mixing Different Populations Together

(A) Neutral Red-stained adults (0.005% for 3 hours) imaged with Cy3 and FITC excitations and filters, and merged with DIC.

(B) An example of the relative intensities of fluorescence displayed as a histogram with the chemical structure of Neutral Red.

(C) CellTracker Green BODIPY (Thermo)-stained adults (50 μM for 3 hours) imaged with Cy3 and FITC excitations and filters, and merged with DIC.

(D) An example of the relative intensities of fluorescence displayed as a histogram with the chemical structure of CellTracker Green BODIPY.

(E) Combined worms from Neutral Red and CellTracker Green BODIPY staining on the same slide, merged with DIC.

(F) Age-dependent functional pheromone assay: experimental juveniles were stained with neutral red and challenged with CellTracker Green BODIPY-stained juveniles or adults on standard condition Nematode Growth Media (NGM) agar plates seeded with 300 μL OP50 *E. coli*. Three days later, only red-positive and green-negative adults were phenotyped.

With a total of 200 dauers and 500 adults, 25.7% of dauers became Eu, whereas only 1.8% of dauers become Eu on a plate containing 700 dauers (and no adults) (Figure 3F). Collectively, these data indicate that adult crowding specifically induces the Eu mouth form. However, it should be noted that because of the difficulty in obtaining a pure J4 culture from RSC017s, we cannot rule out that crowding by large numbers of J4s could also induce the Eu morph.

Even though we did not detect a mouth-form switch in large populations of J2s or dauers, and food was still visible on plates containing the most animals (500 "crowders"), we could not completely rule out the possible effect of food availability on mouth form. As a proxy for starvation, we conducted assays with greatly increased numbers of juveniles from 1,000 to 10,000 that would rapidly deplete bacterial food. We noticed a stark cliff in the fraction of animals that reach adulthood at 4,000–5,000 juveniles, arguing that food is a limiting resource at this population density (Figure 3G). Importantly, however, in these plates we still did not see a shift in mouth form (Figure 3H) ($p = 0.99$, binomial regression, Table S1). With an overwhelming 10,000 worms on a plate, 5.8% were Eu, compared with 48% in the presence of only 500 adults. Although longer-term starvation may have an impact on mouth form, under our experimental conditions it appears to be negligible.

Late-Stage Secretions Induce the Eu Mouth Form

As the mouth-form decision in *P. pacificus* can be influenced by NDMMs (Bose et al., 2012), we wondered if the difference in Eu induction between adults and juveniles resulted from differences in secreted pheromones. To test this hypothesis, we added secretions from 24- and 72-hr cultures of RSC017 and the laboratory strain RS2333 (which is highly Eu) to RSC017 juveniles. We found that the 72-hr (late juvenile stage

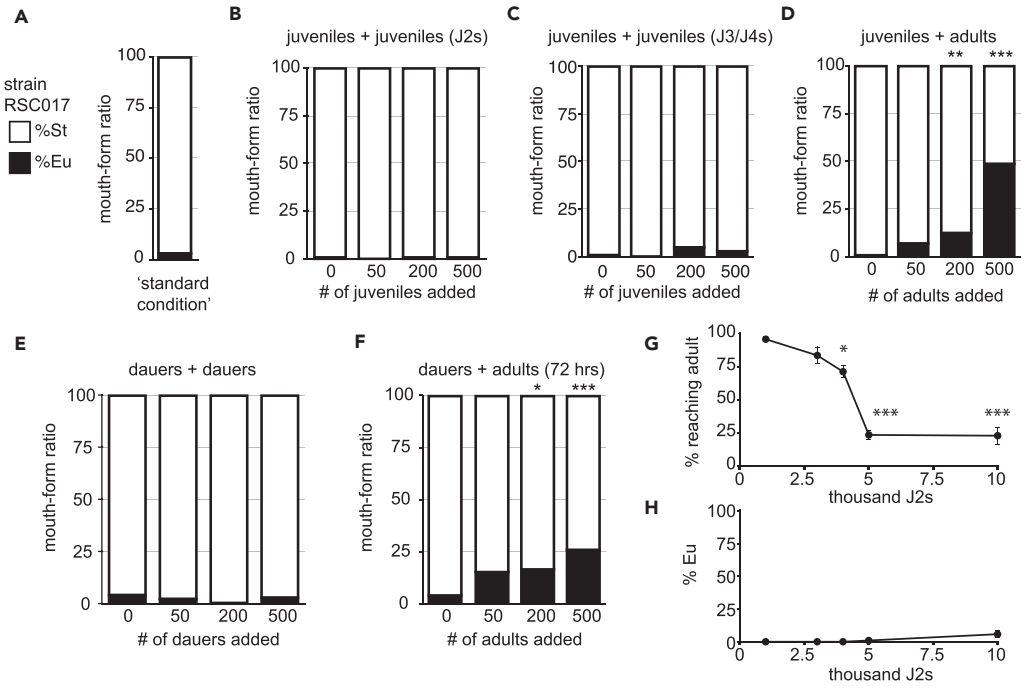

Figure 3. Vital Dye Method Demonstrates Adult-Specific Density Effect on Mouth Form

(A–F) (A) The wild isolate RSC017 grown in standard conditions (5 young adults passed to fresh plates, progeny phenotyped 4 days later) are highly stenostomatous (<10%, *n* = 102). Mouth form ratios of neutral red-stained J2s (B–D) and dauers (E and F), with increasing number of CellTracker Green-stained competitors (total number of animals *n* > 100 per experiment, with 3–5 independent biological replicates for J2 and adult crowding, and 2 for J3/J4s). Overall significance between strain and age was determined by a binomial linear regression (see Transparent Methods), and pairwise comparisons were assessed by Fisher's exact test on summed Eu counts (***p < 0.001, **p < 0.01, *p < 0.05). Mouth forms were phenotyped at 40–100× on a Zeiss Axio Imager 2 light microscope.

(G and H) (G) Percent reaching adulthood and percent Eu of those that reached adulthood (H) after increasing numbers of J2s were added to standard 6-cm Nematode Growth Media (NGM) agar plates with 300 μL OP50 *E. coli* bacteria (n = 2 biological replicates, with total *n* > 200 for percent reaching adulthood, and total n > 100 for mouth form. Significance was determined by a binomial regression; Error bars represent standard deviation of the two biological replicates).

4/adult, Figure S4H; Werner et al., 2017) secretions from both strains led to a significant increase in the Eu morph relative to the 24-hr (early juvenile J2) secretions ($p = 5.27 \times 10^{-6}$, 1.33×10^{-3}, respectively, Fisher's exact test on Eu counts relative to S-medium controls, n = 2-4 biological replicates; for display, summed percentages are shown in Figure 4). To confirm that the effect was caused by ascaroside pheromones, we exposed RSC017 juveniles to supernatant from a *P. pacificus daf-22.1;daf-22.2* double mutant, which exhibits virtually no ascaroside production in both *C. elegans* and *P. pacificus* (Golden and Riddle, 1985; Markov et al., 2016). Again, early juvenile secretion had no impact on Eu frequency, but in contrast to wild-type supernatants, we observed no significant increase in Eu frequency with the 72-hr secretions (p = 0.8324, Fisher's exact test, Figure 4). Thus, late-stage NDMMs induce development of the Eu mouth form.

Developmental-Staged NDMM Profiles Reveal Age-Specific Synthesis of dasc#1

Next, we investigated whether the different effects of early and late pheromones are ones of dosage, or of identity. To determine the potential age-specific differences in pheromones, we profiled *P. pacificus* NDMM levels in two strains and at three time points throughout development with high-performance liquid chromatography-mass spectrometry (Figures 5A, 5B, and S5). We performed a linear regression with the area under the curve of each NDMM chromatogram (Figure S5A) as the response variable. Stage and strain were modeled as fixed effects, and because we performed separate regression analyses for each pheromone, we adjusted the resulting p values for multiple testing using false discovery rate (FDR) (see Table S2 for p and FDR values between stage and strain). We observed that among developmental stages there were significant differences in the levels of ascr#9, ascr#12, npar#1, and dasc#1, and that dasc#1,

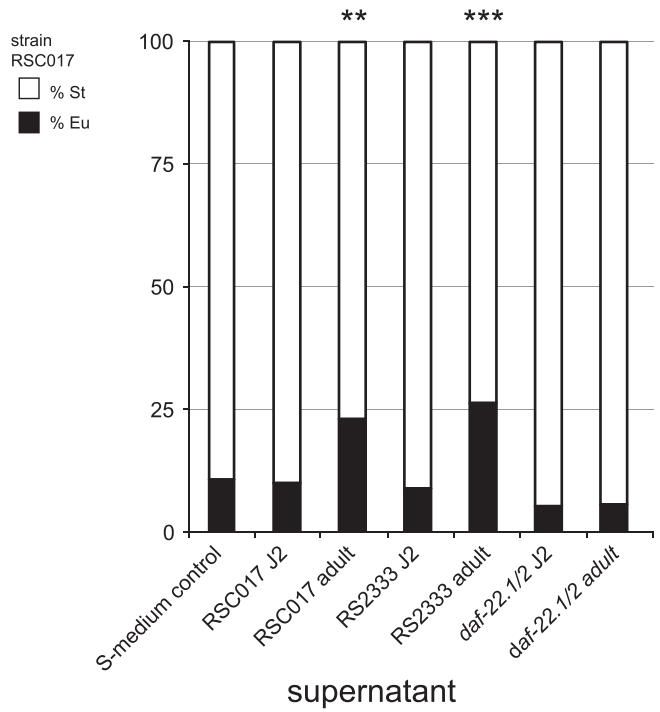

Figure 4. Late-Stage Secretions Induce Predatory Morph in Juveniles

Highly St strain RSC017 juveniles were exposed to 24- and 72-hr supernatants of its own strain, and to the 24- and 72-hr supernatants of the highly Eu strain RS2333. Mouth form was phenotyped 3 days later. Worms exposed to 24-hr secretions remained highly St, whereas worms exposed to 72-hr secretions had a small but significant increase in Eu morphs ($p < 0.05$, Fisher's exact test). Supernatants from the double mutant *daf-22.1/2*, which has deficient ascaroside pheromone production (Golden and Riddle, 1985; Markov et al., 2016), did not elicit increases in Eu from either 24- or 72-hr supernatants. Worms exposed to the S-media control also remained highly St. n = 4 independent biological replicates for RS2333 and *daf-22.1/2* secretions, and n = 2 independent biological replicates for RSC017 secretions, with an average count of 55 animals per replicate. For display, total Eu and St counts are presented as percentages (*** $p < 0.001$, ** $p < 0.01$).

ubas#1, and ubas#2 are affected by both stage and strain (FDR<0.05). Interestingly, dasc#1 is the most potent known Eu-inducing pheromone when tested as a single synthesized compound, whereas npar#1 is both an Eu- and a dauer-inducing pheromone (Bose et al., 2012). Closer inspection revealed dasc#1, npar#1, and ascr#9 increase throughout development in both strains, and dasc#1 peaks at 72 hr in RS2333 (Figures 5C and 5D and 5F–5I, $p < 0.05$, Student's two-tailed t test between 72 and 24 hr for each NDMM in both strains, and 72 and 48 hr for dasc#1 in RS2333, Table S3). Intriguingly, the trajectory of dasc#1 appeared "binary/off-on" in both strains; in some replicates dasc#1 levels were undetectable, whereas others were high and virtually no replicates exhibited intermediate levels (Figures 5F and 5G). In fact, our statistical model for dasc#1 fits better if we assume cubic rather than linear growth (model difference Akaike information criterion, $\Delta AIC = 3.958$). In contrast, ascr#9, which was also statistically increased but does not affect known plastic phenotypes (Bose et al., 2012), displayed a more gradual increase in both strains (Figures 5E, 5J, and 5K), and the model fits better with linear growth ($AIC_{linear} - AIC_{cubic} = -1.208$). Meanwhile, the induction pattern of npar#1 appears particular to each strain, although our linear model did not detect significant strain effects. Thus the kinetics of induction appears to be NDMM specific, which may be related to their roles in phenotypic plasticity.

We were interested to know if there was a transcriptional signal that would correlate with the increase in NDMMs throughout development. An analysis of previously published RNA sequencing data (Baskaran et al., 2015) reveals an ~5-fold increase in transcription of the thiolase *Ppa-daf-22.1* (Figure S6A) between J2 and J4/adults, the most downstream enzyme in the β-oxidation pathway of ascaroside synthesis. However, this enzyme is responsible for the last step in synthesizing many NDMMs in addition to dasc#1, npar#1, and ascr#9, so other enzymes must also be involved, and identifying them is an area of active research.

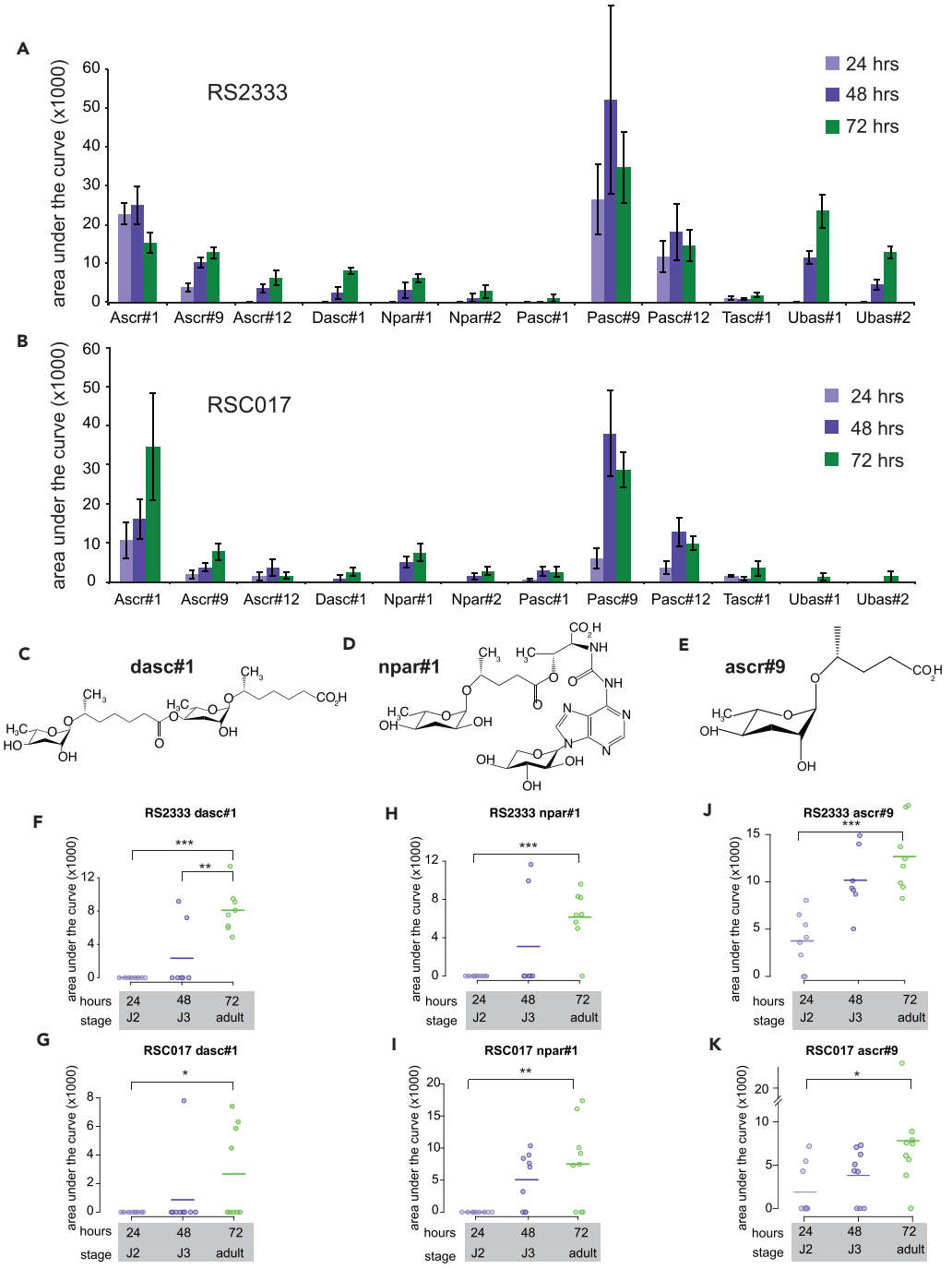

Figure 5. Time-Resolved Nematode-Derived Modular Metabolites (NDMMs) in *Pristionchus pacificus*

(A and B) (A) Time-resolved secretion profile of nematode-derived modular metabolites from the wild-type laboratory strain RS2333 and (B) wild isolate RSC017. In both strains, at 24 hr cultures represent predominantly J2 stage worms, at 48 hr a mix of J2–J4, and at 72 hr predominantly adults in RSC017 (90%, Figure S4H) and a mix of J4/adults in RS2333 (Werner et al., 2017). Data are presented as the mean of 8 (RS2333) and 9 (RSC017) biological replicates, and error bars represent the standard error of mean (SEM).

(C–E) Chemical structures of age-specific NDMMs (C) dasc#1, (D) npar#1, and (E) ascr#9, as described in the Small Molecule Identifier Database (http://www.smid-db.org/), produced in ChemDraw.

(F–K) Time-resolved abundance of (F and G) dasc#1, (H and I) npar#1, and (J and K) ascr#9 NDMMs in RS2333 and RSC017. Each data point represents a biological replicate, and for comparison with (A and B) lines represent mean abundance. p values calculated by a 2-tailed Student's t test (***p < 0.001, **p <0.01, *p <0.05).

In principle, the increase in abundance of dasc#1, npar#1, and ascr#9 throughout development could be a result of a concomitant increase in body mass. We used WormSizer (Moore et al., 2013) to measure the size of RSC017 animals from each time point and then normalized NDMM abundances by volume. We found a 1.1-fold difference in body volume between 24- and 48-hr samples, and a 1.3-fold difference between 24- and 72-hr samples. However, normalizing by these factors did not affect the significance of dasc#1, npar#1, or ascr#9 between time points (Tables S3 and S4; Figures S6B–S6D). We also suspect that size is not the only factor because no other compounds significantly increased throughout development in our linear model. Finally, we profiled the endo-metabolome of eggs and found appreciable amounts of ascr#1, #9, and #12 and pasc#9, but little to no traces of other ascaroside derivatives (Figure S5C), suggesting age-specific synthesis, rather than release from ascarosides already present in eggs/J1. Together, these results suggest that the observed increase in ascr#9, npar#1, and dasc#1 over time corresponds to age-specific production. The observation that dasc#1 is produced specifically during the juvenile-to-adult transition is especially intriguing because adults are no longer able to switch mouth forms, hinting at cross-generational signaling.

DISCUSSION

Here, we introduce a novel dye-based method that allowed us to assess cross-generational influence on mouth form. Our results demonstrate that adult crowding induces the Eu predatory morph, and that this effect is, at least partially, a result of age-specific pheromones. In doing so, we provide the first multi-stage time series of pheromone production in *P. pacificus*, which shows that dasc#1 exhibits a surprising switch-like induction pattern. Collectively, our results suggest that adults represent a "critical age group" with respect to phenotypic plasticity. The fact that adults also represent the critical age group with respect to population density (Charlesworth, 1972) may explain their outsized contribution to induction of the Eu morph. The presence of adults may indicate rapidly decreasing bacterial resources, and thus developing the Eu morph will allow worms to exploit additional resources and kill competitors.

Our developmental profiling revealed an increase in two NDMMs that affect plastic phenotypes. Given that J4s can produce dasc#1/npar#1, we believe the lack of effect of the J3/J4 stage compared with adults in our mixed-culture assay simply reflects the more consistently present and higher amounts of dasc#1/npar#1 produced at 72 hr and experienced for longer periods of time. The observation that this trend occurs regardless of body size implies that these molecules are programmed for stage-specific production. The "off-on" induction kinetics might reflect a population-level feedback loop, wherein the production of excess pheromones is based on a threshold level of previously produced pheromones. The variability observed at 48 hr for dasc#1/npar#1 might reflect biological variability in developmental timing and/or technical variation in staging. It is also worth noting that although npar#1 is the major dauer-inducing pheromone in *P. pacificus* (Bose et al., 2012), we did not observe dauer juveniles in any of our dye-crowding assays. Thus, it seems that mouth-form phenotype is the first-level plastic response to population density. Presumably higher concentrations are required for dauer induction, reflecting a calculated response strategy depending on the level of crowding or duration of starvation. Interestingly, the effect of 72-hr supernatants was noticeably less (23%–26% Eu) than the physical presence of adult worms (up to 48% with only 500 adults). It is difficult to compare pheromone concentrations between experiments, but presumably worms in the vital dye assay experienced a greater local concentration as they were in direct contact with each other for longer periods of time, and were also older than the 72-hr supernatant assayed in our pheromone profiling. However, it is also formally possible that other factors, like increased physical contact, can induce the Eu morph.

The maximum levels of Eu reached in our mixed culture experiment was ~50% with 500 adults, begging the question if this could be pushed further by using greater levels of crowding. However, this proved technically difficult due to food constraints with excess worms. Adding more food (OP50 LB) began to decrease the integrity of the agar, which made recovering animals for phenotyping difficult. Importantly, adults do not seem capable of eating other adults, which might otherwise push the Eu frequency even higher as a defense strategy. We also suspect that there are unknown trade-offs between the Eu and St mouth forms, which may manifest in a "ceiling" of the Eu frequency even under more crowded conditions.

Among the many environmental influences on mouth form (Werner et al., 2017), population density and starvation are perhaps the most ecologically relevant. However, teasing apart these two factors has

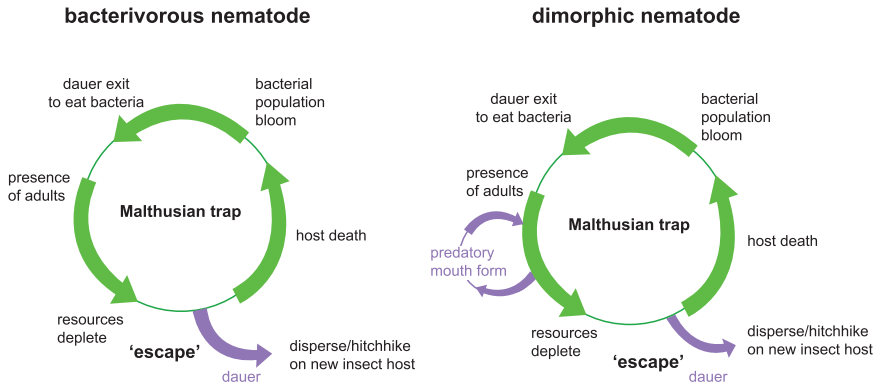

Figure 6. Conceptual Model of the Role of Critical Age Classes in Mouth-Form Phenotypic Plasticity

Conceptual life cycle models of monomorphic or dimorphic mouth-form nematodes. In an isolated niche such as a decaying insect carcass, at some point microbial food supplies will run out, leading to a Malthusian catastrophe. Nematodes escape this trap by entering the dauer state and dispersing, and re-starting the cycle. Dimorphic nematodes may sense the impending "catastrophe" earlier by recognizing an abundance of adults in the population, and switching to the Eu morph to exploit new resources and kill competitors. By analogy to economic models, the mouth-form switch is a technological innovation to temporarily escape a Malthusian resource trap.

been a challenge (Bento et al., 2010). Here, we demonstrate that whereas a strong shift is observed with age-specific pheromones, no such effect was seen under limited resource conditions. Thus, age-specific crowding is sufficient to induce the Eu mouth form. Nevertheless, this does not preclude that long-term starvation could also have an effect. Determining the relative contributions of these factors to mouth form will be important to better understand the sophisticated ecological response strategies of *P. pacificus*, nematodes, and phenotypic plasticity in general.

Why do adults and not juveniles affect mouth form? For now we can only speculate, but given that St animals develop slightly faster (Serobyan et al., 2013), there may be a "race" to sexual maturation in emergent populations at low densities. However, as the nematode population increases, there will likely be a commensurate decrease in bacterial populations. When faced with competition from other nematodes, *P. pacificus* has a particular advantage in developing the Eu morph; their expanded dietary range includes other nematode competitors. Indeed, when nematode prey is the only available food source, animals with the Eu morph have longer lifespans and more progeny than animals with the St morph (Serobyan et al., 2014). When resources become depleted as the population size increases, *C. elegans* and other monomorphic nematodes may enter dauer and disperse (Frézal and Félix, 2015). However, in St-biased dimorphic strains of *P. pacificus*, juveniles may switch to the Eu morph in response to adults as a first-level indication of rapidly increasing population size (Figure 6). Then, after prolonged starvation and crowding, worms will presumably enter dauer. By analogy to economic models of population growth (Malthus, 1826; Trewavas, 2002) mouth-form plasticity is a "technological innovation" to temporarily escape a Malthusian resource trap.

The evolution of dimorphic mouth forms is one among myriad nematode ecological strategies. For example, entomopathogenic nematodes release their symbiont bacteria in insect hosts to establish their preferred food source, and the bacteria can release antibiotics to kill off competing bacteria and fungi (Griffin, 2012). Some free-living species, like those of the genus *Oscheius*, may refrain from combat and stealthily feed and reproduce amid warring entomopathogenic species (Campos-Herrera, 2015a). Interspecific killing also occurs in gonochoristic species, in which both mated and virgin males are killed, implying fighting not just for mates but for resources as well (O'Callaghan et al., 2014; Zenner et al., 2014). Different reproductive strategies also exist, and hermaphroditic species have an advantage over gonochoristic species when colonizing a new niche, such as an insect carcass (Campos-Herrera, 2015b). Meanwhile, insect hosts and colonizing nematodes have their own distinct pheromone-based attraction and toxicity (Cinkornpumin et al., 2014; Renahan and Hong, 2017). Finally, the renaissance of *C. elegans* sampling from around the world (Cook et al., 2017; Evans et al., 2016; Félix et al., 2013; Petersen et al., 2014; Poullet and Braendle, 2015) is rapidly building a resource of wild isolates that will almost certainly have different and fascinating ecologies. We hope our method for labeling and then combining different

nematode populations on the same plate will aid in studies to identify these strategies. Perhaps the time is also ripe to complement these studies with more sophisticated ecological modeling that can lead to testable hypotheses.

Although beyond the scope of this manuscript, the cross-generational communication we observed could in principle reflect an intended signal from adults to juveniles, i.e., kin selection (Bourke, 2014). However, we favor a more simplistic view that juveniles have evolved to recognize late-stage metabolites. Regardless of these interpretations, our results argue that age classes are a critical factor in density-dependent plasticity, as has been theorized in density-dependent selection (Charlesworth, 1994).

Limitations of the Study

Given the ubiquity of certain traits in reproductive adults and their contribution to population growth, we suspect similar results will be found in other systems. However, it may depend on the phenotype and system being studied. For example, the population dynamics of this nematode (fast hermaphroditic reproduction) may be sufficiently different from other species such that our findings have limited generalizability. In addition, our method of staining different populations, although fast and easy, is particular to nematodes. Finally, to what extent our ecological interpretations exist in nature remains to be determined.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Transparent Methods, six figures, and four tables and can be found with this article online at https://doi.org/10.1016/j.isci.2018.11.027.

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AUTHOR CONTRIBUTIONS

M.S.W. and R.J.S. conceived of the project. M.H.C. conducted pheromone profiling with help from M.S.W. and T.R. M.S.W. and T.R. designed and conducted dye-labeling experiments. T.R. and M.H.C. performed supernatant experiments. M.D. and M.S.W. considered ecological implications. M.S.W. and T.R. wrote the manuscript with input and edits from all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES

Baskaran, P., Rödelsperger, C., Prabh, N., Serobyan, V., Markov, G.V., Hirsekorn, A., and Dieterich, C. (2015). Ancient gene duplications have shaped developmental stage-specific expression in *Pristionchus pacificus*. BMC Evol. Biol. *15*, 185.

Ben Hamouda, A., Tenaka, S., Ben Hamouda, M.H., and Bouain, A. (2011). Density-dependent phenotypic plasticity in body coloration and morphometry and its transgenerational changes in the migratory locust, Locusta migratoria. J. Entomol. Nematol. *3*, 105–116.

Bento, G., Ogawa, A., and Sommer, R.J. (2010). Co-option of the hormone-signalling module dafachronic acid–DAF-12 in nematode evolution. Nature *466*, 494–497.

Bose, N., Meyer, J.M., Yim, J.J., Mayer, M.G., Markov, G.V., Ogawa, A., Schroeder, F.C., and Sommer, R.J. (2014). Natural variation in dauer pheromone production and sensing supports intraspecific competition in nematodes. Curr. Biol. *24*, 1536–1541.

Bose, N., Ogawa, A., von Reuss, S.H., Yim, J.J., Ragsdale, E.J., Sommer, R.J., and Schroeder, F.C. (2012). Complex small-molecule architectures regulate phenotypic plasticity in a nematode. Angew. Chem. Int. Ed. *51*, 12438–12443.

Bourke, A.F.G. (2014). Hamilton's rule and the causes of social evolution. Philos. Trans. R. Soc. Lond. B Biol. Sci. *369*, 20130362.

Butcher, R.A. (2017). Small-molecule pheromones and hormones controlling nematode development. Nat. Chem. Biol. *13*, 577–586.

Butcher, R.A., Fujita, M., Schroeder, F.C., and Clardy, J. (2007). Small-molecule pheromones that control dauer development in *Caenorhabditis elegans*. Nat. Chem. Biol. *3*, 420–422.

Butcher, R.A., Ragains, J.R., Kim, E., and Clardy, J. (2008). A potent dauer pheromone component in *Caenorhabditis elegans* that acts synergistically with other components. Proc. Natl. Acad. Sci. U S A *105*, 14288–14292.

Campos-Herrera, R. (2015a). Traditional and molecular detection methods reveal intense interguild competition and other multitrophic interactions associated with native entomopathogenic nematodes in Swiss tillage soils. Plant Soil *389*, 237–255.

Campos-Herrera, R. (2015b). Nematode Pathogenesis of Insects and Other Pests (Springer).

Charlesworth, B. (1994). Evolution in Age-Structured Populations (Cambridge Univ. Press).

Charlesworth, B. (1972). Selection in populations with overlapping generations. III. Conditions for genetic equilibrium. Theor. Popul. Biol. *3*, 377–395.

Chasnov, J.R., So, W.K., Chan, C.M., and Chow, K.L. (2007). The species, sex, and stage specificity of a *Caenorhabditis* sex pheromone. Proc. Natl. Acad. Sci. U S A *104*, 6730–6735.

Chen, B., Li, S., Ren, Q., Tong, X., Zhang, X., and Kang, L. (2015). Paternal epigenetic effects of population density on locust phase-related characteristics associated with heat-shock protein expression. Mol. Ecol. *24*, 851–862.

Choe, A., von Reuss, S.H., Kogan, D., Gasser, R.B., Platzer, E.G., Schroeder, F.C., and Sternberg, P.W. (2012). Ascaroside signaling is widely conserved among nematodes. Curr. Biol. *22*, 772–780.

Cinkornpumin, J.K., Wisidagama, D.R., Rapoport, V., Go, J.L., Dieterich, C., Wang, X., Sommer, R.J., and Hong, R.L. (2014). A host beetle pheromone regulates development and behavior in the nematode *Pristionchus pacificus*. Elife *3*, e03229.

Cook, D.E., Zdraljevic, S., Roberts, J.P., and Andersen, E.C. (2017). CeNDR, the *Caenorhabditis elegans* natural diversity resource. Nucleic Acids Res. *45*, D650–D657.

Dantzer, B., Newman, A.E.M., Boonstra, R., Palme, R., Boutin, S., Humphries, M.M., and McAdam, A.G. (2013). Density triggers maternal hormones that increase adaptive offspring growth in a wild mammal. Science *340*, 1215–1217.

Diaz, S.A., Brunet, V., Lloyd-Jones, G.C., Spinner, W., Wharam, B., and Viney, M. (2014). Diverse and potentially manipulative signalling with ascarosides in the model nematode *C. elegans*. BMC Evol. Biol. *14*, 46.

Dong, C., Reilly, D.K., Bergame, C., Dolke, F., Srinivasan, J., and von Reuss, S.H. (2018). Comparative ascaroside profiling of *Caenorhabditis* exometabolomes reveals species-specific (ω) and (ω – 2)-hydroxylation downstream of peroxisomal β-oxidation. J. Org. Chem. *83*, 7109–7120.

Dudley, S.A., and Schmitt, J. (2015). Testing the adaptive plasticity hypothesis: density-dependent selection on manipulated stem length in impatiens capensis. Am. Nat. *147*, 445–465.

Evans, K.S., Zhao, Y., Brady, S.C., Long, L., McGrath, P.T., and Andersen, E.C. (2016). Correlations of genotype with climate parameters suggest *Caenorhabditis elegans* niche adaptations. G3 (Bethesda) *7*, 289–298.

Falcke, J.M., Bose, N., Artyukhin, A.B., Rödelsperger, C., Markov, G.V., Yim, J.J., Grimm, D., Claassen, M.H., Panda, O., Baccile, J.A., et al. (2018). Linking genomic and metabolomic natural variation uncovers nematode pheromone biosynthesis. Cell Chem. Biol. *25*, 787–796.e12.

Félix, M.-A., Jovelin, R., Ferrari, C., Han, S., Cho, Y.R., Andersen, E.C., Cutter, A.D., and Braendle, C. (2013). Species richness, distribution and genetic diversity of *Caenorhabditis* nematodes in a remote tropical rainforest. BMC Evol. Biol. *13*, 10.

Fielenbach, N., and Antebi, A. (2008). *C. elegans* dauer formation and the molecular basis of plasticity. Genes Dev. *22*, 2149–2165.

Frézal, L., and Félix, M.-A. (2015). The natural history of model organisms: *C. elegans* outside the Petri dish. Elife *4*, e05849.

Golden, J.W., and Riddle, D.L. (1982). A pheromone influences larval development in the nematode *Caenorhabditis elegans*. Science *218*, 578–580.

Golden, J.W., and Riddle, D.L. (1985). A gene affecting production of the *Caenorhabditis elegans* dauer-inducing pheromone. Mol. Gen. Genet. *198*, 534–536.

Greene, J.S., Brown, M., Dobosiewicz, M., Ishida, I.G., Macosko, E.Z., Zhang, X., Butcher, R.A., Cline, D.J., McGrath, P.T., and Bargmann, C.I. (2016). Balancing selection shapes density-dependent foraging behaviour. Nature *539*, 254–258.

Griffin, C.T. (2012). Perspectives on the behavior of entomopathogenic nematodes from dispersal to reproduction: traits contributing to nematode fitness and biocontrol efficacy. J. Nematol. *44*, 177–184.

Hastings, A. (2013). Population Biology: Concepts and Models (Springer).

Herrmann, M., Mayer, W.E., and Sommer, R.J. (2006). Nematodes of the genus *Pristionchus* are closely associated with scarab beetles and the Colorado potato beetle in Western Europe. Zoology *109*, 96–108.

Herrmann, M., Mayer, W.E., Hong, R.L., Kienle, S., Minasaki, R., and Sommer, R.J. (2007). The nematode *Pristionchus pacificus* (Nematoda: Diplogastridae) is associated with the oriental beetle exomala orientalis (Coleoptera: Scarabaeidae) in Japan. Zoolog. Sci. *24*, 883–889.

Izrayelit, Y., Srinivasan, J., Campbell, S.L., Jo, Y., Reuss, von, S.H., Genoff, M.C., Sternberg, P.W., and Schroeder, F.C. (2012). Targeted metabolomics reveals a male pheromone and sex-specific ascaroside biosynthesis in *Caenorhabditis elegans*. ACS Chem. Biol. *7*, 1321–1325.

Jeong, P.-Y., Jung, M., Yim, Y.-H., Kim, H., Park, M., Hong, E., Lee, W., Kim, Y.H., Kim, K., and Paik, Y.-K. (2005). Chemical structure and biological activity of the *Caenorhabditis elegans* dauer-inducing pheromone. Nature *433*, 541–545.

Kaplan, F., Srinivasan, J., Mahanti, P., Ajredini, R., Durak, O., Nimalendran, R., Sternberg, P.W., Teal, P.E.A., Schroeder, F.C., Edison, A.S., et al. (2011). Ascaroside expression in *Caenorhabditis elegans* is strongly dependent on diet and developmental stage. PLoS One *6*, e17804.

Ludewig, A.H., Gimond, C., Judkins, J.C., Thornton, S., Pulido, D.C., Micikas, R.J., Döring, F., Antebi, A., Braendle, C., and Schroeder, F.C. (2017). Larval crowding accelerates *C. elegans* development and reduces lifespan. PLoS Genet. *13*, e1006717.

MacArthur, R.H. (1962). Some generalized theorems of natural selection. Proc. Natl. Acad. Sci. U S A *48*, 1893–1897.

Maeno, K., and Tanaka, S. (2008). Maternal effects on progeny size, number and body color in the desert locust, *Schistocerca gregaria*: density- and reproductive cycle-dependent variation. J. Insect Physiol. *54*, 1072–1080.

Malthus, T.R. (1826). An Essay on the Principle of Population (Cambridge University Press).

Markov, G.V., Meyer, J.M., Panda, O., Artyukhin, A.B., Claaßen, M., Witte, H., Schroeder, F.C., and Sommer, R.J. (2016). Functional conservation and divergence of daf-22 paralogs in *Pristionchus pacificus* Dauer development. Mol. Biol. Evol. *33*, 2506–2514.

Meyer, J.M., Baskaran, P., Quast, C., Susoy, V., Rödelsperger, C., Glöckner, F.O., and Sommer, R.J. (2017). Succession and dynamics of *Pristionchus* nematodes and their microbiome during decomposition of *Oryctes borbonicus* on La Réunion Island. Environ. Microbiol. *19*, 1476–1489.

Moore, B.T., Jordan, J.M., and Baugh, L.R. (2013). WormSizer: high-throughput analysis of nematode size and shape. PLoS One *8*, e57142.

O'Callaghan, K.M., Zenner, A.N.R.L., Hartley, C.J., and Griffin, C.T. (2014). Interference competition in entomopathogenic nematodes: male *Steinernema* kill members of their own and other species. Int. J. Parasitol. *44*, 1009–1017.

Pener, M.P., and Simpson, S.J. (2009). Locust phase polyphenism: an update. Adv. Insect Physiol. *36*, 196–201.

Petersen, C., Dirksen, P., Prahl, S., Strathmann, E.A., and Schulenburg, H. (2014). The prevalence of *Caenorhabditis elegans* across 1.5 years in

selected North German locations: the importance of substrate type, abiotic parameters, and *Caenorhabditis* competitors. BMC Ecol. *14*, 4.

Poullet, N., and Braendle, C. (2015). Sampling and isolation of *C. elegans* from the natural habitat. Methods Mol. Biol. *1327*, 221–229.

Renahan, T., and Hong, R.L. (2017). A species-specific nematocide that results in terminal embryogenesis. J. Exp. Biol. *220*, 3238–3247.

Sanghvi, G.V., Baskaran, P., Röseler, W., Sieriebriennikov, B., Rödelsperger, C., and Sommer, R.J. (2016). Life history responses and gene expression profiles of the nematode *Pristionchus pacificus* cultured on Cryptococcus yeasts. PLoS One *11*, e0164881.

Serobyan, V., Ragsdale, E.J., and Sommer, R.J. (2014). Adaptive value of a predatory mouth-form in a dimorphic nematode. Proc. R. Soc. Lond. B Biol. Sci. *281*, 20141334–20141989.

Serobyan, V., Ragsdale, E.J., Müller, M.R., and Sommer, R.J. (2013). Feeding plasticity in the nematode *Pristionchus pacificusis* influenced by sex and social context and is linked to developmental speed. Evol. Dev. *15*, 161–170.

Simpson, S.J., Despland, E., Hägele, B.F., and Dodgson, T. (2001). Gregarious behavior in desert locusts is evoked by touching their back legs. Proc. Natl. Acad. Sci. U S A *98*, 3895–3897.

Simpson, S.J., and Miller, G.A. (2007). Maternal effects on phase characteristics in the desert locust, *Schistocerca gregaria*: a review of current understanding. J. Insect Physiol. *53*, 869–876.

Sloggett, J.J., and Weisser, W.W. (2002). Parasitoids induce production of the dispersal morph of the pea aphid, *Acyrthosiphon pisum*. Oikos *98* (2), 323–333.

Sommer, R.J., and Mayer, M.G. (2015). Toward a synthesis of developmental biology with evolutionary theory and ecology. Annu. Rev. Cell Dev. Biol. *31*, 453–471.

Sommer, R.J., and McGaughran, A. (2013). The nematode *Pristionchus pacificus* as a model system for integrative studies in evolutionary biology. Mol. Ecol. *22*, 2380–2393.

Sommer, R.J., Dardiry, M., Lenuzzi, M., Namdeo, S., Renahan, T., Sieriebriennikov, B., and Werner, M.S. (2017). The genetics of phenotypic plasticity in nematode feeding structures. Open Biol. *7*, https://doi.org/10.1098/rsob.160332.

Srinivasan, J., von Reuss, S.H., Bose, N., Zaslaver, A., Mahanti, P., Ho, M.C., O'Doherty, O.G., Edison, A.S., Sternberg, P.W., and Schroeder, F.C. (2012). A modular library of small molecule signals regulates social behaviors in *Caenorhabditis elegans*. PLoS Biol. *10*, e1001237.

Sutherland, O.R.W. (1969). The role of crowding in the production of winged forms by two strains of the pea aphid, *Acyrthosiphon pisum*. J. Insect Physiol. *15*, 1385–1410.

Thomas, M.C., and Lana, P.D.C. (2008). Evaluation of vital stains for free-living marine nematodes. Braz. J. Oceanogr. *56*, 249–251.

Travis, J., Leips, J., and Rodd, F.H. (2013). Evolution in population parameters: density-dependent selection or density-dependent fitness? Am. Nat. *181*, S9–S20.

Trewavas, A. (2002). Malthus foiled again and again. Nature *418*, 668–670.

von Reuss, S.H., Bose, N., Srinivasan, J., Yim, J.J., Judkins, J.C., Sternberg, P.W., and Schroeder, F.C. (2012). Comparative metabolomics reveals biogenesis of ascarosides, a modular library of small-molecule signals in *C. elegans*. J. Am. Chem. Soc. *134*, 1817–1824.

Werner, M.S., Sieriebriennikov, B., Loschko, T., Namdeo, S., Lenuzzi, M., Dardiry, M., Renahan, T., Sharma, D.R., and Sommer, R.J. (2017). Environmental influence on *Pristionchus pacificus* mouth form through different culture methods. Sci. Rep. *7*, 7207.

Wilecki, M., Lightfoot, J.W., Susoy, V., and Sommer, R.J. (2015). Predatory feeding behaviour in *Pristionchus* nematodes is dependent on phenotypic plasticity and induced by serotonin. J. Exp. Biol. *218*, 1306–1313.

Zenner, A.N.R.L., O'Callaghan, K.M., and Griffin, C.T. (2014). Lethal fighting in nematodes is dependent on developmental pathway: male-male fighting in the entomopathogenic nematode *Steinernema longicaudum*. PLoS One *9*, e89385.

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Supplemental Information

Adult Influence on Juvenile Phenotypes by Stage-Specific Pheromone Production

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Supplemental Information

Transparent Methods

Nematode strains and husbandry

P. pacificus Wild-type RS2333 (California) and RSC017 (La Réunion) strains were kept on 6 cm nematode growth media (NGM) plates seeded with OP50 and kept at 20°C. RSC017 is highly St and does not predate on other nematodes, and thus was used for biological assays instead of the highly Eu, predatory RS2333. To induce dauer, mixed-stage plates with little to no OP50 were washed with M9 and the resulting worm pellets were used in a modified 'White Trap' method. Worm pellets were placed on killed *Tenebrio molitor* grubs and dispersing dauers were collected in surrounding MilliQ water. Age of dauers ranged from one week to one month.

Dye staining

A stock solution of Neutral Red was prepared by dissolving 0.5 mg in 10 ml 5% acetic acid and stored at -20°C. Working solutions were prepared by 100x dilution in M9, aliquoted, stored at -20°C, and thawed directly before use. Working solutions were kept for approximately 1 month. Stock solutions of 10 mM CellTracker Green BODIPY were made in DMSO and stored at -20°C. J2s were prepared from 20-40 x 6 cm plates 6 days after passaging 5 worms to each plate on 300 µl OP50. Worms were washed from plates with M9 into a conical tube, and then filtered through 2 x 20 µM filters (Millipore) placed between rubber gaskets. The flow-through contained mostly J2 and some J3, which were pelleted by centrifugation, 8 seconds on a table-top eppendorf centrifuge 5424, reaching approximately 10,000 x g. The older/larger adult worms

remained on the filters, and were washed into a 50 ml conical tube with ~2 ml M9. Adults were then isolated by transferring worms to a 15 ml conical, and allowing them to swim/sink to the bottom of the tube. Adults reach the bottom faster than younger stages do, and after 3-5 rounds of removing supernatant and re-suspending in 2-3 ml M9, the pellet contains almost exclusively adults, which were re-suspended in 1 ml M9/50 µM Green BODIPY (Thermo Fisher). The J2 pellet was either directly re-suspended in 1 ml Neutral Red working solution, or in 1 ml M9 and split to two tubes, re-centrifuged, and re-suspended in 1 ml working solution Neutral Red (0.005% in M9) or 1 ml M9/50 µM Green BODIPY (Thermo Fisher). For the intermediate time point juveniles (J3s and some J4s), J2s isolated from 20 µM filtering were placed back on agar plates containing 300 µl OP50 bacterial food and grown for another 24 hours, and then washed from plates in M9 and re-filtered through 5 µM filters, then re-suspended in 1 ml 50 µM Green BODIPY (Thermo Fisher). Each tube was rotated for 3 hours in the dark at 20°C, then washed by centrifugation as before, and re-suspended in 1 ml M9. This was repeated 3-4x until the dye was no longer visible in the worm pellet. Then, the concentration of worms per microliter was determined by aliquoting 2 µl onto a glass coverslip in 5 technical replicates, and counted under a dissecting microscope. Finally the appropriate number of animals was added to 6 cm plates that had been previously seeded with 300 µl OP50, and incubated at 20°C. After 3 days, 100% of worms exhibited Neutral Red staining (*n*=50, Supplementary Figure 3). Dauers and J2s recovered after Neutral Red staining developed at the same developmental speed (3-4 days) and with the same mouth-form ratio as control worms recovered side-by-side (100% St for both, Supplementary Figure 4, *n*=30). Dauers and J2s stained with CellTracker Green BODIPY (50 µM) (Thermo) were similar, although less efficiently stained compared to Neutral Red. On day 4, 90% retained intestinal fluorescence (Supplementary Figure 3), and brightness decreased with the number of days. J2s in +/- 50 µM CellTracker Green BODIPY also developed at equivalent rates and mouth-form ratios (Supplementary Figure 4). Lower than 25 µM did not yield strongly

fluorescent worms after three hours. CellTracker Blue CMAC (Thermo Fisher) was also used at 50 µM and imaged 3 days post-staining for *P. pacificus*, and one day post-staining for *C. elegans*. However, due to the higher fluorescent background in the blue light spectrum in both *P. pacificus* and *C. elegans*, we performed all experiments using only Neutral Red and CellTracker Green BODIPY.

Microscopy

All images were taken on a Zeiss Axio Imager 2 with an Axiocam 506 mono, and processed using Zen2 pro software. Image brightness and contrast were enhanced in ImageJ with a minimum displayed value of 10 and maximum of 100 for all images in Figure 2, and Supplementary Figures 1 and 2, and a minimum of 21 and maximum of 117 for Supplementary Figure 3. The following exposure times were used for all images: Cy3 (peak emission = 561, exposure = 80 ms), FITC (peak emission = 519, exposure = 150 ms), Dapi (peak emission = 465, exposure = 80 ms), DIC (exposure = 80-140 ms).

Mixed culture experiments and statistical analysis

We performed the mixed culture experiments presented in Figure 3 with a minimum total number of counts $n > 100$, from three to five independent biological replicates for J2/24 hr, dauer, and adult competitor experiments, and two for the intermediate (J3/4) juvenile experiment (median counts per replicate for J2/24 hr=29, dauers=27, and adults=21, and avg. J3/4 counts was 75). J2 or dauers were stained with Neutral Red, then added to green-stained J2, dauer, J3/4, or adult populations as described in the 'Dye Staining' method section, on 6 cm plates with 300 µl OP50 and incubated at 20°C. To ensure consistent bacterial food supply, we added 1 ml more overnight OP50-LB to each plate on the following day, then air-dried under a chemical fume hood for 1 hour, then returned the plates to 20°C. On days three to four, we

phenotyped 'red' adults that exhibited no 'green' staining. To assess whether the age of the 'green' surrounding population affects the mouth form of the dependent variable 'red' J2s we performed a binomial regression on Eu counts (i.e. "successes") weighted by the number of counts per replicate, and the stage (juveniles vs. adults) and number added as a fixed effects, using a generalized linear model from the standard statistical package in R:

glm(formula=cbind(Eu,total)~'stage_added' * '#_added', data='J2/Da', family="binomial"))

See Supplementary Table S1 for a table containing the resulting *p* values. The AIC for our models (85.52 for juveniles and 72.32 for dauers) was substantially lower than the null hypothesis (220.16 for J2s and 147.29 for dauers), arguing a reasonable fit. For pair-wise comparisons of the effect of age for a given number of added animals, we performed a post-hoc Fisher's exact test on a contingency table containing the summed counts (n>100) of Eu and St observations against control plates (no added crowding animals). For display, we converted Eu counts into percent of total in Figure 3, with the *p* values for the number of animals added indicated over the relevant column (Significance codes: 0 '***' 0.001, '**' 0.01, '*' 0.05).

Measuring the effect of food depletion on mouth form

To verify that starvation was not a factor in our mixed culture experiments, we added increasing number of J2s to standard 6 cm plates with 300 µl OP50 to rapidly consume bacterial food, and measured both the amount of animals that reached adulthood, and the percent Eu in each population for two biological replicates. To assess the affects of added J2s to each dependent variable we performed a binomial regression with count data weighted by the total number of counts for each replicate:

glm(formula = cbind(reached_adult, total)~thousand_J2s, data=data_2, family="binomial"))

p values indicate a significant difference in percent reaching adult as a function of J2s added, but not in percent Eu (Table S1 bottom frame).

Supernatant collection and assays

Strains RS2333, RSC017, and RS2333-*daf-22.1;22.2* were raised in 10 ml liquid culture as in the time-resolved NDMM collections (see below). For each time point, 9 ml of the supernatant was lyophilized overnight, extracted again overnight with 90% ethanol (diluted in Millipore water) while being stirred, and centrifuged (4000 x g, 10 min, 4°C). The solvent was evaporated and the solid re-dissolved with 1 ml Millipore water. This clear extract was then directly used for the assays. One ml of the supernatant was cleaned for HPLC-MS analysis for quality control, as described in HPLC-MS sample preparation below. For the assays, RSC017 was synchronized by bleaching (Werner et al., 2017) and added to plates seeded with 300 µl OP50. The supernatants were added to the RSC017 J2s in two 500 µl increments (for a total of 1 ml supernatant) and dried for 30 minutes in a sterile hood after each addition. Plates were kept at 20°C and adult mouth forms were screened three days later. To determine significance a Fisher Exact test was performed on summed count data relative to S-medium control contingency tables, and the data are presented for representation as percentages in Figure 4.

HPLC-MS sample preparation for exo-metabolome and time resolved analysis

To collect staged pheromone profiles, we seeded 35 x 6 cm plates with 5 worms each, and bleached 5-6 days later when gravid to collect eggs/J1s. These were then added to 6 x 10 ml flasks with OP50 as described in Werner et al., 2017 (Werner et al., 2017). Then at 24, 48, or 72 hr time intervals, supernatants were obtained by centrifugation (>4,000 x g, 4°C for 10 minutes). 1 ml supernatant was adsorbed onto a SPE-C8 cartridge (Thermo Scientific Hypersep C8 100 mg/1ml), conditioned with 1 ml MeOH followed by 2 ml Millipore water. The adsorbed material was then washed with 200 µl water and subsequently eluted with 200 µl MeOH. This extract was then measured directly via HPLC-qTof MS (Bruker ImpactII).

125

126 **HPLC-MS measurement**

127 20 µl extract was injected into a Thermo UltiMate 3000 HPLC equipped with a Sigma-Aldrich
128 Ascentis Express C18 2.7 µm 10 mm x 4.6 mm column at 20°C with a flow of 500 µl/min. All MS
129 measurements have been performed in negative ion mode and molecules are detected as [M-
130 $H]^-$ Ions. The solvent gradient started with 5% acetonitrile (ACN)/ 95% water (both containing
131 0.1% formic acid) for 2 minutes. After this equilibration step, the ACN proportion was increased
132 to 65% over 8 min, then to 100% ACN in 1.2 minutes followed by a hold step for 8.8 minutes.
133 Afterwards, the system was flushed to 5% ACN with 2 minutes equilibration for a total of 22
134 minutes. For calibration, a sodium formate cluster building solution was automatically injected in
135 the first 2 minutes of each run. Data analysis was performed with TASQ version 1.0 from Bruker
136 Daltonics. Extracted ion chromatograms for each well-known compound with a mass width of
137 0.1 m/z and time slices of 0.5 minutes around the expected retention time were produced after
138 calibrating and baseline correction. Assignment errors were corrected with the provided MRSQ
139 value, and areas under the curve were calculated from the integral of each peak.

140

141 **Statistical analysis of NDMMs**

142 NDMM levels were compared simultaneously against strains and developmental stages by a
143 linear model in R: lm('NDMM' ~ 'developmental stage' * 'strain', data='data.frame')). In essence,
144 the linear model regressed the abundance of NDMMs against stage and strain as fixed effects.
145 *P* values between stages and strains were adjusted for multiple testing by a false discovery rate
146 correction (FDR). The level of fit between linear vs. exponential growth was determined by the
147 Akaike information criterion (AIC). The lowest AIC for iterations of different exponents
148 (n=1,2,3…) was used for comparison to the simple linear model. While significant in both cases,
149 for consistency we present the original *p* values from the original linear model in Table S2.

Supplemental Figure Legends

Figure S1, related to Figure 2. Vital dye staining of *Pristionchus pacificus*.

(A) Control *P. pacificus* imaged with Cy3, FITC, and DAPI filters, and a merge with Differential Interference Contrast (DIC). Histogram on the right represents quantification of intensity with each filter. (B) Same as (A) but stained with 0.005% Neutral Red, (C), 50 µM CellTracker Green BODIPY (Thermo Fisher), or (D) 50 µM CellTracker Blue CMAC Dye (Thermo Fisher). J2s were stained (see Transparent Methods), and ensuing adult animals were imaged 3 days later on a Zeiss Axio Imager 2 with an Axiocam 506 mono, and processed using Zen2 pro software. Image brightness and contrast were enhanced in ImageJ for display, with a minimum displayed value of 10 and maximum of 100 for all images. Note that while Neutral Red and CellTracker Green staining are bright and specific to their respective channels, CellTracker Blue is indistinguishable from background fluorescence.

Figure S2, related to Figure 2. Vital dye staining of *Caenorhabditis elegans.*

(A-D) Same as Supplementary Figure 1, but with *C. elegans*.

Figure S3, related to Figure 2. Vital dye staining of *P. pacificus* dauers, and duration of staining. (A) Control *P. pacificus* dauer imaged with DIC, Cy3, and FITC filters. (B) Dauers stained with either 0.005% Neutral Red or 50 µM CellTracker Green BODIPY and imaged immediately after staining with DIC, Cy3, and FITC filters and merged with DIC. Images were taken using Zeiss Axio Imager 2 with an Axiocam 506 mono, processed using Zen2pro software, and adjusted in ImageJ, with a display value minimum of 21 and maximum of 117.

(C-G) 50 µM CellTracker Green BODIPY and 0.005% Neutral Red-stained J2s were imaged every day for five days. Percent of individuals retaining the dyes are shown in panels next to each microscope image for each day. Both stains are seen in all organisms for three days; Neutral Red (NR) persists for at least five, while the number of Green BODIPY (GB) –stained worms drops on day four. All images are merged with DIC, n=31 GB, 63 NR day 1, 68 GB, 56 NR day 2, 50 GB, 50 NR day 3, 50 GB, 50 NR day 4, 50 GB, 50 NR day 5.

Figure S4, related to Figure 2. Vital dye staining does not affect *P. pacificus* mouth form or development.

(A) Neutral Red and CellTracker Green BODIPY-stained J2s reach adulthood at the same rate as unstained J2s (3 days). (B) All of the J2s stained retain the dye in adulthood in the intestine. (C) Neither dye affects mouth form; both unstained and stained worms remain 100% St (n=30). (D-F) Same as for (A-C) except with dauers instead of J2s, and only with Neutral Red. (G) Developmental rate of J2 unstained, Neutral Red-stained (NR), and CellTracker Green BODIPY-stained (GB) RSC017 every 12 hours post-J2 staining. Two biological replicates, n=60. To see if there were significant differences between stained and un-stained, a Fisher's Exact test was performed on summed counts of each stage (all $p>0.05$) (H) Staging of RSC017 worms from liquid culture at the relevant time points, 24 hrs, 48 hrs, and 72 hrs. Error bars represent standard error of the mean for 3 biological replicates, n>100 animals counted per replicate.

Table S1, related to Figure 3. Table of binomial regression *p* values for crowding assays.

Significance *p* values from binomial regression of vital-dye method for age and number added, and from binomial regression of number-reaching-adult and Eu counts, for each number of individuals added relative to 1,000 individuals added (see Transparent Methods for details).

Figure S5, related to Figure 5. Pheromone profiling quality control.

(A) Extracted ion traces (width 0.1 m/z) of 11 of the 12 NDMMs used in this publication from a seven-day mixed-stage sample, double peak of 247.12 m/z indicate isomeric structures (Part#9/Ascr#9). (B) Example of an averaged spectrum over a calibration segment; sodium-formate cluster building solution was used to ensure high mass accuracy in each run. (C) Comparison of an endometabolome sample from a seven- day mixed-stage cultured compared to the endometabolome of eggs, produced by using bleached eggs from 80 x 60 mm plates.

Table S2, related to Figure 5. Table of linear regression *p* values with FDR corrections for strain and stage comparison of NDMM levels. FDR-corrected and uncorrected *p* values from linear regression of *P. pacificus* NDMMs (alternating grey background between NDMMs for clarity). Red values indicate FDR<0.05.

Table S3, related to Figure 5. P values from pairwise comparison of dasc#1, npar#1, and ascr#9 throughout development. Significance assessed with a two-tailed student's *t*-test. Top table indicates comparison of raw pheromone levels experienced by worms, and the bottom table indicates comparison of volume-normalized pheromone levels (normalized data from WormSizer (Moore et al., 2013), Fig. S6B-D).

Figure S6, related to Figure 5. Enzyme that synthesizes NDMMs is transcriptionally regulated during development, and volume normalization of pheromones. (A) Comparison of *daf-22.1* (FPKM) by RNA-seq through different stages of development, data from Baskaran et al., 2015 (Baskaran et al., 2015). A two-sided students *t*-test was performed between 56-68

hours (J4-adults) and 22 hours (J2s) (Significance codes: 0 '***' 0.001, '**' 0.01, '*' 0.05). (B) Representative images of worms raised in liquid culture at 24 hrs, 48 hrs, and 72 hrs. (C) Comparison of worm volumes (picoLiters) for 24 hrs, 48hrs, and 72 hrs, using WormSizer (Moore et al., 2013). (D) Time-resolved NDMM levels of RSC017 normalized by worm volume (upper graph) and unnormalized (lower graph, also shown in Figure 5B). Data is presented as the mean of nine biological replicates and error bars represent standard error of the mean (SEM). In the upper graph, levels were normalized to worm volume based on the data shown in (C).

Table S4, related to Figure 5. Raw and volume-normalized data of RSC017 pheromones, in absolute value of area under the curve. Normalization of 48 hr and 72 hr time point abundances relative to 24 hrs. Average volumes obtained by WormSizer (Moore et al., 2013)(Figure S6B-C).

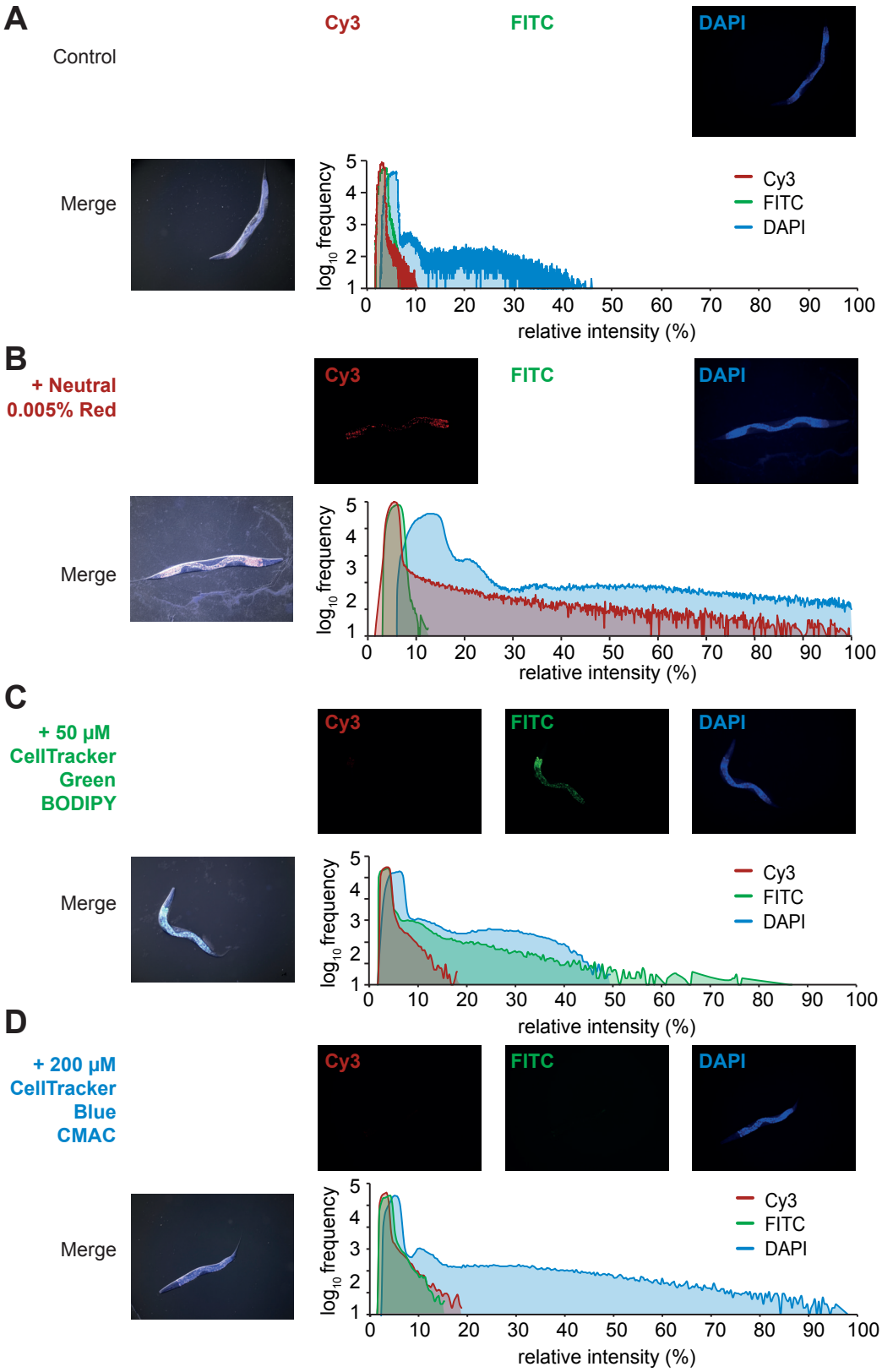

Figure S1, related to Figure 2. Vital dye staining of *Pristionchus pacificus*.

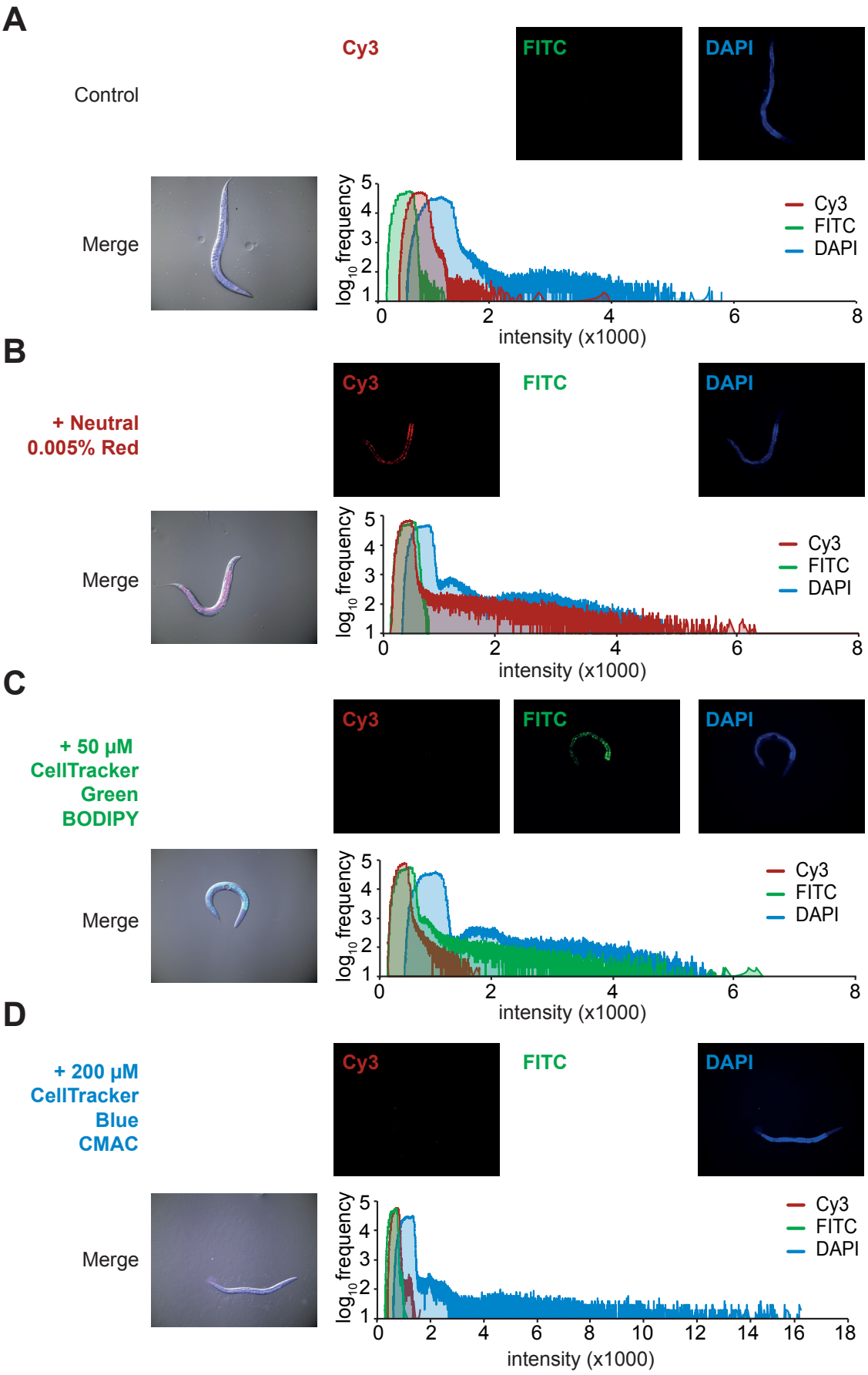

Figure S2, related to Figure 2. Vital dye staining of *Caenorhabditis elegans*.

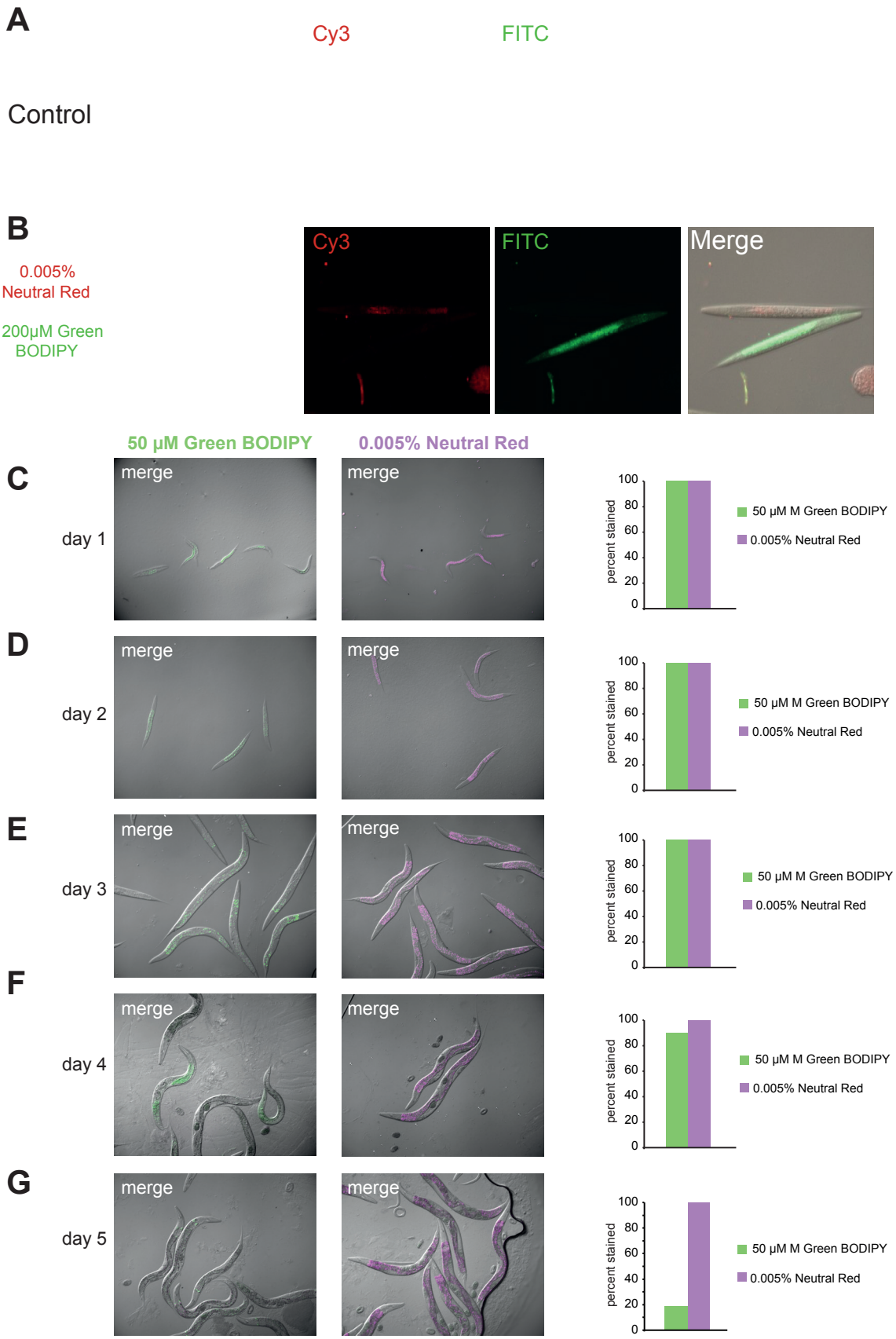

Figure S3, related to Figure 2. Vital dye staining of *Pristionchus pacificus* dauers, and duration of staining.

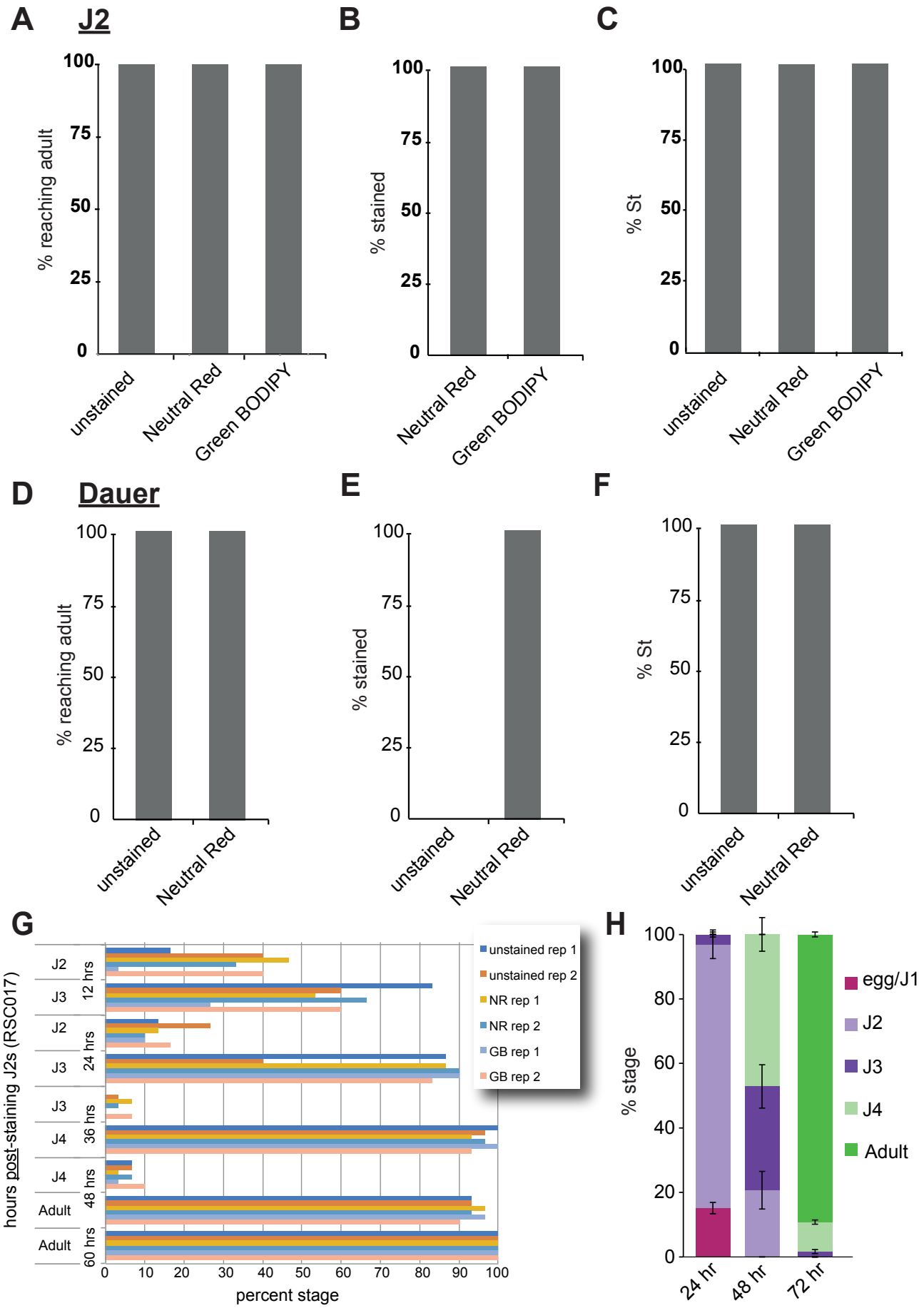

Figure S4, related to Figure 2. Vital dye staining does not affect P. pacificus mouth form or development.

effect of population age on mouth form of developing juveniles

binomial regression	*p* value red-stained J2s	*p* value red-stain dauers
stage added (adults vs. juveniles)	0.0132	0.002955
number added	4.28e-13	0.000404

effect of number of peers on development and mouth form (proxy for potential starvation effects on mouth form)

binomial regression	*p* value for development (relative to 1,000)	*p* value for Eu (relative to 1,000)
3,000 J2s added	0.3408	1.0
4,000 J2s added	0.0424	1.0
5,000 J2s added	6.06E-14	0.99
10,000 J2s added	4.09E-14	0.99

Table S1, related to Figure 3. Table of binomial regression p values for vital-dye method and excess crowding.

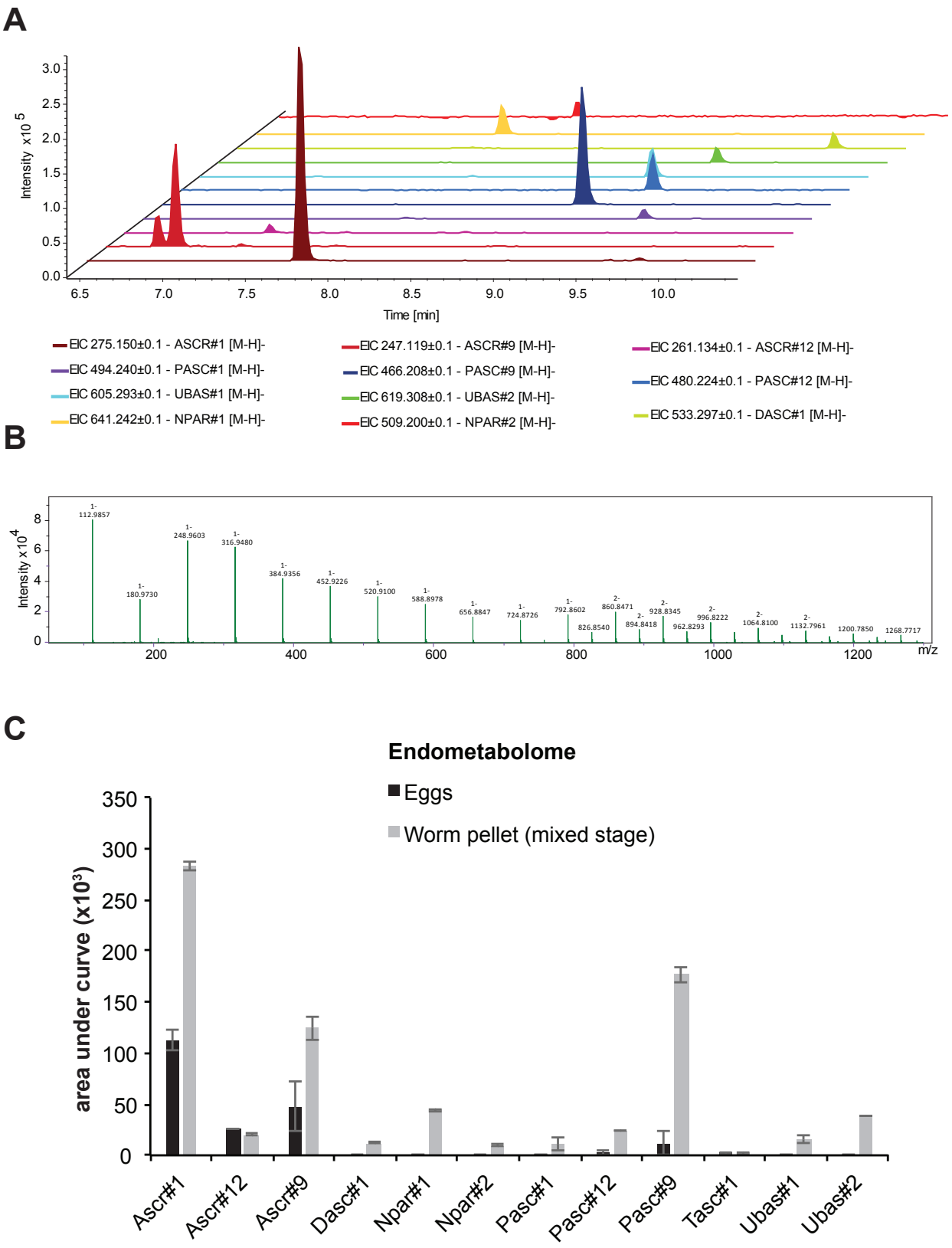

Figure S5, related to Figure 5. Pheromone profiling quality control

NDMM comparison	pvalue	fdr corrected
ascr1_stage	0.4733	0.774490909
ascr1_strain	0.0429	0.110314286
ascr1_stage:strain	0.031	0.085846154
ascr9_stage	3.79E-05	0.0002274
ascr9_strain	0.651	0.778064516
ascr9_stage:strain	0.272	0.50148
ascr12_stage	0.0029	0.01404
ascr12_strain	0.0897	0.201825
ascr12_stage:strain	0.0302	0.085846154
dasc1_stage	9.62E-08	8.66E-07
dasc1_strain	0.11363	0.240628235
dasc1_stage:strain	0.00351	0.01404
npar1_stage	0.0033	0.01404
npar1_strain	0.9426	0.984
npar1_stage:strain	0.6355	0.778064516
npar2_stage	0.0516	0.12384
npar_2strain	0.984	0.984
npar2_stage:strain	0.9716	0.984
pasc1_stage	0.449	0.769714286
pasc1_strain	0.753	0.847125
pasc1_stage:strain	0.564	0.778064516
pasc9_stage	0.616	0.778064516
pasc9_strain	0.267	0.50148
pasc9_stage:strain	0.523	0.778064516
pasc12_stage	0.6122	0.778064516
pasc12_strain	0.2786	0.50148
pasc12_stage:strain	0.67	0.778064516
tasc1_stage	0.522	0.778064516
tasc1_strain	0.862	0.940363636
tasc1_stage:strain	0.57	0.778064516
ubas1_stage	3.13E-12	1.13E-10
ubas1_strain	0.00538	0.019368
ubas1_stage:strain	6.69E-08	8.03E-07
ubas2_stage	1.34E-11	2.41E-10
ubas2_strain	0.00711	0.023269091
ubas2_stage:strain	6.18E-07	4.45E-06

Table S2, related to Figure 5. Table of linear regression *p* values with *FDR* correction for strain and stage comparison of NDMM levels.

RS2333	dasc#1	npar#1	ascr#9
72 hrs compared to 24 hrs	5.75E-07	3.47E-05	1.03E-04
72 hrs compared to 48 hrs	5.71E-03	1.76E-01	1.97E-01
RSC017	dasc#1	npar#1	ascr#9
72 hrs compared to 24 hrs	2.55E-02	3.66E-03	2.03E-02
72 hrs compared to 48 hrs	2.12E-01	3.66E-01	1.04E-01

Volume normalized

RS2333	dasc#1	npar#1	ascr#9
72 hrs compared to 24 hrs	5.75E-07	3.47E-05	1.02E-03
72 hrs compared to 48 hrs	1.44E-02	2.92E-01	6.21E-01
RSC017	dasc#1	npar#1	ascr#9
72 hrs compared to 24 hrs	2.55E-02	3.66E-03	4.34E-02
72 hrs compared to 48 hrs	2.71E-01	5.46E-01	1.70E-01

Table S3, related to Figure 5. P values from pairwise comparison of dasc#1, npar#1, and ascr#9 throughout development.

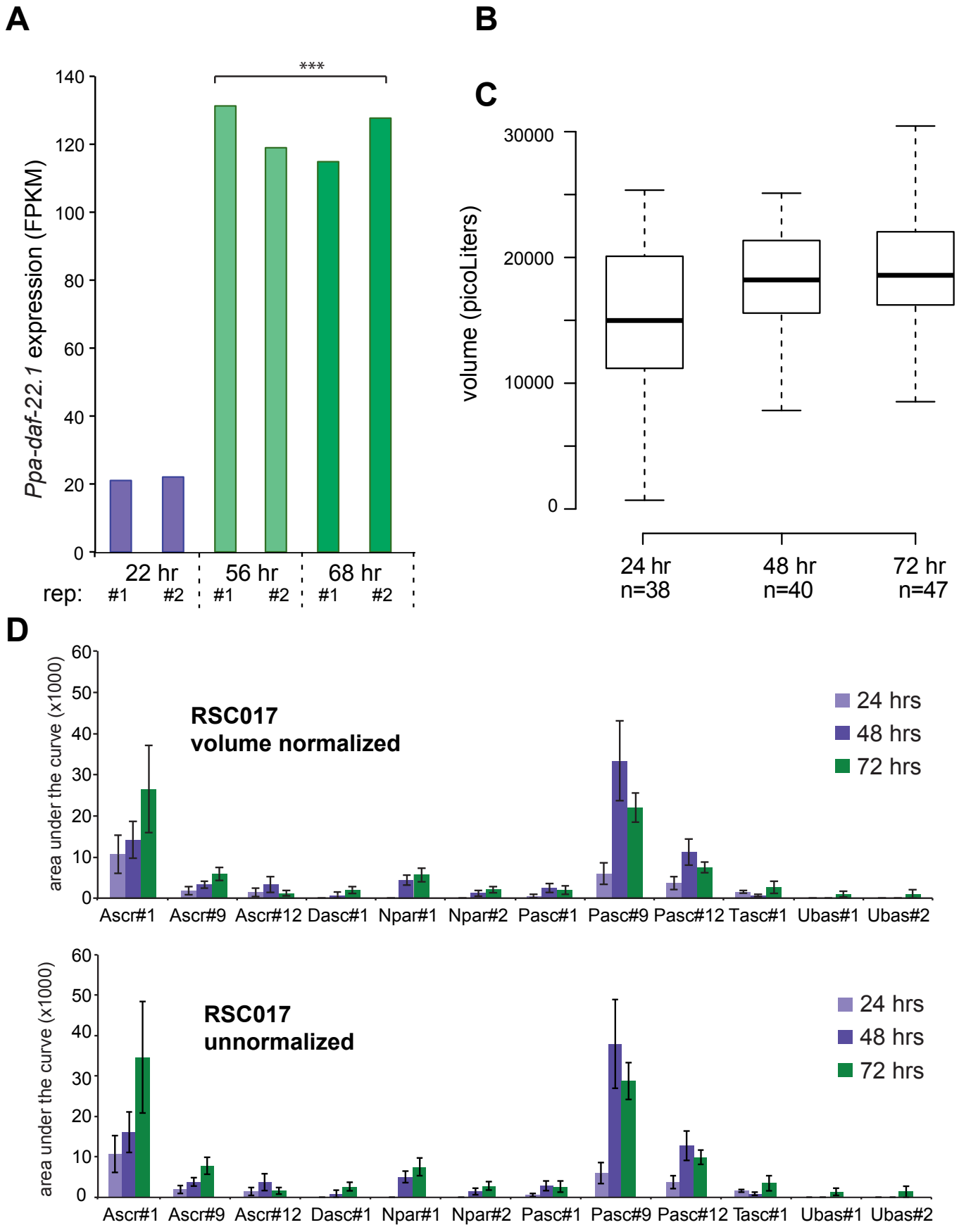

Figure S6, related to Figure 5. Enzyme that synthesize NDMMs is transcriptionally regulated during development, and volume normalization of pheromones.

STAGE	DASC1	NPAR1	Pasc9	Ascr1	Ascr12	Ascr9	Npar2	Pasc1	Pasc12	Tasc1	Ubas1	Ubas2
24	0	0	0	0	0	0	0	0	0	1610	0	0
24	0	0	0	4489	0	0	0	0	0	1214	0	0
24	0	0	0	0	0	0	0	0	0	1769	0	0
24	0	0	0	22265	0	4301	0	0	0	1169.5	0	0
24	0	0	0	28319.5	0	5450	0	0	0	1871.5	0	0
24	0	0	7193.5	35197.5	8299.5	7177	0	0	9476	3918	0	0
24	0	0	16048.5	3929.5	5028.5	0	0	0	8318	969.5	0	0
24	0	0	19293.5	2386.5	0	0	0	1657.5	11094	999	0	0
24	0	0	11623.5	0	0	0	0	3667.5	4799.5	949.5	0	0
48	7800	8901	111298	7866	0	4250	6050	8486	35583.5	2827	0	0
48	0	8393	54479	7660	0	7077	5605	6699	19222.5	1047.5	0	0
48	0	10347	32381.5	11133	0	4339	0	6513	11901.5	2324	0	0
48	0	0	13819	34084	16659.5	5087	0	0	5916	1217	0	0
48	0	0	6893	40108	12167	7298	0	0	0	0	0	0
48	0	0	6766	32972	5415	6235.5	0	0	0	0	0	0
48	0	7635.5	56471.5	6725	0	0	1522	3957	17663.5	400.5	0	0
48	0	7036	29685.5	4781	0	0	0	0	13964.5	0	0	0
48	0	3205.5	29656	0	0	0	0	0	10977	0	0	0
72	0	16111.5	45664.5	9007	0	7593.5	5065	10394.5	17614.5	2243	0	12581
72	6321	9157.5	36161.5	7275	0	5649	5062	8322	13492	562.5	0	0
72	4475.5	17381	51388	7354.5	0	7472	7192	5269.5	12932	1192	0	0
72	7400.5	10075	25671	93903	6060	22877	6485	0	8342	12416	6377.5	0
72	0	0	9248.5	61584	3670.5	7879	0	0	0	14621	0	0
72	5861	0	13904	107297	4907.5	8875	0	0	5734	0	5697.5	0
72	0	0	20159.5	12767.5	0	0	0	0	9426	0	0	0
72	0	7294.5	28800	6249	0	3823	0	0	7802	544	0	0
72	0	7454.5	28094	6695.5	0	6082	1696.5	0	13884.5	201	0	0

RSC017 pheromone levels

STAGE	DASC1	NPAR1	Pasc9	Ascr1	Ascr12	Ascr9	Npar2	Pasc1	Pasc12	Tasc1	Ubas1	Ubas2
24	0	0	0	0	0	0	0	0	0	1610	0	0
24	0	0	0	4489	0	0	0	0	0	1214	0	0
24	0	0	0	0	0	0	0	0	0	1769	0	0
24	0	0	0	22265	0	4301	0	0	0	1169.5	0	0
24	0	0	0	28319.5	0	5450	0	0	0	1871.5	0	0
24	0	0	7193.5	35197.5	8299.5	7177	0	0	9476	3918	0	0
24	0	0	16048.5	3929.5	5028.5	0	0	0	8318	969.5	0	0
24	0	0	19293.5	2386.5	0	0	0	1657.5	11094	999	0	0
24	0	0	11623.5	0	0	0	0	3667.5	4799.5	949.5	0	0
48	6859.790284	7828.076066	97882.17167	6917.834663	0	3737.706244	5320.734771	7463.100045	31294.27533	2486.234248	0	0
48	0	7381.310238	47912.11729	6736.665843	0	6223.940492	4929.374941	5891.504502	16905.42548	921.2346567	0	0
48	0	9099.77565	28478.24347	9791.03144	0	3815.97821	0	5727.924887	10466.89667	2043.86572	0	0
48	0	0	12153.26179	29975.52462	14651.36875	4473.81451	0	0	5202.887092	1070.303176	0	0
48	0	0	6062.119798	35273.39342	10700.39338	6418.301217	0	0	0	0	0	0
48	0	0	5950.428341	28997.56477	4762.277486	5483.874656	0	0	0	0	0	0
48	0	6715.119066	49664.44193	5914.370469	0	0	1338.538566	3480.024379	15534.34688	352.2238473	0	0
48	0	6187.88262	26107.21852	4204.69966	0	0	0	0	12281.22326	0	0	0
48	0	2819.109969	26081.27444	0	0	0	0	0	9653.835634	0	0	0
72	0	12340.85154	34977.427	6899.050356	0	5816.358263	3879.61475	7961.827348	13492.09753	1718.060392	0	9636.610695
72	4841.667292	7014.328149	27698.45781	5572.398284	0	4326.938544	3877.316854	6374.364057	10334.40517	430.8555374	0	0
72	3428.078147	13313.24461	39361.42997	5633.292533	0	5723.293467	5508.823155	4036.254674	9905.46455	913.0307566	0	0
72	5668.526941	7717.101403	19663.09778	71926.44894	4641.750323	17522.99045	4967.285618	0	6389.683365	9510.226404	4884.944337	0
72	0	0	7084.031	47171.21318	2811.476	6035.041385	0	0	0	11199.18011	0	0
72	4489.323208	0	10649.98292	82185.7895	3758.9752	6797.942923	0	0	4392.045603	0	4364.087865	0
72	0	0	15441.47948	9779.463242	0	0	0	0	7219.989859	0	0	0
72	0	5587.334609	22059.80351	4786.517783	0	2928.285723	0	0	5976.062049	416.6851775	0	0
72	0	5709.889073	21519.03194	5128.521334	0	4658.601562	1299.460301	0	10635.04659	153.9590454	0	0

RSC017 volume normalized pheromone levels

Table S4, related to Figure 5. Raw and normalized data of RSC017 pheromones, in absolute value of area under the curve.

COMMENTARY

Mechanism of murderous mushrooms paves path for parasitic helminth halt

Tess Renahan[a] and Ralf J. Sommer[a,1]

"In the animal kingdom, the rule is, eat or be eaten" (ref. 1, p. 20). We will expand academic Thomas Szasz's statement to include the fungi, because in the multifaceted interactions of fungi and nematodes, this is often a steadfast rule. Indeed, soil systems that appear inert or lifeless to the naked human eye in reality teem with microscopic life dominated by interactions between nematodes and fungi. Numerous free-living *Bursaphelenchus* nematodes are mycophagous (2), and beetle-associated *Pristionchus pacificus* has expanded its dietary range to include consumption of fungi in addition to bacteria and other nematodes (3). Parasitic *Deladenus* nematodes are even commercially used for their mycophagous phase to keep fungus *Amylostereum* in check; however, the fungus has responded with hyphal invasion through the worms' vulvae (4). Indeed, *P. pacificus* has also been on the receiving end of predation: Myriad mushrooms have evolved various capabilities to capture worms, often when times are dire and nitrogen contents are low. These predation techniques include adhesive and ring traps that target different nematode developmental stages, such as in the case of *Arthrobotrys*, a well-studied nematode-trapping fungus (5). Other fungi use infective spores that either bind to the nematode cuticle or are ingested (6). As explored in PNAS by Lee et al. (7), the eatable oyster mushroom *Pleurotus ostreatus* of the Basidiomycota uses yet another mechanism by employing toxin-induced paralysis to kill its prey. While the predatory capacities of fungi are well established, the execution mechanisms are gradually being elucidated. Lee et al. delve into a cilia-dependent killing mechanism that instigates an inundation of calcium and hypercontraction of head and pharyngeal muscle cells. The study by Lee et al. not only reveals the mechanism behind an imperative ecological advantage but also provides an opportunity for anthelmintic drug development, an indispensable opportunity given the rise of resistance to current treatment and high rates of infected humans and agricultural destruction, roughly 1.5 billion people worldwide (8) and $80–120 billion in crop damages (9), respectively.

Intact Cilia of Head Sensory Neurons Needed for Instantaneous Paralysis

Using carnivorous *Pleurotus ostreatus* and free-living *Caenorhabditis elegans*, Lee et al. (7) uncover a striking mechanism of a predation tactic that differs from other well-described nematophageous fungi and successfully acts against a diversity of nematodes. Fungi relying on trapping mechanisms have co-opted the use of nematode ascaroside pheromones to lure them into their snares. Among the worms, ascarosides are used for various behaviors and communication, including mating and sensing population size (10). While these wedged worms wiggle for hours, *P. ostreatus* immobilizes *C. elegans* almost immediately upon contact with fungal hyphae, as Lee et al. observe in their study in PNAS. The authors found a substantial influx of calcium levels in the pharynx and head muscles using calcium indicator GCaMP6 under two promoters, one expressing in neurons and the pharynx and the other in various muscles. They traced this calcium source to the endoplasmic reticulum but also recognized that downstream factors participating are different from the currently known ones involved in channel-mediated neurotoxicity. Specifically, a ryanodine receptor r mutant displayed decreased calcium levels in the pharynx compared to wild type yet experienced the same ciliated sensory neuronal necrosis. This leaves the door open for further investigation.

With the power of unbiased genetic screens, Lee et al. found 12 *C. elegans* mutants displaying normal locomotion on *P. ostreatus* hyphae. Single-nucleotide polymorphism mapping and whole-genome sequencing revealed nine independent, loss-of-function mutations in genes, all of which are essential for the development of ciliated sensory neurons or amphid channel morphogenesis, the latter representing the nose of the worm, the most crucial sensory organ of these blind creatures. Using these mutants along with previously established lines with known defects in either the function of ciliated sensory neurons or impaired signaling, Lee et al. (7) surprisingly found that the intact cilia structure, but not sensory neural function,

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is needed to elicit paralysis. However, mutants with defects in ciliated sensory neuron development displayed a range of paralysis upon hyphae contact, suggesting that several factors are at play. Together, these findings reveal that the oyster mushroom successfully evolved a hitchhiking mechanism of the essential communication systems of their nematode prey, a strategy commonly used by predators throughout all domains of life.

However, what really does happen in the victim after the attack has been initiated? Using the powerful list of genetically engineered reporter lines available in *C. elegans*, the authors found neuronal degeneration across the nervous system within 10 min after being in contact with *P. ostreatus* hyphae. Similarly, muscle cells exhibit rapid necrotic cell death. Is that effect different from those elicited by anthelmintic drugs, such as ivermectin, levamisole, and aldicarb, which are known to act at the neuromuscular junctions and to also paralyze their worm victims (11)? Lee et al. found only *Pleurotus* to cause neuronal necrosis; thus, this mechanism is different from that of current anthelmintic drugs. The authors also ascertained that a *P. ostreatus* toxin known to induce paralysis in nematodes (12) is not a response for executing the reaction they observed; therefore, this system can also be utilized to unearth the chemical compounds employed by the predator.

Findings Suggest Capacity for Innovative Anthelmintic Drugs

Potential application, however, would require a conserved mode of action of *Pleurotus* across nematodes. Indeed, the general neuroanatomy of nematodes is largely conserved even though variation is observed across species (13, 14). Reassuring therefore, Lee et al. found that *Pleurotus* has a common effect across nematodes tested, although none of them was parasitic. Two main factors contribute to the feasibility of these findings shedding light on anthelmintic drugs: the conserved effects across the nematode phylum and sensitivity of all stages. The authors maintain that all developmental stages are equally susceptible to paralysis, including the arrested dauer larvae. Dauers are a premediated response to stressful environmental conditions, have an occluded opening, and display remodeled neurons, including, in *C. elegans*, cilia retraction of a neuron in the amphid pore (14). While neuronal restructuring is common among nematode dauers, dauer entry and exit rely on chemosensation, and thus ciliated sensory neurons (15), suggesting this mechanism will be effective across nematode dauers. In terms of developing anthelmintic drugs, this is especially critical given that the analogous stage in parasitic nematodes is often the infective stage.

Lee et al. uncover a beautiful example of an intricate predator–prey relationship using a widespread fungus and ubiquitous nematodes indicating the sophisticated interactions within soil ecosystems. Of course, in our Red Queen world, these nematodes may develop an antagonistic response to the paralysis-inducing mushroom, ceasing to be the eaten and becoming the eater. Thus, soil systems reveal ever-increasing multifaceted interactions among bacteria, fungi, protozoans, and nematodes, and likely others. The time is ripe for more researchers to study them in greater detail, and not only because of rewarding application prospects.

1 T. S. Szasz, "Language" in *The Second Sin* (Anchor Press, 1973), p. 20.
2 N. Kanzaki, T. Ekino, R. M. Giblin-Davis, Feeding dimorphism in a mycophagous nematode, *Bursaphelenchus sinensis*. *Sci. Rep*. **9**, 13956 (2019).
3 G. V. Sanghvi *et al*., Life history responses and gene expression profiles of the nematode *Pristionchus pacificus* cultured on *Cryptococcus* yeasts. *PLoS One* **11**, e0164881 (2016).
4 A. E. Hajek, E. E. Morris, T. A. Hendry, Context-dependent interactions of insects and defensive symbionts: Insights from a novel system in siricid woodwasps. *Curr. Opin. Insect Sci.* **33**, 77–83 (2019).
5 K. Q. Zhang, K. D. Hyde, Eds., *Nematode-Trapping Fungi* (Springer Science and Business, 2014), vol. 23.
6 F. E. de Freitas Soares, B. L. Sufiate, J. H. de Queiroz, Nematophagous fungi: Far beyond the endoparasite, predator and ovicidal groups. *Agric. Nat. Resour. (Bangk.)* **52**, 1–8 (2018).
7 C.-H. Lee *et al*., Sensory cilia as the Achilles heel of nematodes when attacked by carnivorous mushrooms. *Proc. Natl. Acad. Sci. U.S.A.* **117**, 6014–6022 (2020).
8 World Health Organization, "Soil-transmitted helminth infections." Newsroom. Available at https://www.who.int/news-room/fact-sheets/detail/soil-transmitted-helminth-infections. Accessed 12 February 2020.
9 G. C. Bernard, M. Egnin, C. Bonsi, Data from "The impact of plant-parasitic nematodes on agriculture and methods of control, nematology—concepts, diagnosis and control." IntechOpen. Available at https://www.intechopen.com/books/nematology-concepts-diagnosis-and-control/the-impact-of-plant-parasitic-nematodes-on-agriculture-and-methods-of-control. Deposited 16 August 2017.
10 A. H. Ludewig, F. C. Schroeder, Ascaroside signaling in *C. elegans*. *WormBook* **2013**, 1–22 (2013).
11 A. K. Jones, S. D. Buckingham, D. B. Sattelle, Chemistry-to-gene screens in *Caenorhabditis elegans*. *Nat. Rev. Drug Discov.* **4**, 321–330 (2005).
12 O. C. H. Kwok, R. Plattner, D. Weisleder, D. T. Wicklow, A nematicidal toxin from *Pleurotus ostreatus* NRRL 3526. *J. Chem. Ecol*. **18**, 127–136 (1992).
13 Z. Han, S. Boas, N. E. Schroeder, Unexpected variation in neuroanatomy among diverse nematode species. *Front. Neuroanat.* **9**, 162 (2016).
14 R. L. Hong *et al*., Evolution of neuronal anatomy and circuitry in two highly divergent nematode species. *eLife* **8**, e47155 (2019).
15 P. S. Albert, S. J. Brown, D. L. Riddle, Sensory control of dauer larva formation in *Caenorhabditis elegans*. *J. Comp. Neurol*. **198**, 435–451 (1981).

PLOS PATHOGENS

RESEARCH ARTICLE

Rhabditophanes diutinus a parthenogenetic clade IV nematode with dauer larvae

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Abstract

Comparative studies using non-parasitic model species such as *Caenorhabditis elegans*, have been very helpful in investigating the basic biology and evolution of parasitic nematodes. However, as phylogenetic distance increases, these comparisons become more difficult, particularly when outside of the nematode clade to which *C. elegans* belongs (V). One of the reasons *C. elegans* has nevertheless been used for these comparisons, is that closely related well characterized free-living species that can serve as models for parasites of interest are frequently not available. The Clade IV parasitic nematodes *Strongyloides* are of great research interest due to their life cycle and other unique biological features, as well as their medical and veterinary importance. *Rhabditophanes*, a closely related free-living genus, forms part of the Strongyloidoidea nematode superfamily. *Rhabditophanes diutinus* (= *R.* sp. KR3021) was included in the recent comparative genomic analysis of the Strongyloididae, providing some insight into the genomic nature of parasitism. However, very little is known about this species, limiting its usefulness as a research model. Here we provide a species description, name the species as *R. diutinus* and investigate its life cycle and subsequently gene expression in multiple life stages. We identified two previously unreported starvation induced life stages: dauer larvae and arrested J2 (J2A) larvae. The dauer larvae are morphologically similar to and are the same developmental stage as dauers in *C. elegans* and infective larvae in *Strongyloides*. As in *C. elegans* and *Strongyloides*, dauer formation is inhibited by treatment with dafachronic acid, indicating some genetic control mechanisms are conserved. Similarly, the expression patterns of putative dauer/infective larva control genes resemble each other, in particular between *R. diutinus* and *Strongyloides* spp. These findings illustrate and increase the usefulness of *R. diutinus* as a non-parasitic, easy to work with model species for the Strongyloididae for studying the evolution of parasitism as well as many aspects of the biology of *Strongyloides* spp, in particular the formation of infective larvae.

Competing interests: The authors have declared that no competing interests exist.

Author summary

Parasitic worms are an issue of great medical, veterinary, agricultural and economic importance, yet little is known about how worms become parasites. Comparative studies with non-parasitic model species like *C. elegans* have been useful, however, this usefulness decreases as the evolutionary distance between the species increases. One way to combat this is by having more well-studied closely related species to parasites of interest. To address this, we provide information about *Rhabditophanes diutinus*, a free-living nematode that is part of the same superfamily as the medically and veterinary important *Strongyloides* parasites. We provide analysis on its life cycle, in particular on two starvation induced life stages, along with gene expression data. Overall, this important information illustrates and improves the use of *R. diutinus*, as a non-parasitic model species for studying parasite evolution and basic biology within *Strongyloides*.

Introduction

Parasitic nematodes are of great medical, agricultural, veterinary and economic importance, yet their study and control has been often neglected [1]. As a result, multiple parasitic nematodes are causative agents of Neglected Tropical Diseases (NTDs) [2] and human parasitic nematodes alone cause annually a loss of over 5 million disability adjusted life years [3]. While parasitism is generally ubiquitous [4], it is known to have evolved independently within nematodes on upto 18 separate occasions [5], meaning it can only be studied at specific defined transitions. Nematodes themselves consist of 5 major clades [6], all of which contain parasites, yet there is still a lack of understanding of how parasitism has arisen. Since each transition to parasitism was an independent historical event, each transition must therefore be studied independently. To what extent some or all of these separate evolutionary transitions followed common general principles is unclear.

The so-called dauer hypothesis for nematode parasitism [7] was proposed as one such common principle that might have been involved in multiple transitions to parasitism. This hypothesis states that dauer larvae, which exist in a number of non-parasitic nematodes, served as a pre-adaptation to and eventually evolved into infective larvae. Dauer larvae themselves are a specialized long lasting life stage produced in response to extreme environmental conditions, allowing the population to survive [8]. Their formation has been well-studied in the model nematode *C. elegans* and for an overview of the genetic pathways involved in their formation and dauers themselves, we encourage the reader to consult [7–10].

Recent attempts to understand the biology and the evolution of parasitic nematodes through comparative studies between parasitic species and *C. elegans*, have begun to identify conserved canonical dauer signaling pathways involved in the formation of infective larvae, providing support to the dauer hypothesis [11,12]. In particular, the functions of the nuclear hormone receptor DAF-12 and its ligand dafachronic acid which are the most downstream effectors of dauer development appear highly conserved across nematodes [13–20], with suppression of DAF-12 resulting in a loss of dauer or infective development or a redirection towards free-living stages. However, clear differences such as the absence of key dauer control genes in parasitic species [21] or changes in functions or logic of conserved genes and their interactions [10,22], means substantial further analysis is required. The use of *C. elegans* in comparative studies is often a problem, as while it has a substantial knowledge base, it is often too evolutionarily remote from the studied parasitic species, resulting in phylogenetic separation being the likeliest cause of any observed differences [5,6]. Therefore, it is through studying

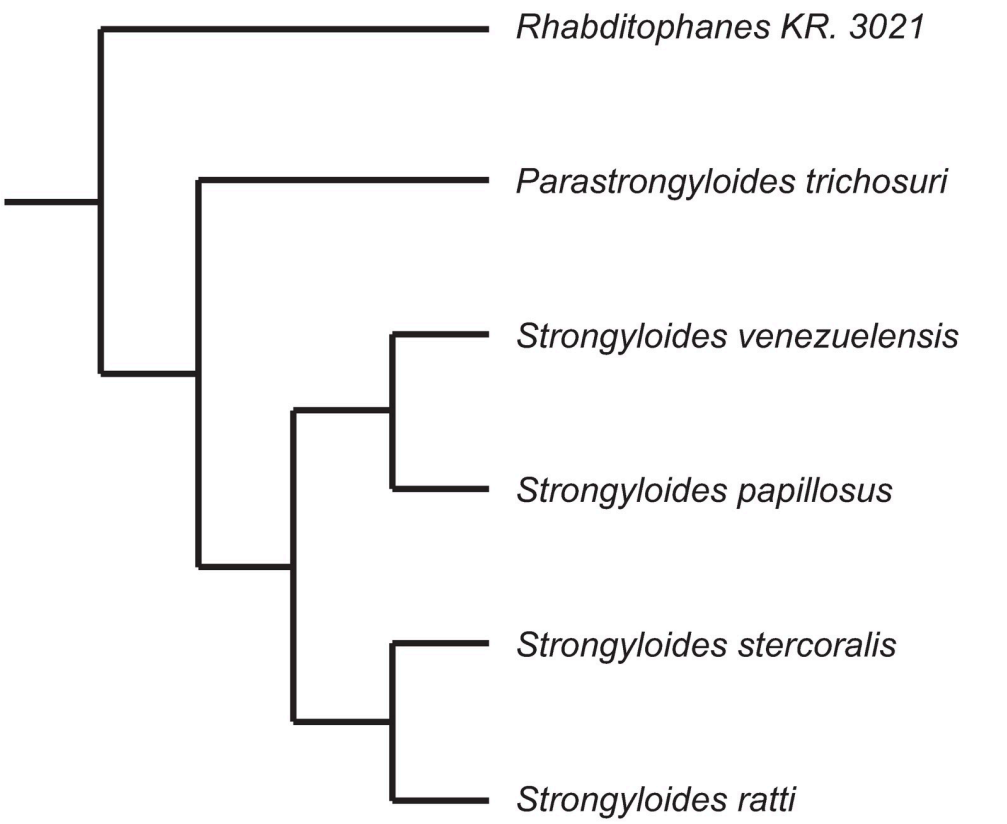

Fig 1. Phylogenetic relationship of *Rhabditophanes* and selected Strongyloididae species based on(5, 25). Only the tree topology is shown. The branch lengths are not informative.

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closely related free-living and parasitic species, that the biology and the evolution of parasitic nematodes can possibly be better understood. For the clade IV Strongyloididae parasites, who are more divergent from the clade V *C. elegans* than humans are from zebrafish [23], this is particularly true.

The Strongyloididae together with the Alloinematidae form the Strongyloidoidea superfamily [24], which is a highly attractive model for studying the evolution of parasitism and testing the dauer hypothesis, as it contains the obligate *Strongyloides* parasites, the facultative *Parastrongyloides* parasites and the free-living *Rhabditophanes* nematodes [25]. The phylogeny of these taxa can be found in Fig 1. However, this superfamily is also of great interest in its own right because it contains *Strongyloides stercoralis*, a small intestinal parasite estimated to infect more than 600 million people worldwide [26]. Although frequently asymptomatic, infections can result in deadly disease, particularly in immuno compromised patients [27]. The progeny of host dwelling parasitic *S. stercoralis* females can develop to infective third stage larvae (iL3) while still within the host and infect that same host individual (auto-infective cycle). This is in addition to the "normal" life cycle present in all species of *Strongylodies*, whereby these progeny leave the host as young larvae and continue their development into iL3 (direct development), or free-living adults which reproduce sexually (indirect development) [28]. The auto-infective cycle is mostly responsible for severe cases of strongyloidiasis, as it enables low-level infections to persist asymptomatically for many years before self-enhancing [27]. This enhancement results in hyperinfection syndrome and dissemination, which is lethal in most cases if not treated in time [27]. Therefore, investigating the developmental switch between (auto-)

infective larvae and free-living worms is important in order to understand the pathogenicity of *S. stercoralis*.

As the closest non-parasitic relative of *Strongyloides* spp., *Rhabditophanes* is of particular interest not only with respect to the study of the evolution of parasitism [29] but also as a possible non-parasitic model species to study other aspects of *Stronglyoides* spp. biology, such as the formation of dauer / (auto-) infective larvae. Although it has been used in a small number of comparative studies [25,30–33], relatively little is known about *Rhabditophanes*, as evidenced by the lack of a species name for its most cultured and well-studied member (sp. KR3021).

As *Rhabditophanes* sp. KR3021 fits the niche of being free-living and phylogenetically close to parasites of interest, we aim to provide some much needed basic information of this species. We suggest naming the species *Rhabditophanes diutinus*, in light of both its multiple survival strategies (see below) and the long period of time from its isolation to naming. We provide a formal species description including measurements in the supplement (S1 File). We found that this species can form two long-lived stages in response to starvation, namely a J2 arrested stage (J2A) and dauer larvae, both of which could be recovered with the addition of food. To our knowledge this is the first example of a clade IV nematode that can be induced to enter and exit the dauer stage under laboratory conditions. Its SDS resistance, developmental stage, morphology, longevity, gene expression patterns and inhibition of dauer formation with dafachronic acid strongly suggests that *R. diutinus* dauers are homologous to *C. elegans* dauers and *Strongyloides* infective larvae. Our results make it highly unlikely that *R. diutinus* is secondarily free-living upon reverting from a parasitic life style as had been previously proposed [34], based in part however on the erroneous phylogenetic placement of *Rhabdias bufonis* [35]. Overall, our results illustrate that *R. diutinus* is not only an attractive system to study the evolutionary transition to parasitism but also as a non-parasitic model for the study of a group of nematodes that contains important pathogens without the need for host animals.

Results

Life Cycle of *Rhabditophanes diutinus*

Previous descriptions of *R. diutinus* (KR3021) state it has a simple free-living cycle consisting of four larval molts from embryos through adults with reproduction occurring by meiotic parthenogenesis [25] (Fig 2A). In other species of *Rhabditophanes*, dauer larvae (identified solely through morphology) in association with arthropods had been previously described, but not in *R. diutinus* [36,37]. When examining overgrown *R. diutinus* plates, we saw worms of two previously undescribed stages: a dauer-like larvae (Fig 2D) and a small arrested stage larvae (Fig 2C). If we assume that *Rhabditophanes* spp. is secondarily free-living [34], we would expect it to have lost its original dauer stage. As a result, should a functional dauer stage exist, then we would expect this to be newly gained after the reversal to a non-parasitic lifestyle. To determine if *R. diutinus* does indeed form dauer larvae that are homologous to the dauer larvae of clade V nematodes, we further investigated the two starvation induced stages. In other nematodes, only dauer larvae which have both a thickened cuticle and buccal plug are able to survive treatment with SDS [38,39]. We found that also in *R. diutinus*, only dauer-like larvae were able to survive this treatment and could be recovered on an NGM plate. Examination of these larvae under DIC microscopy, revealed that they contain a 3 part buccal plug, consisting of a plug in the mouth (Fig 2F), a further larger plug located approximately one third of the way down in the intestinal lumen (Fig 2G) and a final series of plugs located throughout the posterior portion of the intestinal lumen.

The arrested small stage larvae (Fig 2C) (from hereon referred to as J2A) were unable to survive SDS treatment. Microscope examination revealed they lack a buccal plug (Fig 2H) or

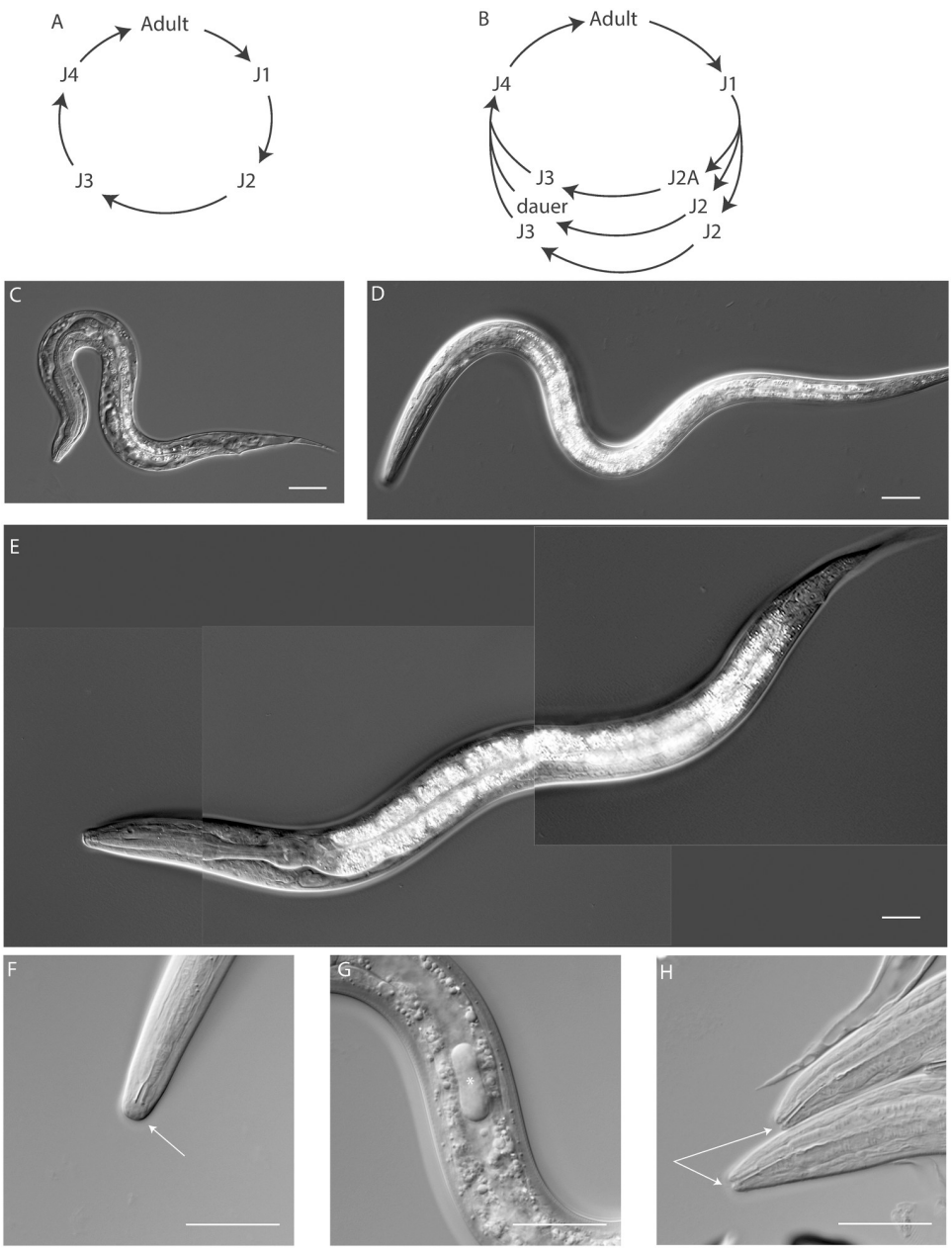

Fig 2. Life cycle and developmental stages of *R. diutinus*. (A) previously described simple life cycle according to [25]. (B) Updated life cycle for *R. diutinus*. (C) J2A larva. (D) dauer larva. (E) J3 larva for comparison. (F) buccal plug (arrow) and (G) the uppermost of several intestinal plugs (star). (H) Front ends of J2A, lacking a buccal plug but featuring an ordinary narrow open mouth (arrows). All scale bars are 25μm.

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any other dauer specific morphological features and instead appear more similar to J2 larvae, although they have a smaller germline, thickened intestinal lumen and increased lifespan (a full morphological description of adults, J2As, J2s and dauers including measurements can be found in S1 File). For comparative purposes, a J3 worm is included as Fig 2E. Type material will be submitted to the CNRC, the Swedish Museum of Natural History and the State Museum of Natural History in Karlsruhe upon acceptance of the manuscript.

Dauer and J2A recovery

To determine if J2A is a developmental stage of the dauer development route, we transferred J2A larvae onto bacteria-free NGM plates. When recovered without food, J2A larvae were never able to develop into dauer larvae suggesting that they are an alternative to dauer formation. This suggests that *R. diutinus* contains multiple strategies for survival when under environmental stress. To confirm that the presumed J2A and dauer larvae were really second and third larval stages respectively, we transferred them onto NGM plates supplemented with *Escherichia coli* OP50 (OP50) bacteria to trigger exit from the enduring stage. Dauer larvae underwent two molts (from dauers into J4 and J4 into adults (Fig 3)), confirming that the

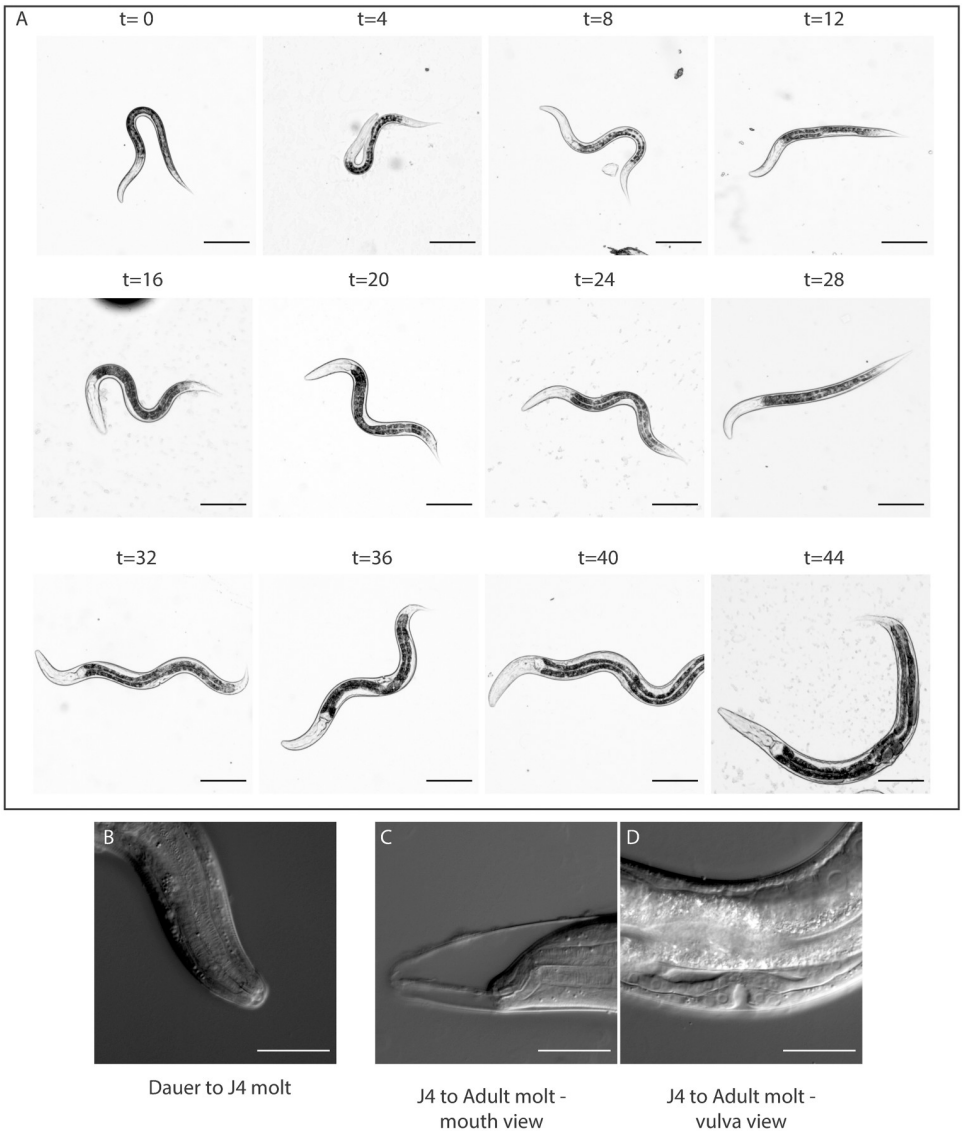

Fig 3. Recovery of dauers into adults in *R. diutinus*. (A) Time course of dauer recovery. Images are taken from a time course experiment. Images are shown at 4 hours intervals from dauers (t = 0 hours) through to fully developed reproducing adults (t = 44 hours). (B-D) High magnification DIC images of the two molts, at 16 and 36 hours are shown. Scale bars indicate 100μm. The images shown here are a composite of the experiment and do not constitute a single worm as it develops.

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dauers are a third stage larvae as in *C. elegans* [8]. It took nearly 2 hours for the buccal plug to disappear and a further 4 hours for the intestinal plugs to be removed, perhaps explaining why recovery following SDS treatment took longer than is typical in *P. pacificus* or *C. elegans*. J2A larvae required 3 molts and nearly 24 hours longer than dauers to become adults. This, in addition to the observation that J2As cannot form dauers confirms that they represent different survival strategies. An updated version of the *R. diutinus* life cycle can be seen in Fig 2B. As we have functional dauer larvae within this species, we asked if parts of the regulatory machinery that controls the dauer switch might also be conserved in *R. diutinus*.

Identification of orthologs of known dauer genes in *R. diutinus*

We identified orthologs for 53 of the 102 known *C. elegans* dauer pathway genes (Table 1) [7,10,11]. Of those present, 45 are one-to-one orthologs. Interestingly, there are striking differences in conservation between different signaling pathways. While the TGF-β (8 of 13) and steroid hormone (12 of 14) signaling pathways are well conserved, much of the insulin signaling pathway is missing (26 of 63 present). As most of these genes were previously shown to be restricted to the genus *Caenorhabditis* based on phylostratigraphic analysis [40], we also identified orthologs of the same genes within all other members of the Strongyloidoidea that have a published genome (Table 2) and found the same genes to be missing across all Strongyloidoidea genomes tested. The full list of genes present in all Strongyloidoidea species tested can be found in S2 File.

Comparison of the transcriptomes of different *R. diutinus* developmental stages

Next, we determined the transcriptional profile of the orthologs of *C. elegans* dauer genes in different stages of *R. diutinus* (adult, J2/J3, J2A and dauer)(Fig 4). In *C. elegans*, these genes are involved in dauer formation but many of them remain expressed in dauers and are also involved in dauer exit while the presence and expression of their orthologs has also been investigated in various parasitic nematodes [7,8,10,11,15,16,41] The majority of these genes are upregulated in one or both of the two long living life stages (41 in J2A, 37 in dauer), with only 7 genes downregulated in both stages. The substantial differences in gene expression combined with the separate PCA grouping of the different stages (S1 Fig), further confirms that J2A and J2 represent different stages, as do J2As and dauers.

In terms of the different dauer development pathways, we found that the cGMP and TGF-β pathways have similar expression in both dauers and J2As, with only *daf-21* being upregulated in J2As but downregulated in dauers. The insulin signaling pathway is substantially differentially expressed across all life stages. Similarly, there are large differences in the steroid

Table 1. Genes known to be involved in the dauer signaling pathway in *C. elegans* that are present within the published *Rhabditophanes diutinus* genome.

Pathway	Total Genes in *C. elegans* pathway	1 to 1	1 to Many	Many to 1	Missing	Total Present in *R. diutinus*
cGMP	12	6	1	0	5	7
TGF-β	13	8	0	0	5	8
Insulin	63	22	0	4	37	26
Steroid Hormone	14	9	1	2	2	12
Total	102	45	2	6	49	53

Genes are grouped according to the pathway in which they belong, and whether they are 1-to-1, 1-to-Many, Many-to-1 orthologs or are missing compared to *C. elegans*. An extended version of this table including information on how each ortholog was identified can be found in S2 File.

https://doi.org/10.1371/journal.ppat.1009113.t001

Table 2. Genes present within 5 tested Strongyloididae genomes that are part of the *C. elegans* dauer signaling pathway.

Pathway	Orthology	*P. trichosuri*	*S. stercoralis*	*S. ratti*	*S. papillosus*	*S. venezuelensis*
cGMP	1 to 1	8	7	6	7	7
	1 to Many	0	0	0	0	0
	Many to 1	0	1	1	1	1
	Missing	4	4	5	4	4
	Total present	8	8	7	8	8
TGF-β	1 to 1	11	11	12	12	11
	1 to Many	0	0	0	0	0
	Many to 1	0	0	0	0	0
	Missing	2	2	1	1	2
	Total present	11	11	12	12	11
Insulin	1 to 1	22	22	22	21	22
	1 to Many	0	0	0	0	0
	Many to 1	4	4	4	4	4
	Missing	37	37	37	38	37
	Total present	26	26	26	25	26
Steroid Hormone	1 to 1	9	9	9	9	9
	1 to Many	1	1	1	1	1
	Many to 1	2	2	2	2	2
	Missing	2	2	2	2	2
	Total present	12	12	12	12	12
TOTAL		57	57	57	57	57

Orthologs of known *C. elegans* genes were identified through a variety of bioinformatics analyses (see Methods). Genes are grouped according to their pathway, species and what state of orthology they exist in. An extended version of this table with full gene information including the method used to identify each ortholog can be found in S2 File.

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hormone pathway including between J2A and dauers. *daf-12*, the most downstream receptor of this pathway, is only upregulated in dauers.

To identify other genes potentially involved in dauer and J2A production or maintenance, we analysed the transcriptomes of J2As, dauers and J2/J3s. Between J2/J3s and J2As, 1371 (10.16%) (Table 3) genes were significantly differentially expressed, further supporting that they are different stages. To identify genes potentially involved in dauer production or maintenance, we compared the transcriptome of dauers and J2/J3s and found that 1409 (10.44%) genes were significantly differentially expressed, with 523 of these being upregulated in dauers. When comparing J2As and dauers, we found only 318 (2.36%) genes were significantly differentially expressed, of which 123 were upregulated only in dauers. Of these, 43 genes were significantly upregulated in dauers compared to both J2A and J2/J3 (S2 File).

Comparison of the transcriptomes of *R. diutinus* dauers and *Strongyloides* infective larvae

To determine whether *R. diutinus* dauers might resemble dauer larvae of the phylogenetically remote *C. elegans* or infective larvae of the closely-related *S. papillosus*, we compared the expression levels of *C. elegans* dauer genes for which orthologs are present within all three species (Fig 5). We only studied genes known to be dauer genes in *C. elegans* as opposed to global gene expression, as previous research has shown that even between much more phylogenetically close species (*P. pacificus and C. elegans*), global gene expression is vastly different in

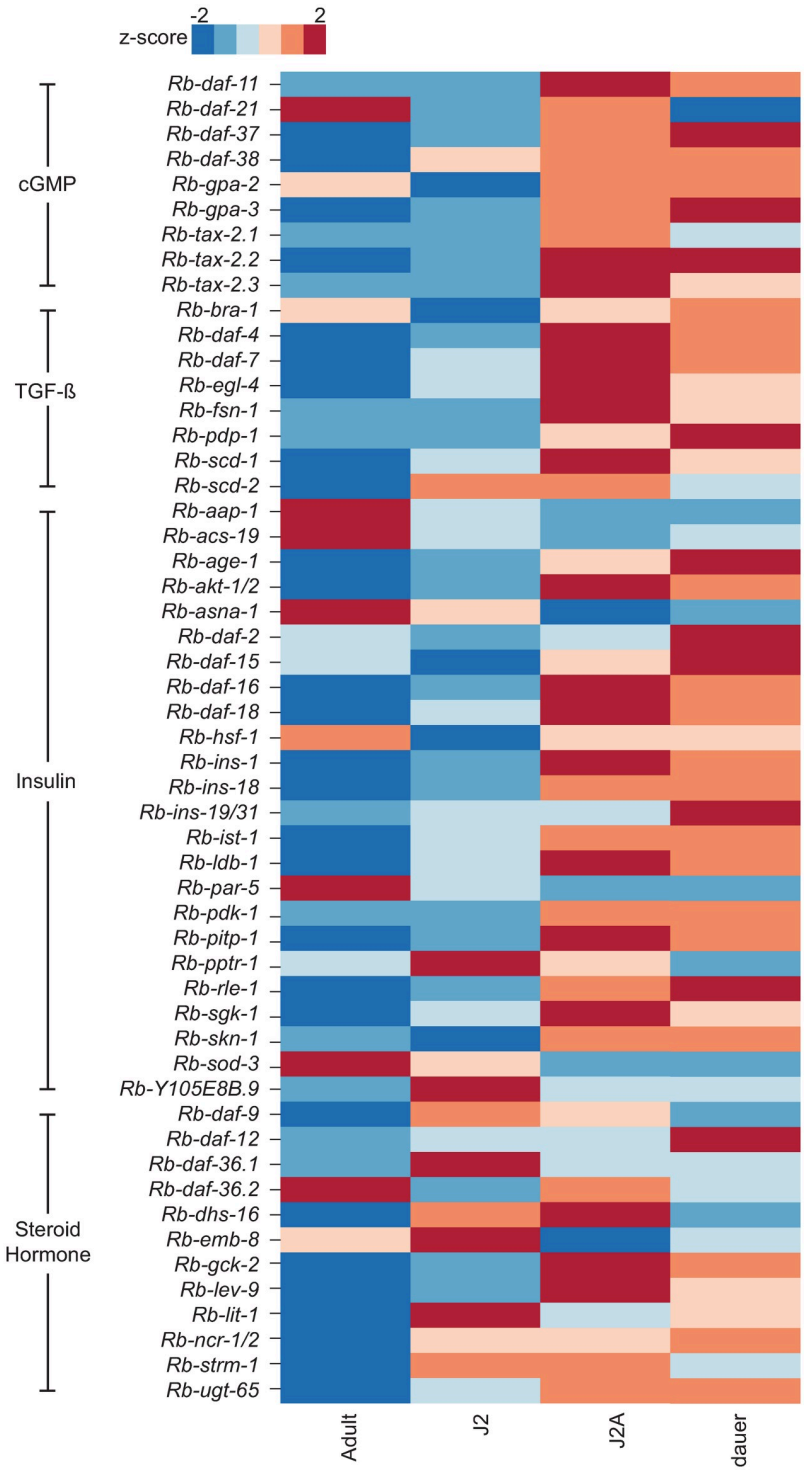

Fig 4. Heatmap of orthologs of known dauer genes in different life stages of *R. diutinus*. Transcriptome profile of orthologs of known dauer genes in Adults, J2/J3 larvae, J2As and Dauers. Values shown are mean z score from all 3 biological replicates as replicates were determined to be similar (see S1 Fig). Z score indicates difference in expression between the different stages, with blue indicating a negative z score (-2 to 0) and decreased expression and red indicating a positive z score (0 to 2) and increased expression.

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Table 3. Significantly differentially expressed genes in *R. diutinus* between J2/J3s, J2As and dauers.

Stage Comparison	Significantly differentially expressed genes	Non-differentially expressed genes	Upregulated in dauers	Upregulated in J2As	Upregulated in J2/J3s
Dauer vs J2A	318	13178	123	195	
Dauer vs J2/J3	1409	12087	523		886
J2A vs J2/J3	1371	12125		656	715

Total numbers of genes that were significantly expressed between different life stages in *R. diutinus*. All genes identified in the transcriptome were compared between different life stages, their fold change determined, and the significance between the two samples. Only those with a log fold change either greater than 2 or lower than -2 and a FDR-corrected p-value of less than 0.05 were determined to be significantly differentially expressed. Dauers were compared with J2/J3 as this represents a similar life stage in terms of molts, and J2As as they are both long living. J2As were compared with J2/J3s as they represent a similar life stage in terms of molts. The complete list of genes for each of these comparisons can be found in S2 File.

https://doi.org/10.1371/journal.ppat.1009113.t003

dauers [42]. The cGMP pathway appears well conserved in terms of expression profile between all three species, the only difference being *daf-21* which is strongly upregulated in *C. elegans* but not in the other two species. The TGF-β pathway is also broadly consistent among the three species, but shows differences in expression of *pdp-1* (upregulated in Strongyloidoidea) and *scd*-2 (upregulated in *C. elegans*). The insulin signaling pathway is differentially expressed between all three species. It should be said that large amounts of this pathway are not present within the *R. diutinus* or *S. papillosus* genomes, however for the genes that are present, there appears to be greater similarity in the profiles of *R. diutinus* and *S. papillosus* (10 genes up/downregulated in both species only) than *R. diutinus* and *C. elegans* (3 genes up/downregulated in both species only). Interestingly, *asna-1* which functions as a regulator of insulin secretion [43] is upregulated in *C. elegans* but downregulated in *R. diutinus* and *S. papillosus*. Of the insulin signaling ligands present within *R. diutinus* and *S. papillosus*, *ins-19/31* is upregulated only in *R, diutinus* while *ins-18* is upregulated in all three species. The steroid hormone signaling pathway is differentially expressed among the three species, however *daf-12*, the most downstream gene known to control dauer formation, is strongly upregulated in all three species, suggesting a conserved function within all three species.

Dafachronic acid prevents dauer formation but does not prevent J2A formation

As the conserved function of *daf-12* in dauer/infective larvae formation has been experimentally demonstrated for *C. elegans* and *Strongyloides* spp. [13,15,16], we tested whether the function of *daf-12* is also conserved in *R. diutinus*. *R. diutinus* larvae were treated with dafachronic acid (DA) or ethanol as a negative control (DA is diluted with ethanol and so all worms were exposed to this solvent) and their developmental stage monitored 14 days later as in [13]. No dauer larvae were seen when plates were supplemented with DA indicating that dauer development was prevented by the DA (Table 4). However, there was no effect on the formation of J2A larvae (95.90% in the negative control compared to 94.40–94.53% when treated with DA) confirming that their development is regulated by a different pathway (Table 4).

Taken together the results presented so far, we conclude that *R. diutinus* forms dauer larvae which most likely are homologous to dauer larvae of *C. elegans* and to iL3s of *Strongyloides* spp.

Dauer and J2A survival

To determine how long both types of stress induced larvae could survive, dauers and J2A were transferred separately onto bacteria-free NGM plates and observed daily for upto 3 weeks. As a

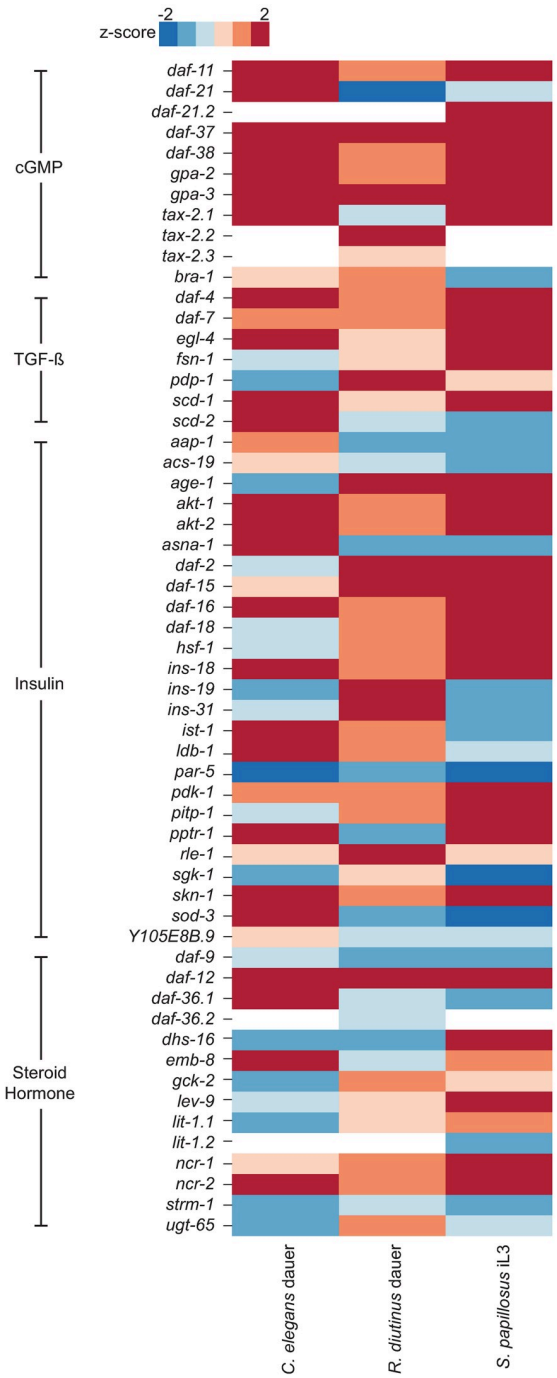

Fig 5. Heatmap of known dauer genes present in all three species (*R. diutinus*, *S. papillosus* and *C. elegans*). Z scores were determined and used to measure differences in expression for known dauer genes in all three species. Only genes that are present in all three species are shown. White indicates that the copy is not present within the genome, blue indicates a negative z score (-2 to 0) and decreased expression and red indicates a positive z score (0 to 2) and increased expression.

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Table 4. Dafachronic acid treatment prevents the formation of Dauer but not J2A larvae.

	Treatment		
Worm Stage (%)	**Ethanol**	**10μM Δ7 DA**	**100μM Δ7 DA**
Dauers	2.60	0.0 (**)	0.0 (**)
J2A	95.93	94.40	94.53

Young larvae were transferred on new plates supplemented with either Δ7 dafachronic acid (DA) or ethanol (solvent) and incubated for 14 days, after which the amount of dauer and arrested J2 larvae were counted. DA is diluted using Ethanol so all worms were exposed to the same carrier. On each plate, 200 larvae were chosen at random and staged. On plates were no dauer larvae could be counted within the 200, the whole plate was examined to determine if it truly was free from dauer larvae or not. For each treatment, 5 plates were used, and the experiment was repeated three times. Data shown here is a mean of all samples. Mann-Whitney U was performed to determine statistical significance between the different treatments ($p = <0.0001$ for both).

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control, normal J2/J3 larvae were also included. As seen in Fig 6, J2/J3 larvae had the shortest lifespan (median 3 days) with all the larvae dead after 12 days. Interestingly, the transfer of these larvae into a foodless environment resulted in them stopping development. The J2A larvae were able to survive significantly longer than the J2 larvae (p-value 0.017) with a median lifespan of 4 days, but all had died after 14 days. As expected, the dauer larvae had a significantly longer lifespan than both the J2 (p-value <0.0001) and J2A larvae (p-value <0.0001) with a median survival of 12 days and 24% were still alive at the end of the 3 weeks. It is currently unknown how long these dauer larvae may be able to last for but they have been seen in plates for upto 3 months.

The offspring of individuals that developed through dauer are not more likely to form dauers

Next we asked if the progeny of an individual who underwent dauer development are more likely to form dauers themselves. Such an effect could either be caused by genetic differences within the population predisposing some genotypes for dauer development, or it could be due to an epigenetic transgenerational effect. To this end, dauers, J2A and J2/J3 were transferred in groups onto NGM plates supplemented with OP50 and incubated at 15˚C. After 2 days of producing offspring, the initial worms were removed from the plates and the total number of offspring produced counted. As seen in Table 5, there was no difference in the number of offspring produced between J2/J3s and recovered dauers and J2A (78.5 for J2/J3, 73.9 for dauer and 73.8 for J2A). After further incubation for 2 weeks, the percentage of dauer larvae present on each plate was then counted. There was no difference in dauer production between J2/J3s (2.95%), recovered J2A (2.90%) and recovered dauers (2.92%). These results suggest that the larvae undergoing dauer development are not genetically different and that there are no transgenerational effects present from being dauers, at least none that perdure for longer than one generation.

R. diutinus alters its reproductive output in response to crowding

While there was no difference in fecundity between never starved adults and recovered either J2A or dauer larvae (Table 5), it was noticed during a pilot study that there was a substantial difference in fecundity based upon how many larvae were initially plated. To confirm this, J3 were transferred onto fresh NGM plates with OP50 either singly or in groups of 5 or 10. They were then allowed to develop for 2 days until they started laying embryos. The

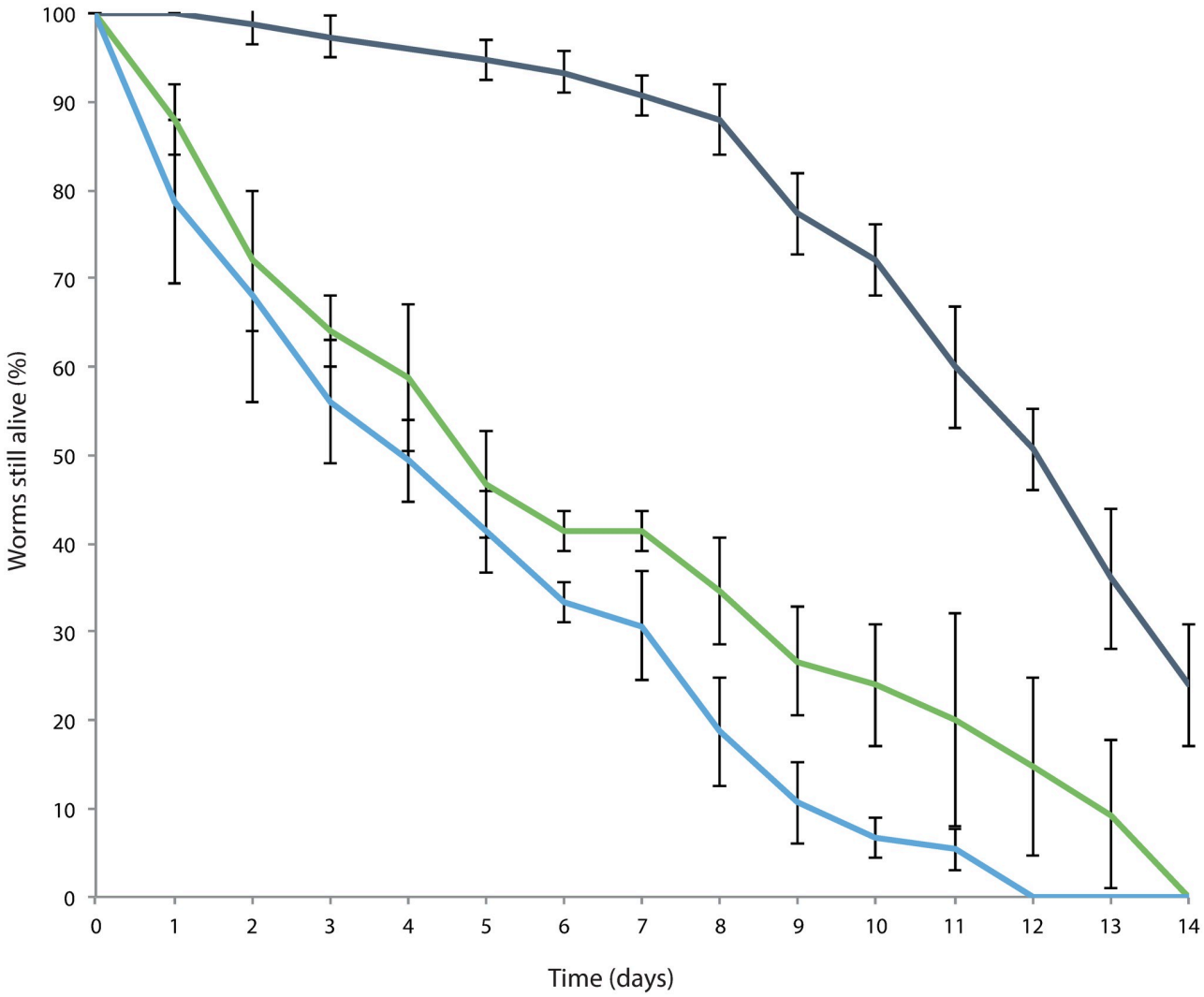

Fig 6. Dauers survive longer than J2A or J2/J3 when on fresh foodless plates. Survival curves of Dauers (dark blue line), J2As (green line) and J2/J3s (light blue line) when transferred onto fresh plates to induce starvation. Per treatment, 75 worms were picked in groups of 5 and checked daily for survival. The experiment was performed at three time points such that each time 25 worms per treatment were analyzed in parallel. The data points shown here are the daily mean of all 75 worms. Error bars are standard deviation.

https://doi.org/10.1371/journal.ppat.1009113.g006

Table 5. No transgenerational effects of being a dauer or J2A larvae compared to a J2/J3 in terms of offspring produced and likelihood to become dauers in the next generation.

Worm Stage Plated	Offspring after 48h of laying	Dauers after 14 day incubation (%)
J2/J3	78.46 ± 7.25	2.95 ± 0.36
Dauer	73.87 ± 5.31	2.90 ± 0.52
J2A	73.80 ± 4.76	2.92 ± 0.46

There is no difference in production of offspring or dauer formation between any of the starting populations. For each starting population, 10 plates were picked and the experiment was repeated three times. Data shown here is a mean of all samples plus the standard deviation. A students t test and Mann Whitney U were carried out to determine statistical significance between the different treatments, none was found.

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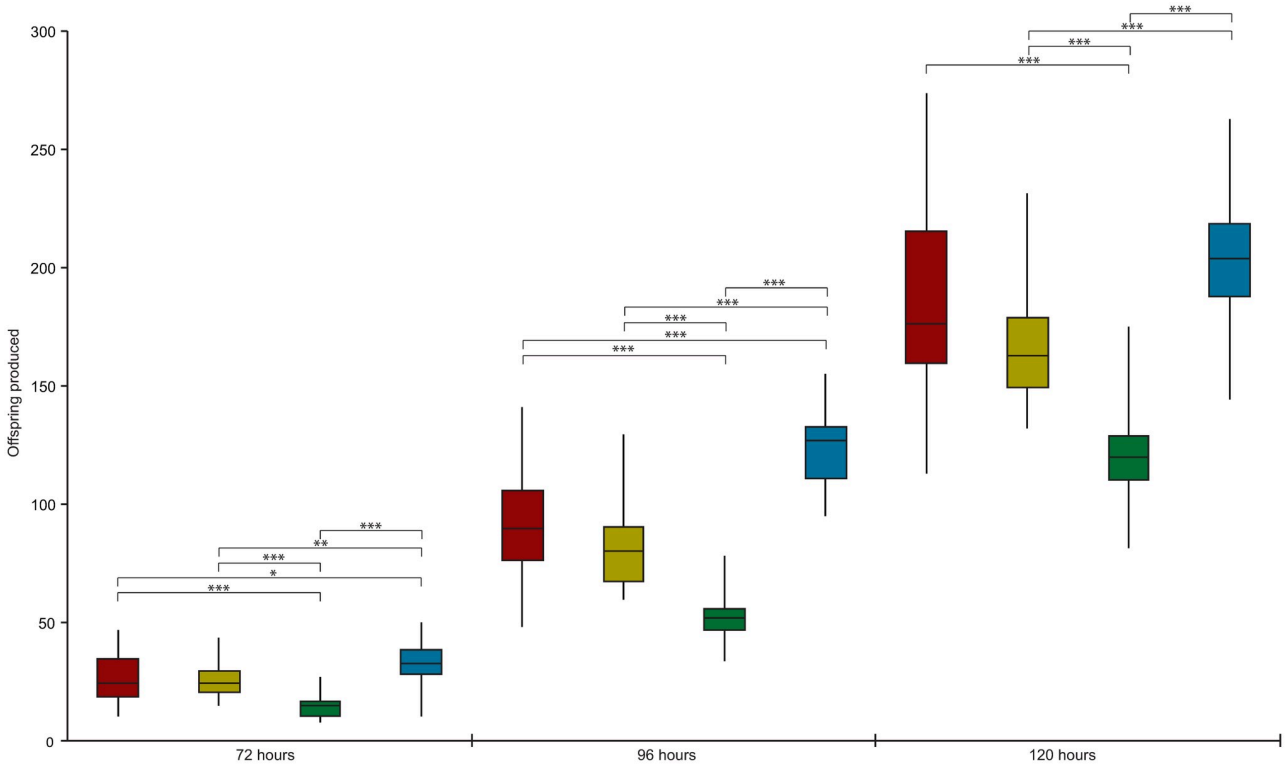

Fig 7. *R. diutinus* alters its reproductive output in response to density. *R. diutinus* J2s were picked either singularly (red), in groups of 5 (yellow) or 10 (green) onto plates and maintained for 120 hours or picked singularly onto plates and transferred daily to a new plate (blue). Offspring produced after 72, 96 and 120 hours was recorded. ANOVAs were performed between the different samples at each time point. For each density, at least 10 plates were picked, and the experiment was repeated three times. Data is shown as median (line), interquartile range (box) and range of values (whiskers). * indicates a statistically significant value of between 0.05 and 0.01, ** indicates a statistically significant value of between 0.01 and 0.001 and *** indicates a statistically significant value of lower than 0.001.

https://doi.org/10.1371/journal.ppat.1009113.g007

numbers of embryos laid along with any hatched offspring were then counted for 3 days. For some of the single worm plates, the adult was transferred daily to a new plate. As seen in Fig 7, there is a statistically significant decrease in the number of offspring produced on the first day among the different populations. Whilst there is no statistically significant difference between the single worm and 5 worm plates, there is a strong decrease between 1 worm and 10 worms, with worms maintained in groups of 10 producing nearly half as many eggs on average (14.49) as compared to worms alone on a plates (26.77) (p-value <0.0001). 24 hours later, this pattern of decreased fecundity in response to crowding still persists, with worms kept in groups of 10 producing 52.30 offspring on average compared to 82.29 offspring for groups of 5 and 90.39 worms for single worms (p-values both <0.0001). Most surprisingly however, there is a strong significantly significant decrease in the number of offspring produced between single worms kept on the same plate (90.39) versus those transferred to a new plate daily (123.16) (p-value <0.0001). 24 hours later this pattern remains, with single worms transferred daily having produced an average of 203.48 offspring compared to single worms kept on the same plate (188.10), worms kept in groups of 5 (167.22) and 10 (120.45). This strong correlation between crowding and a reduction in offspring, suggests that *R. diutinus* senses its offspring and adjusts its reproductive output based upon how many offspring are already present.

Discussion

Rhabditophanes diutinus life cycle

Rhabditophanes diutinus produces a very low amount (~3% in our experimental settings, Table 5) of dauers in response to starvation. It is unclear as to why dauers in this species have not been previously reported, particularly seeing as dauers were originally reported for other species in this genus over 80 years ago [36]. To our knowledge, this study provides the first example of a fully free-living Clade IV nematode and the closest free-living nematode to any parasite that can be induced to enter and exit a dauer stage under laboratory conditions.

In addition to the production of dauers, *Rhabditophanes diutinus* uses the formation of arrested J2As as a second strategy to survive environmental stress such as lack of food. While other free-living nematodes can also arrest their development at an earlier stage than dauer upon starvation (i.e. *C. elegans* [44] or *P. pacificus* (R. Sommer, personal communication)), this function is performed by specialised forms of the developmental stage that leaves the egg shell (J1 in *C. elegans*, J2 in *P. pacificus*) and can therefore develop with yolk as the only food source. In these cases, the survival stages are only physiologically but not morphologically different from their rapidly developing counterparts. In contrast, *R. diutinus* forms a physiologically and morphologically different specialized version of J2s, even though J1s are the stage that emerges from the egg shell. In our experiments, J2As survived starvation only moderately longer than J2s. However, J2As were induced by starvation, such that these individuals had been starving prior to the experiment while the J2s were picked from well-fed plates. Therefore, the increase of starvation tolerance was underestimated in our experiments. Nevertheless, one could question if this relatively small benefit would be sufficient for the evolution of a different developmental stage. One could also speculate that the development into J2A does not primarily occur in order to prolong survival after reaching the stage, but because it is easier to enter under starvation conditions. It is imaginable that J2A formation might be less energy consuming than development into a normal J2, for example due to its under-replicated germ line. As such, development to J2A instead of J2 might therefore allow a larva to complete an already initiated J1 to J2 transition upon starvation, thereby avoiding short-term death.

Dauer larvae survive starvation clearly longer than the J2A or J2 larvae but their survival in our experiments was much shorter than was described by [45] for *C. elegans* or *P. pacificus* dauer larvae, albeit under different conditions. Together with our observation that only a small proportion of *R. diutinus* worms formed dauer larvae, as a result of high population density associated with food deprivation, this may suggest that survival of starvation is not the most important purpose of the dauer stage in *R. diutinus*. Response to other environmental stresses (i.e. temperature, salinity, osmolarity, oxygen concentration) or dispersal through phoretic associations with other organisms, as was observed in other species of *Rhabditophanes* [36,37] may be more important functions of dauer larva in *R. diutinus*. It remains to be tested if such stressors or the presence of putative phoretic partners are more potent inducers of dauer development than starvation or population density. In the closely related facultative parasite *P. trichosuri* an iL3-inducing pheromone, as well as an influence of temperature on iL3 development, have already been described [46].

Potential of *Rhabditophanes diutinus* as a research model

Like other free-living species, *R. diutinus* can be easily maintained on standard NGM plates supplemented with OP50, can be frozen following standard protocols for other nematodes [47] and has a high quality genome [25], for which the gene annotations can be improved using the transcriptome data presented in this manuscript. The conservation of function

(survival of starvation), morphology, developmental stage (J3), physiology (SDS resistance) and a crucial endocrine regulatory module (DAF-12) strongly suggests that the *R. diutinus* dauers are homologous with *Strongyloides* spp. infective larvae and are not an analogous survival stage newly acquired upon reversal from parasitism. Of course, we cannot completely exclude that infective larvae reverted to being dauers, but we think that our results along with [35] make the hypothesis of a reversion from parasitism very unlikely to be true and suggests that the Strongyloididae represent one of multiple independent events of transitioning to a parasitic life style within nematodes, that occurred within the Strongyloidoidea after the split of the families Strongyloididae and Alloionematidae. All species (parasitic, facultative parasitic or non-parasitic) of Strongyloidoidea examined lack almost the same dauer pathway genes from *C. elegans*, illustrating that the vast majority of the genomic differences between *C. elegans* and *Strongyloides* spp. are due to the enormous phylogenetic distance and not the fact that one taxon is parasitic and forms infective larvae while the other is free-living and makes dauer larvae. The consistent lack of the same insulin signaling pathway genes in Strongyloidoidea together with the recent finding that most of these genes are restricted to the genus *Caenorhabditis* [48] rather points towards recent duplication events in the *Caenorhabditis* lineage than towards a loss in Strongyloididae. While our gene expression analyses are consistent with the hypothesis that some elements of the genetic control of dauers are conserved because the genes are expressed at the right time, the transcriptomic results cannot be interpreted as evidence that these genes do indeed participate in any part of dauer control in *R. diutinus*, be it formation, maintenance or exit. For this, functional studies need to be done.

As opposed to comparisons between *Strongyloides* and *C. elegans*, it is far likelier that differences in gene content or expression between *Rhabditophanes* and *Strongyloides* are due to the change in lifestyle, although some differences may still be due to phylogeny.

The presence of closely related, cultivable, experimentally tractable parasitic, facultative parasitic and free-living species renders the Strongyloidoidea an excellent example for studying the evolution of parasitism. The dauer hypothesis is an attractive and plausible hypothesis for the evolution of this taxon, which therefore represents a test case for the further study and testing of this hypothesis, that may also apply to several other transitions to parasitism within nematodes [7]. In addition to studying evolutionary parasitism, the Strongyloidoidea are also of interests for studying other fundamental biological processes. For example, it contains two independent transitions to parthenogenetic reproduction, one within the genus *Rhabditophanes* (females of *R. diutinus* are parthenogentic [25] while males do exist in other species of *Rhabditophanes* [36,37]) and the second after the split of *Parastrongyloides* (which reproduce sexually in the parasitic generation) and *Strongyloides* (which is parthenogenetic as a parasite) [29].

Finally and most importantly, given *R. diutinus*'s phylogenetic proximity to *Strongyloides*, it will be much more similar than *C. elegans* is both genomically [25] and with respect to many biological aspects as has previously been shown for vulva development [31] or gonad structure [32]. Therefore *R. diutinus* represents an easy to work with, non-parasitic model species for *Strongyloides*, that allows safe experimentation without the need for laboratory animals to study many aspects of these important veterinary and human pathogens.

Methods

Species and strain

Rhabditophanes sp. KR. 3021 (*diutinus*) was originally isolated in 1994, from a conifer forest floor soil sample by one of us (AR), near the Bamfield Marine Station on the West coast of Vancouver Island, British Columbia. The strain was sent to our lab by Dee Denver (Oregon

State University) in July 2014. It has been since maintained in the lab at 15˚C on NGM plates supplemented with OP50 bacteria as a food source or as frozen stock (frozen according to [47]). This strain is the same as that in the recent *Strongyloididae* genome paper [25].

Staging of worms and determination of dauer molts

Worms were picked manually from mixed stage culture plates into 5μl of water on a 4% agarose slide and visualized under a microscope using DIC optics [49]. For the dauer staging, dauers were isolated from 2 week old plates that had been starved of food for at least a week and then visualized directly using DIC microscopy on a Zeiss Imager M2 microscope with a Zeiss Axiocam 506 mono camera. To determine the dauer recovery and number of molts, dauers were picked from the same plates onto fresh NGM plates supplemented with OP50 in groups of 5 and then incubated at 15˚C. A plate was removed every 2 hours (upto 44 hours) and the worms on the plate were examined under 40x DIC microscopy to determine stage and changes in development between time points. High magnification images of the different stages was taken using a Zeiss Image Z1 with a Zeiss Axiocam 506 mono camera.

SDS treatment of dauers and other stages

Old (minimum 2 weeks) plates that had run out of food supply and contained dauers, J2As and a small number of long-living adults were washed off into 1% SDS and incubated gently shaking for 20 mins. Following this, the worms were washed multiple times with water and then recovered on NGM plates with OP50 at 15˚C. 1 hour later, plates were examined for surviving larvae and their developmental stage was scored.

Dauer survival determination

Dauers J2As (from starved plates) and J2/J3s (from well fed plates) were picked in groups of 5 onto NGM plates without any bacteria and incubated for 15 days at 15˚C. The plates were checked daily and the number of surviving worms counted. There were 3 biological replicates for each life stage, with each replicate consisting of 5 plates.

Test for transgenerational effects from dauers

To examine for any transgenerational effects, dauers, J2As and J2/J3s were picked onto fresh NGM plates with a lawn of OP50 and incubated at 15˚C for upto 15 days. Plates were checked daily until they began producing offspring. From this day, the original worms were allowed to lay embryos for a further 2 days at which point they were removed. Plates were checked daily to count the number of offspring produced. Once the worms had been removed, the plates were then incubated at 15˚C for 10 days after which the amount of dauers and J2A were counted on every plate. Worms were picked onto plates originally in groups of 10, with 10 plates per life stage. This experiment was repeated three times.

Fecundity determination

To examine fecundity changes, J3 worms were picked either singularly, in batches of 5 or 10 onto fresh NGM plates with a lawn of OP50 and incubated for 48 hours at 15˚C to allow development to adults. Following this, plates were examined and total number of offspring (laid eggs and hatched larvae) were counted along with the number of adult worms still alive on the plate. For plates with multiple original larvae, the adults were left on the plate overnight before counting the offspring again. For plates with single worms, the adults were either left on the plate or transferred to a fresh plate and incubated again overnight before counting the

offspring. This was repeated once more so that 3 days of offspring production were counted (total of 120h from the original picking of the J3s). At least 10 plates were picked per treatment, with this experiment being repeated three times.

Bioinformatics analysis of dauer pathway in Strongyloidoidea

To screen for the orthologs of candidate *C. elegans* genes in six species of Strongyloidoidea [25], we obtained protein and genome data sets for *C. elegans* (WBPS13), *S. ratti* (WBPS13), *S. stercoralis* (WBPS14), *S. venezuelensis* (WBPS14), *S. papillosus* (WBPS13), *P. trichosuri* (WBPS14), and *Rhabditophanes diutinus* (WBPS14) from WormBase ParaSite [50]. We further selected a broadly sampled set of high quality nematode genomes consisting of *T. spiralis* (WBPS13), *B. malayi* (WBPS13), and *P. pacificus* (El Paco version 2) [40]to be included in the homology and phylogenetic analysis. In case of multiple isoforms for a given gene, the isoform with the longest protein product was chosen as a representative sequence. We then applied a set of complementary approaches to derive the orthology relationships. These included all-against-all blastp searches, orthologous clustering, best-reciprocal hit (BRH) identification, phylogenetic analysis, phylostratigraphy and manual inspection. First, we defined orthologous groups based on a Markov clustering algorithm as implemented in the software orthAgogue which takes the results of all-against-all blastp searches (E-value$<$0.00001) as input [51]. Protein sequences corresponding to orthologous groups were aligned by the program MUSCLE (version 3.8.31) and phylogenetic trees were computed by RAxML (version 8.2.11, with the PROTGAMMAILG model and fast bootstrapping -f a -N 100) and the resulting trees were manually inspected to test whether their topology roughly corresponded to the species phylogenies [52,53] which is a reliable signature of one-to-one orthologs. In the case that no orthologous sequence for the most robust outgroup species *T. spiralis* could be found, we tested if the protein sequences from Strongyloidoidea formed a monophyletic group with regard to all other species. The phylogenetic tree was also useful to disentangle inparalogs from outparalogs if the orthologous cluster contained more than one sequence per species. Second, if a candidate gene from *C. elegans* was not part of any orthologous cluster, we screened for BRHs of *C. elegans* candidates in all other species and repeated the phylogenetic analysis as described above to identify further one-to-one orthologs. Third, for the remaining candidates we investigated estimates of gene ages as inferred by a recent phylostratigraphic analysis [40,54] to detect recently evolved *C. elegans* genes that consequently do not have a homolog in Strongyloidoidea. Notably, many of the insulin related peptides fall under this category. They could be identified by searches with profile hidden Markov Models as implemented by the hmmsearch program of the HMMER package (version 3.0, e-value $<$ 0.001) and extracting hits for the PFAM profile PF03488 (Nematode insulin-related peptide). Even though some level of sequence similarity exists with related sequences in Strongyloidoidea, the amount of sequence divergence prohibits recognition as homologs by BLASTP, which is a commonly used criteria to define novel genes [55]. Finally, for all remaining candidates, we performed phylogenetic analyses based on a set of manually extracted best BLASTP matches in all investigated genomes. For the complete orthology analysis, it was extremely helpful to have multiple genomes of Strongyloidoidea as these serve as independent biological replicates to support gene gains and losses which were usually phylogenetically consistent as they could be explained by a single evolutionary event [48]. A handful of inconsistencies such as secondary losses in individual species could be explained by problems in genome assembly and gene annotation as additional homology searches with the program exonerate (version 2.2.0) [56] could identify at least partial matches with the candidate *C. elegans* genes. A complete

orthology list including the method using to determine the orthology relationship for each Strongyloidoidea species examined can be found in S2 File.

Transcriptomic analysis of *R. diutinus*

Dauers, J2As, J2/J3 larvae and Adults were isolated and had their RNA extracted using Trizol (Zymo Research) for transcriptome analysis. All stages were picked manually onto bacteria-free NGM plates to reduce excess bacterial contamination. Once a minimum of 200 worms had been transferred, the plates were washed off with H_2O and the worm pellet concentrated by slow centrifugation (4000g, 2 mins). The worm pellet was then removed in 20μl and transferred to a new RNA free tube, 500μl Trizol added, and frozen instantly in liquid nitrogen. Samples were then disrupted using a motorized pestle until thawed, vortexed for 15 seconds and refrozen in liquid nitrogen. This freeze-thaw cycle was repeated 6 times to ensure complete disruption of the cuticle and homogenization of the sample. Following the final freezing, samples were incubated at 37˚C for 5 mins, after which 500μl of Trizol was added and incubated for a further 3 mins. 200μl of Chloroform was added, mixed and incubated for 3 mins and then centrifuged at 12000g for 15 mins. Following this, the aqueous phase was removed and the RNA extracted using RNA Clean and Concentrator-25 Kit (ZymoResearch) as per the manufacturers' protocol. The RNA was eluted into a final volume of 30μl of RNase/DNase free water and then immediately examined using NanoDrop (PEQLAB Biotechnologie) and Qubit (Invitrogen) to assess its purity and concentration. For all 4 stages of worms, 3 separate biological samples were generated. RNAseq libraries were then prepared using TruSeq RNA Library preparation kit v2 (Illumina) according to the manufacturer's instructions from 160–1000ng of total RNA in each sample. Between 12 and 15 PCR cycles were used depending upon the original quantity of RNA used. Libraries were quantified by Qubit and Bioanalyzer measurements (Agilent) and normalized to 2.5nM. Samples were then sequenced on a multiplexed lane of an Illumina HiSeq3000 instrument, resulting in 10–20 million 150bp paired end reads for each sample. All sequencing data was submitted to the European Nucleotide Archive under the study accession PRJEB39019. Sequencing adapter sequences were removed using the bcl2fastq software (version 2.18.0.12) with user defined parameter barcode-mismatches set to 1. No additional quality filtering was applied. The resulting sequencing data were aligned against the *Rhabditophanes* genome (WBPS14) with the help of TopHat2 (version 2.0.14, -I 10000 option). Expression levels were estimated by cufflinks (version 2.2.1, default options). Differential expression analysis was done with the cuffdiff software (version 2.2.1, default options). Only genes with a $\log_2$ fold change either greater than 2 or lower than -2 and a FDR-corrected p-value of less than 0.05 were determined to be significantly differentially expressed. For visualization purposes, expression values in fkpm were transformed into z-scores using the R function "scale". The z-scores denote the expression difference (in the unit of standard deviations) of a given developmental stage with regard to the average expression level across all stages. Principal component analysis (PCA) was done by the prcomp function (with the scale = T option) of R. Data for the transcriptome profiles of *S. papillosus* and *C. elegans* were taken from [11,57].

Dafachronic acid experiments

Dafachronic acid experiments were performed similarly to as described in [13]. OP50 was grown overnight in LB medium and then centrifuged and resuspended in 1/5th volume of 0.9% NaCl. 90μl of resuspended bacteria and either 10μl of 10 or 100μM Δ7 dafachronic acid (diluted in ethanol) or ethanol were combined and spotted on an NGM plate. L3 worms were

added to the plate and incubated for 14 days at 15˚C after which the plates were examined and all worms staged.

Statistical analysis and figure generation

Appropriate statistical analysis was carried out using Excel and R with statistical significance determined as being reached once the p-value was below 0.05. The exact statistical test used is noted in the figure or table legends. Microscopy images were resized in Photoshop and then annotated in Illustrator. Figures were generated in Excel, R and Illustrator.

Supporting information

S1 File. Species description and stage description of *Rhabditophanes diutinus*—Morphological descriptions for adults, dauers, J2As and J2s including measurements.
(PDF)

S2 File. Full data set with which all figures were generated, list of orthologs present within the Strongyloidoidea and full results of the transcriptomic study.
(XLSX)

S1 Fig. Clustering analysis of transcriptomic biological replicates.
(PDF)

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References

1. Perry RN, Wharton DA. Molecular and Physiological basis of nematode survival: CAB International; 2011:2011.
2. Organisation WH. Neglected Tropical Diseases. 2019. https://www.who.int/neglected_diseases/diseases/.
3. Boatin BA, Basáñez M-G, Prichard RK, Awadzi K, Barakat RM, García HH, et al. A research agenda for helminth diseases of humans: towards control and elimination. PLoS neglected tropical diseases. 2012; 6(4):e1547.
4. Weinstein SB, Kuris AM. Independent origins of parasitism in Animalia. Biol Lett. 2016; 12(7).
5. Viney M. How Can We Understand the Genomic Basis of Nematode Parasitism? Trends in parasitology. 2017; 33(6):444–52.
6. Blaxter ML, De Ley P, Garey JR, Liu LX, Scheldeman P, Vierstraete A, et al. A molecular evolutionary framework for the phylum Nematoda. Nature. 1998; 392(6671):71–5.
7. Crook M. The dauer hypothesis and the evolution of parasitism: 20 years on and still going strong. Int J Parasitol. 2014; 44(1):1–8.
8. Hu PJ. Dauer. WormBook. 2007:1–19.
9. Wolkow CA, Hall DH. Introduction to the Dauer Larva, Overview. In: Herndon LA, editor. WormAtlas2015.
10. Gilabert A, Curran DM, Harvey SC, Wasmuth JD. Expanding the view on the evolution of the nematode dauer signalling pathways: refinement through gene gain and pathway co-option. BMC Genomics. 2016; 17(1):476.
11. Ma G, Wang T, Korhonen PK, Stroehlein AJ, Young ND, Gasser RB. Dauer signalling pathway model for *Haemonchus contortus*. Parasit Vectors. 2019; 12(1):187.
12. Ayoade KO, Carranza FR, Cho WH, Wang Z, Kliewer SA, Mangelsdorf DJ, et al. Dafachronic acid and temperature regulate canonical dauer pathways during *Nippostrongylus brasiliensis* infectious larvae activation. Parasites & Vectors. 2020; 13(1):162.
13. Ogawa A, Streit A, Antebi A, Sommer RJ. A conserved endocrine mechanism controls the formation of dauer and infective larvae in nematodes. Current biology: CB. 2009; 19(1):67–71.
14. Bento G, Ogawa A, Sommer RJ. Co-option of the hormone-signalling module dafachronic acid-DAF-12 in nematode evolution. Nature. 2010; 466(7305):494–7.
15. Dulovic A, Streit A. RNAi-mediated knockdown of *daf-12* in the model parasitic nematode *Strongyloides ratti*. PLoS pathogens. 2019; 15(3):e1007705. https://doi.org/10.1371/journal.ppat.1007705 PMID: 30925161
16. Wang Z, Stoltzfus J, You Y-j, Ranjit N, Tang H, Xie Y, et al. The Nuclear Receptor DAF-12 Regulates Nutrient Metabolism and Reproductive Growth in Nematodes. PLOS Genetics. 2015; 11(3):e1005027.
17. Wang Z, Zhou XE, Motola DL, Gao X, Suino-Powell K, Conneely A, et al. Identification of the nuclear receptor DAF-12 as a therapeutic target in parasitic nematodes. Proceedings of the National Academy of Sciences. 2009; 106(23):9138.
18. Albarqi MMY, Stoltzfus JD, Pilgrim AA, Nolan TJ, Wang Z, Kliewer SA, et al. Regulation of Life Cycle Checkpoints and Developmental Activation of Infective Larvae in *Strongyloides stercoralis* by Dafachronic Acid. PLoS pathogens. 2016; 12(1):e1005358-e. https://doi.org/10.1371/journal.ppat.1005358 PMID: 26727267
19. Motola DL, Cummins CL, Rottiers V, Sharma KK, Li T, Li Y, et al. Identification of ligands for DAF-12 that govern dauer formation and reproduction in C. elegans. Cell. 2006; 124(6):1209–23.
20. Ma G, Wang T, Korhonen PK, Young ND, Nie S, Ang C-S, et al. Dafachronic acid promotes larval development in *Haemonchus contortus* by modulating dauer signalling and lipid metabolism. PLoS pathogens. 2019; 15(7):e1007960.
21. Poulin R, Randhawa HS. Evolution of parasitism along convergent lines: from ecology to genomics. Parasitology. 2015; 142 Suppl 1(Suppl 1):S6–S15.
22. Crook M, Thompson FJ, Grant WN, Viney ME. *daf-7* and the development of *Strongyloides ratti* and *Parastrongyloides trichosuri*. Molecular and Biochemical Parasitology. 2005; 139(2):213–23.
23. IHGC. Comparative genomics of the major parasitic worms. Nat Genet. 2019; 51(1):163–74.
24. Holterman M, van der Wurff A, van den Elsen S, van Megen H, Bongers T, Holovachov O, et al. Phylum-wide analysis of SSU rDNA reveals deep phylogenetic relationships among nematodes and accelerated evolution toward crown clades. Mol Biol Evol. 2006; 23(9):1792–800.
25. Hunt VL, Tsai IJ, Coghlan A, Reid AJ, Holroyd N, Foth BJ, et al. The genomic basis of parasitism in the *Strongyloides* clade of nematodes. Nat Genet. 2016; 48(3):299–307.

26. Buonfrate D, Bisanzio D, Giorli G, Odermatt P, Fürst T, Greenaway C, et al. The Global Prevalence of *Strongyloides stercoralis* Infection. Pathogens (Basel, Switzerland). 2020; 9(6):468.
27. Nutman TB. Human infection with *Strongyloides stercoralis* and other related *Strongyloides* species. Parasitology. 2017; 144(3):263–73.
28. Viney ME, Lok JB. The biology of *Strongyloides* spp. WormBook. 2015:1–17.
29. Streit A. How to become a parasite without sex chromosomes: a hypothesis for the evolution of *Strongyloides* spp. and related nematodes. Parasitology. 2014; 141(10):1244–54.
30. Houthoofd W, Willems M, Jacobsen K, Coomans A, Borgonie G. The embryonic cell lineage of the nematode *Rhabditophanes* sp. Int J Dev Biol. 2008; 52(7):963–7. PMID: 18956326
31. Félix MA, De Ley P, Sommer RJ, Frisse L, Nadler SA, Thomas WK, et al. Evolution of vulva development in the Cephalobina (Nematoda). Dev Biol. 2000; 221(1):68–86. https://doi.org/10.1006/dbio.2000.9665 PMID: 10772792
32. Willems M, Houthoofd W, Claeys M, Couvreur M, Van Driessche R, Adriaens D, et al. Unusual intestinal lamellae in the nematode *Rhabditophanes* sp. KR3021 (Nematoda: Alloinematidae). Journal of Morphology. 2005; 264(2):223–32.
33. Kulkarni A, Lightfoot JW, Streit A. Germline organization in *Strongyloides* nematodes reveals alternative differentiation and regulation mechanisms. Chromosoma. 2016; 125(4):725–45.
34. Dorris M, Viney ME, Blaxter ML. Molecular phylogenetic analysis of the genus *Strongyloides* and related nematodes. Int J Parasitol. 2002; 32(12):1507–17.
35. Blaxter M, Koutsovoulos G, Jones M, Kumar S, Elsworth B. Phylogenomics of Nematoda. In: Cotton JA, Hughes J, Olson PD, editors. Next Generation Systematics. Systematics Association Special Volume Series. Cambridge: Cambridge University Press; 2016. p. 62–83.
36. Fuchs G. Neue an Borken- und Rüsselkäfer gebundene Nematoden, halb parasitische und Wohnungseinmeter. Zoologische Jahrbücher Abteilung für Systematik, Geographie und Biologie der Tiere. 1930.
37. Ćurčić BPM, Sudhaus W, Dimitrijević RN, Makarov SE, Tomić VT. *Rhabditophanes schneideri* (Rhabditida) phoretic on a cave pseudoscorpion. Journal of Invertebrate Pathology. 2008; 99(3):254–6.
38. Karp X. Working with dauer larvae. WormBook: the online review of C elegans biology. 2018; 2018:1–19.
39. Cassada RC, Russell RL. The dauerlarva, a post-embryonic developmental variant of the nematode *Caenorhabditis elegans*. Dev Biol. 1975; 46(2):326–42.
40. Rödelsperger C, Athanasouli M, Lenuzzi M, Theska T, Sun S, Dardiry M, et al. Crowdsourcing and the feasibility of manual gene annotation: A pilot study in the nematode *Pristionchus pacificus*. Scientific Reports. 2019; 9(1):18789.
41. Stoltzfus JD, Bart SM, Lok JB. cGMP and NHR signaling co-regulate expression of insulin-like peptides and developmental activation of infective larvae in Strongyloides stercoralis. PLoS pathogens. 2014; 10 (7):e1004235.
42. Sinha A, Sommer RJ, Dieterich C. Divergent gene expression in the conserved dauer stage of the nematodes Pristionchus pacificus and Caenorhabditis elegans. BMC Genomics. 2012; 13:254.
43. Kao G, Nordenson C, Still M, Rönnlund A, Tuck S, Naredi P. ASNA-1 positively regulates insulin secretion in *C. elegans* and mammalian cells. Cell. 2007; 128(3):577–87.
44. Baugh LR. To grow or not to grow: nutritional control of development during Caenorhabditis elegans L1 arrest. Genetics. 2013; 194(3):539–55.
45. Mayer MG, Sommer RJ. Natural variation in *Pristionchus pacificus* dauer formation reveals cross-preference rather than self-preference of nematode dauer pheromones. Proc Biol Sci. 2011; 278 (1719):2784–90.
46. Stasiuk SJ, Scott MJ, Grant WN. Developmental plasticity and the evolution of parasitism in an unusual nematode, Parastrongyloides trichosuri. Evodevo. 2012; 3(1):1.
47. Pires-daSilva A. *Pristionchus pacificus* protocols. WormBook. 2013:1–20.
48. Rödelsperger C. Comparative Genomics of Gene Loss and Gain in *Caenorhabditis* and Other Nematodes. Methods Mol Biol. 2018; 1704:419–32.
49. Shaham S. Methods in Cell Biology. WormBook. 2006.
50. Howe KL, Bolt BJ, Shafie M, Kersey P, Berriman M. WormBase ParaSite—a comprehensive resource for helminth genomics. Molecular and biochemical parasitology. 2017; 215:2–10. https://doi.org/10.1016/j.molbiopara.2016.11.005 PMID: 27899279
51. Ekseth OK, Kuiper M, Mironov V. orthAgogue: an agile tool for the rapid prediction of orthology relations. Bioinformatics. 2014; 30(5):734–6.
52. Smythe AB, Holovachov O, Kocot KM. Improved phylogenomic sampling of free-living nematodes enhances resolution of higher-level nematode phylogeny. BMC Evol Biol. 2019; 19(1):121.

53. Desjardins CA, Cerqueira GC, Goldberg JM, Hotopp JCD, Haas BJ, Zucker J, et al. Genomics of Loa loa, a Wolbachia-free filarial parasite of humans. Nature Genetics. 2013; 45(5):495–500. https://doi.org/10.1038/ng.2585 PMID: 23525074

54. Domazet-Loso T, Brajković J, Tautz D. A phylostratigraphy approach to uncover the genomic history of major adaptations in metazoan lineages. Trends Genet. 2007; 23(11):533–9.

55. Rödelsperger C, Prabh N, Sommer RJ. New Gene Origin and Deep Taxon Phylogenomics: Opportunities and Challenges. Trends Genet. 2019; 35(12):914–22. PMID: 31610892

56. Slater GS, Birney E. Automated generation of heuristics for biological sequence comparison. BMC Bioinformatics. 2005; 6:31.

57. Baskaran P, Jaleta TG, Streit A, Rödelsperger C. Duplications and Positive Selection Drive the Evolution of Parasitism-Associated Gene Families in the Nematode *Strongyloides papillosus*. Genome biology and evolution. 2017; 9(3):790–801. https://doi.org/10.1093/gbe/evx040 PMID: 28338804

Supplementary File 1: *Rhabditophanes diutinus*, Species Description

Adult: This species consists of only females. Cylindrical body shape, around 900μm long and 60μm wide. Lip regions consists of four sectors, each with a labial sensillum. Narrow buccal cavity with large pharynx consisting of a long narrow cylindrical procorpus, a small rounded metacorpus, a long thin cylindrical isthmus and a large circular well-rounded posterior bulb. Nerve ring sits over isthmus. Grinder appears present within the posterior bulb. Excretory pore present in the isthmus region. Intestine runs length of body, 28μm wide, from directly after the pharynx to the rectum. The intestine is not fixed to the body wall and is wrapped around the gonads. Well- developed sphincter following gonadal loop. Rectum is short and wide, with anus a raised opening in the form of a vertical slit in the cuticle. Vulva located at mid body, with a horizontal slit. Gonads are didelphic and wrap around the intestine, posterior arm is as a result normally hidden by the intestine. The gonad arms extend past the vulva in both directions. Germ cells are arranged in giant nuclei which are easily observable under DIC (10-12μm in size). Following the gonadal loop, undeveloped oocytes are present. These oocytes have a shiny appearance and are rich in cytoplasmic material. Whilst it appears that there is a spermatheca, these cells do not have nuclei. Maturing oocytes pass through them, after which they begin developing into embryos. Embryos are less common in other species and it is rare to see ever more than 2 per gonad arm in development. The exact development stage of the embryo when laid appears inconsistent. Embryos are around 55μm when laid.

Dauer Larvae: Cylindrical body shape, around 450μm long with a large amount of radial constriction (20μm wide at midpoint). Dauers are marked by the presence of a large buccal and intestinal plug consisting of multiple parts. Buccal cavity is narrow and restricted, with a large buccal plug. Further plugs are found in the upper third of the intestine, mid intestine, and lower third of intestine. These plugs appear shiny when viewed under DIC. The rest of the pharynx is constricted, with the posterior bulb appearing more pentagonal in shape than its usual spherical. Gonadal development is limited, consisting of around 10-20 cells, within a small smooth structure. Gut lumen is large and full of bacteria. No vulval development. Anus and rectum appear closed and are much narrower than usual. Cuticle is thicker and striated compared to adult stages.

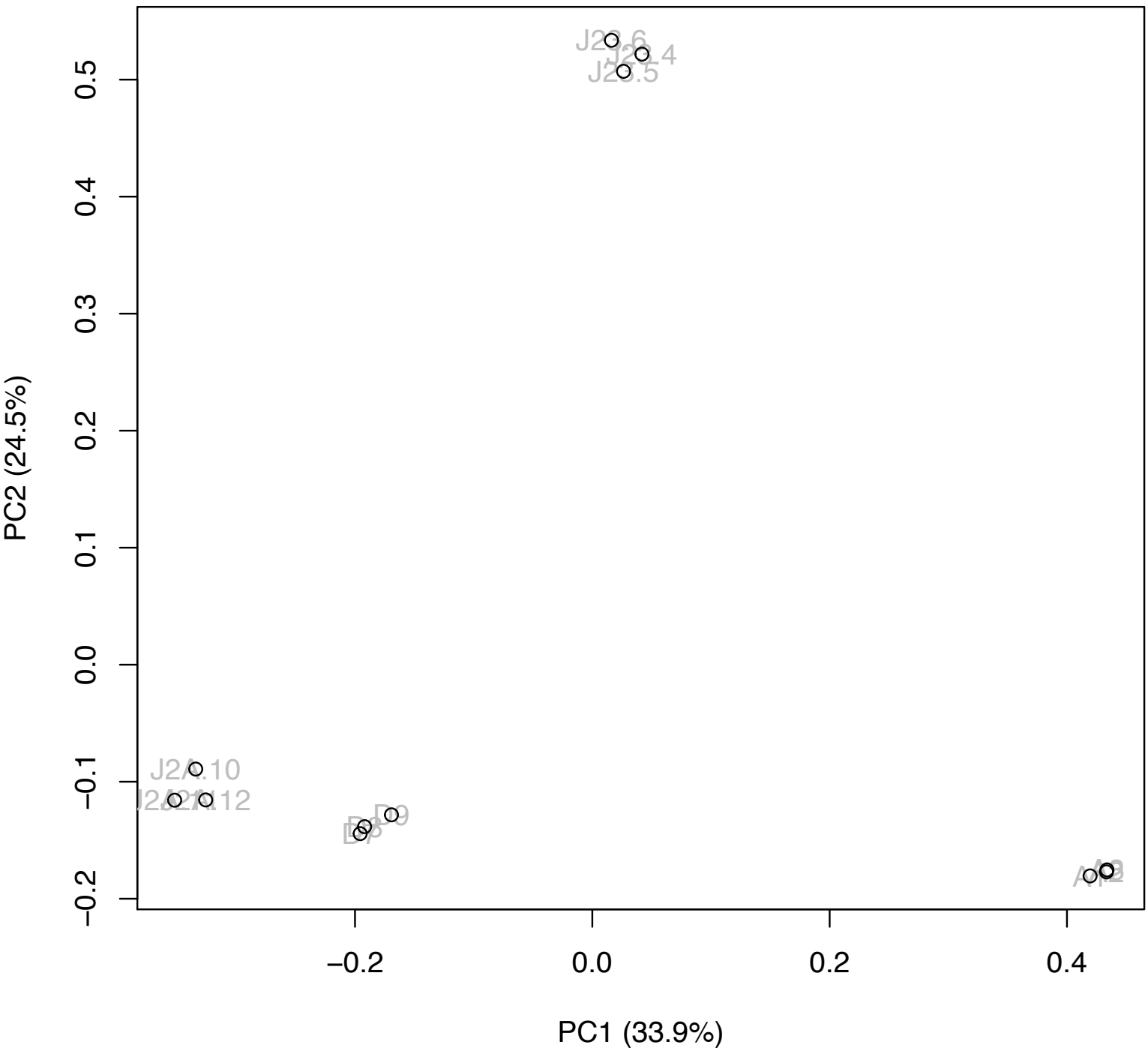
J2A Larvae: Cylindrical body shape, around 300μm in length, proportionally appears as J2 worm. Pharynx consists of 32% of total body length. Buccal cavity is narrow and open, pharynx fully developed. Intestine appears empty, lumen wall is thinner than other stages. Gonad consists of two to four progenitor cells. All other intracellular organelles appear endocytosed dependent on how long the worm has been in this stage for. Tail is short and triangular. Cuticle is striated. When in this stage for a prolonged period of time, the only identifiable features are the mouth, intestine and germline.

J2 Larvae: Cylindrical body shape, around 350μm in length. Pharynx consists of 28% of total body length. Buccal cavity is open and proportional, around 1.14μm in diameter, pharynx otherwise is fully developed. Intestine appears full of food and the lumen is much narrower (2μm) compared to the intestinal walls (6μm). Gonad consists of 4 cells in early J2, yet the gonad undergoes growth during the J2 stage and by the end often consists of around 20 cells. Rectum and anus appear normal and proportional for the stage. No vulval development present.

Measurement	Adult	Dauer	J2A	J2
Body Length	918.50 (836.28-968.77)	460.31 (392.08-512.09)	297.69 (284.88-312.60)	362.32 (329.98-406.43)
Buccal Cavity Width	3.29 (2.50-3.93)	n/a	0.95 (0.80-1.14)	1.14 (0.94-1.64)
Isthmus Width	10.71 (9.26-12.20)	17.02 (14.93-20.21)	4.61 (3.72-5.02)	5.68 (4.48-6.71)
Terminal Bulb Width	29.94 (23.98-34.29)	4.75 (4.28-5.09)	16.87 (15.75-17.75)	13.31 (12.15-14.23)
Body Width at Proximal Bulb	37.40 (34.55-42.38)	10.73 (10.00-12.19)	11.63 (11.13-12.51)	19.60 (18.49-20.89)
Body Width at Grinder	49.23 (43.49-52.20)	20.71 (18.43-23.27)	19.18 (18.39-20.32)	23.32 (21.65-26.83)
Pharynx Length	152.89 (141.38-170.74)	111.77 (103.63-117.51)	94.69 (91.45-97.50)	100.60 (91.84-110.35)
Pharynx Length of Body Length (%)	16.65 (15.61-18.55)	24.41 (22.30-29.27)	31.84 (29.50-34.23)	27.84 (25.36-29.59)
Intestinal Wall Width	11.58 (8.72-14.57)	4.57 (3.81-5.73)	3.39 (2.27-4.03)	6.52 (5.45-8.63)
Intestinal Lumen Width	4.90 (3.29-6.86)	5.42 (4.20-6.90)	3.82 (3.23-4.68)	2.07 (1.32-3.07)
Ratio of	0.42 (0.29-0.57)	1.19 (0.91-1.40)	1.17 (1.00-1.61)	0.32 (0.24-0.50)

Intestinal Lumen to Intestinal Wall				
Vulva/Germ cells from anterior	464.68 (426.18-526.99)	19.81 (17.80-21.92)	123.29 (114.03-137.76)	184.79 (146.50-206.08)
Vulva/Germ cells from anterior as % of body length	50.62 (46.20-55.55)	45.65 (40.93-50.22)	41.44 (38.79-46.82)	51.16 (42.87-61.24)
Body width at Vulva/Germ cells	60.36 (56.59-64.51)	209.28 (184.59-229.26)	15.83 (14.92-16.99)	20.99 (18.52-26.33)
Gonad length (anterior tip to loop)	273.34 (232.99-307.65)	67.35 (57.39-77.54)	12.80 (11.08-14.20)	24.94 (17.94-29.64)
Tail length (anus to tail tip)	102.15 (83.23-128.45)	57.27 (51.20-62.03)	51.32 (43.17-56.05)	57.12 (49.47-71.81)

All measurements are in µm unless stated, mean stated and range shown in brackets. As J2A, dauers and J2 do not have a developed vulva, the midpoint of the germ cell block is used in its place. At least 10 randomly selected individuals for each stage were measured.


PC2 (24.5%)
0.5
0.4
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0.2
0.1
0.0
−0.1
−0.2
−0.2
0.0
0.2
0.4
PC1 (33.9%)

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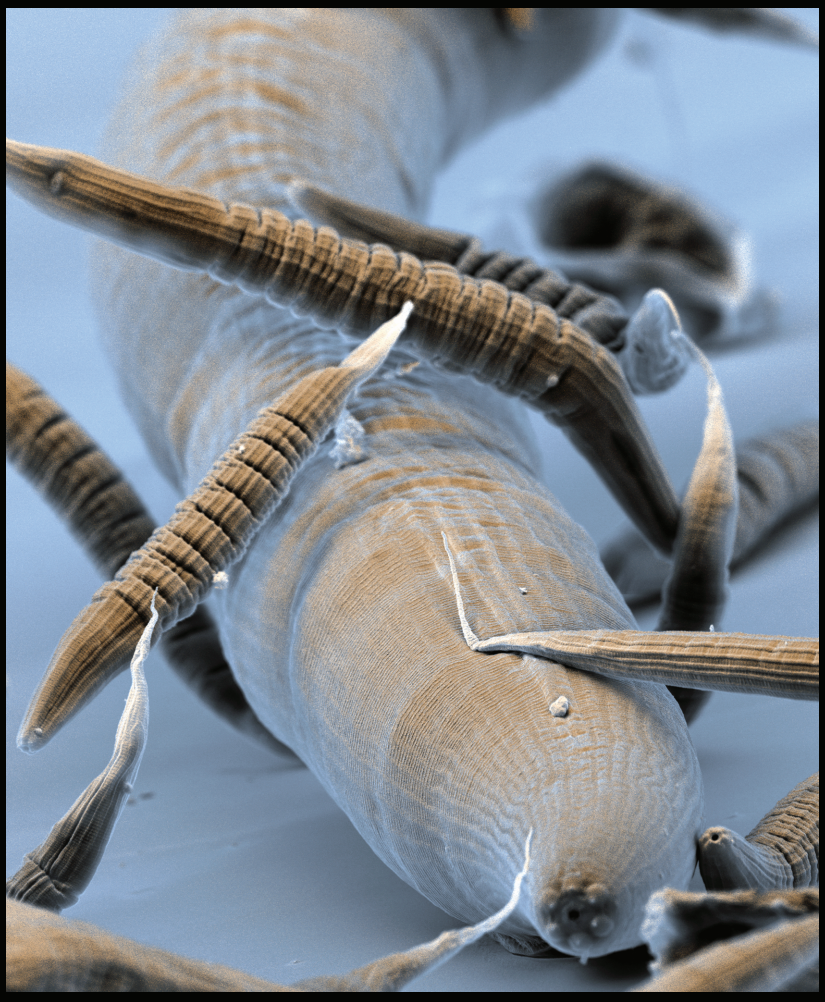

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Nematode biphasic 'boom and bust' dynamics are dependent on host bacterial load while linking dauer and mouth-form polyphenisms

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Abstract

Cross-kingdom interactions involve dynamic processes that shape terrestrial ecosystems and represent striking examples of co-evolution. The multifaceted relationships of entomopathogenic nematodes with their insect hosts and symbiotic bacteria are well-studied cases of co-evolution and pathogenicity. In contrast, microbial interactions in soil after the natural death of insects and other invertebrates are minimally understood. In particular, the turnover and succession of nematodes and bacteria during insect decay have not been well documented - although it represents a rich ecological niche with multiple species interactions. Here, we utilize developmentally plastic nematode *Pristionchus pacificus* and its associated scarab beetles as models. On La Réunion Island, we collected rhinoceros beetle *Oryctes borbonicus*, induced death, and placed carcasses in cages both on the island and in a mock-natural environment in the laboratory controlling for high spatial and temporal resolution. Investigating nematode population density and dispersal dynamics, we were able to connect two imperative plasticities, dauer and mouth form. We observed a biphasic 'boom and bust' dispersal dynamic of dauer larvae that corresponds to bacterial load on carcasses but not bacterial type. Strikingly, all post-dauer adults have the predatory mouth form, demonstrating novel intricate interactions on decaying insect hosts. Thus, ecologically relevant survival strategies incorporate critical plastic traits.

Introduction

'*Let's talk of graves*, *of worms*, *and epitaphs*.' William Shakespeare, 1592.

In King Richard II, William Shakespeare envisioned the necessity of detailing the multifaceted grave environment of a decaying insect host with its myriad bacteria and nematodes. Indeed, there is more than meets the eye in the multilayered interactions among microorganisms, and deconstructing these intricate relationships in an ecological context has proved arduous. It is well established that many genotype–environment interactions do not act in isolation; for example, the host microbiome is often involved in the plasticity of certain phenotypes (Gilbert *et al*., 2015; Bordenstein and Theis, 2015). The importance of the microbiome is deep rooted, as illustrated by the cascade of changes initiated by perturbations of the microbiome, influencing alterations in life history, morphological, and behavioural traits.

Life history strategies towards either longevity or reproduction can be influenced in *Drosophila melanogaster* by altering the microbiome composition (Clark *et al*., 2015; Gould *et al*., 2018). Root morphology in plants has been shown repeatedly to be influenced by mycorrhizal fungi alone or in combination with plant-associated bacteria (Berta *et al*., 1995; Cosme and Wurst, 2013). Social behavioural disorders in mice can be traced to inadequate microbiomes resulting from maternal diet and can be rescued by microbiome reconstitution (Buffington *et al*., 2016). Thus, fluctuations in microbiomes can result from the surrounding environment, creating more layers in studying these interactions.

Increasing interest in the natural ecology of the free-living nematode *Caenorhabditis elegans* has aided in detailing multitiered interactions between microorganisms

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and their environments. Surprisingly, while *C. elegans* is found worldwide in habitats with sundry bacterial communities, its basic microbial composition is conserved (Dirksen *et al*., 2016). The foundational microbiome and habitat bacteria can support or hinder worm growth, in addition to aiding in host immunity by protecting against infection (Zhang *et al*., 2017; Montalvo-Katz *et al*., 2013). However, these studies are somewhat limited in complexity because *C. elegans* lack tight host associations (Archer *et al*., 2020).

The soil nematode *Pristionchus pacificus* is a genetic and evolutionary model used to elucidate the ubiquitous insect–nematode–bacteria ecological niche (Sommer and McGaughran, 2013). Specifically, *P. pacificus* has a facultative necromenic symbiosis with scarab beetles worldwide (Herrmann *et al*., 2006; Herrmann *et al*., 2007). The worms live on the host in an arrested developmental stage, referred to as 'dauer larvae,' and upon host death exit the dauer stage and begin feeding on microbes decomposing the beetle carcass (Dieterich and Sommer, 2009; Ragsdale *et al*., 2015; Sudhaus, 2010). Note that to evade unsuitable environmental conditions, numerous nematodes enter the non-feeding, quasi-stress-resistant juvenile dauer stage (Grant and Viney, 2011; Frézal and Félix, 2015) (Fig. 1A). Indeed, environmental cues triggering dauer induction include extreme temperatures, high population density, and lack of adequate food (Golden and Riddle, 1984; Viney *et al*., 2003). Dauer larvae are also the dispersal stage of many nematodes, regulated by both signals from interspecific and intraspecific worms and environmental factors (Lee, 2002; Kaplan *et al*., 2012). On live beetles, *P. pacificus* is found exclusively in dauer (Ragsdale *et al*., 2015; Herrmann *et al*., 2010), a pattern that is reminiscent of many other insect-associated nematodes (Giblin-Davis, 1993).

Dauer is only one example of a phenotypically plastic trait that critically impacts *P. pacificus* lifestyle. Another vital characteristic is its mouth form: an irreversible decision is made at an early juvenile stage to develop either a strict-bacterial-feeding morph, stenostomatous (St) (Fig. 1B), or an omnivorous morph, eurystomatous (Eu) (Fig. 1C), that in addition to bacterial feeding, allows for predation on both fungi and other nematodes (Susoy and Sommer, 2016; Sudhaus 2010; Ragsdale *et al*., 2013). Like dauer, mouth form is also influenced by population density and environmental conditions, including food availability and age-class-dependent crowding effects (Sanghvi *et al*., 2016; Werner *et al*., 2017; Werner *et al*., 2018). Thus, the decomposing beetle carcass teeming with assorted bacteria and nematodes is an ideal system to study the intricacies of multivariate symbioses and their effects on plasticity and niche construction. However, scrutinizing the life history of *P. pacificus* on beetle carcasses has proved difficult due to the inaccessible aspects of the scarab beetle life cycle: the egg to the pupal stage is below ground in the soil (chafers) or dead wood (stag beetles). Only the adult beetle stage is

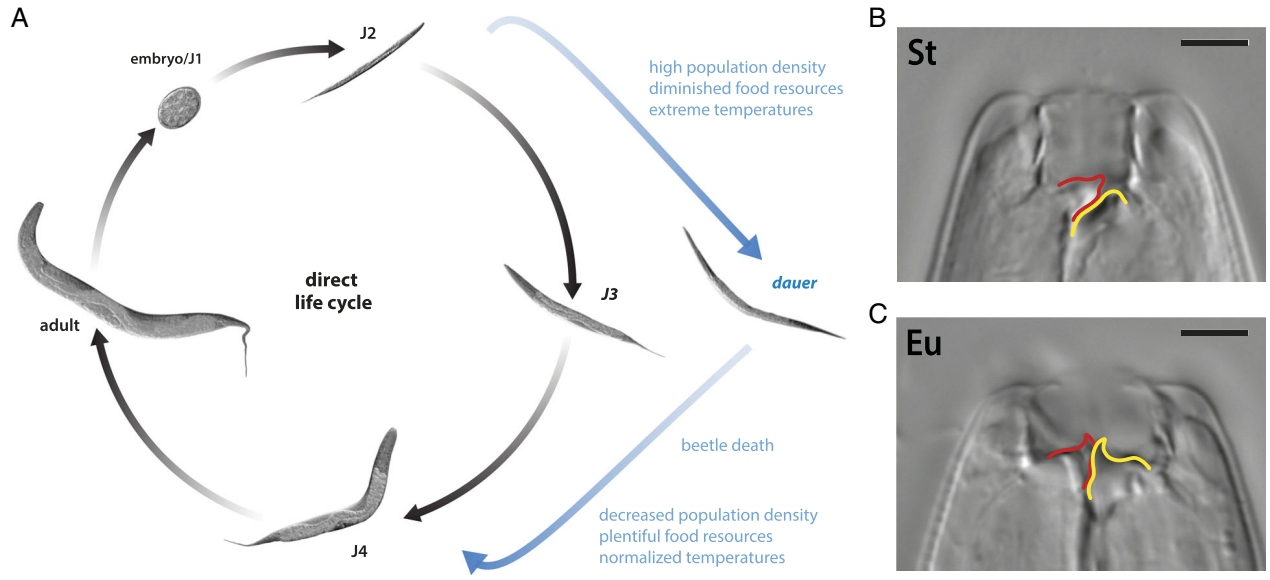

Fig. 1. *Pristionchus pacificus* maintains two critical plastic traits affecting their life cycles and mouth forms. A. The life cycle of *P. pacificus* includes four juvenile stages following embryonic development and concludes with self-fertilizing hermaphrodites (and occasional males). Under particular environmental conditions, worms can enter an arrested developmental stage, dauer, which resume development into reproducing adults when conditions change. B. The narrow stenostomatous (St) mouth only allows for bacterial feeding; it lacks the subventral tooth (yellow) and the dorsal tooth (red) is thin and elongated. C. The wide Eurystomatous (Eu) mouth is omnivorous; the claw-like dorsal (red) and subventral (yellow) teeth enable predation on other nematodes. Scale bar is 5 μM.

freely accessible but often short-lived (Fig. S1). Nonetheless, we have recently begun to study nematode succession on the beetle ecosystem using unique study opportunities on La Réunion Island in the Indian Ocean.

La Réunion harbours a worldwide diversity of *P. pacificus* haplotypes due to multiple invasions with different beetle hosts (Hermann *et al*., 2010; Morgan *et al*., 2014). Genetic studies have revealed a complex population structure with multiple migrations among neighbouring islands, which have provided detailed insight into the evolutionary ecology of this nematode (Morgan *et al*., 2014; Morgan *et al*., 2012; Moreno *et al*., 2016; McGaughran *et al*., 2013; McGaughran *et al*., 2016). In addition, the high abundance of some of the *P. pacificus* insect hosts also allows unprecedented understanding of the life history of the nematode. The rhinoceros beetle *Oryctes borbonicus*, which is endemic to the island, has the highest known infestation rates of *P. pacificus* (Herrmann *et al*., 2010; Meyer *et al*., 2016). Recently, *O. borbonicus* has been used to uncover the dynamics of nematode succession after beetle death (Meyer *et al*., 2017). In these studies, Meyer and coworkers followed the carcass dynamics from day one of beetle death to 12 days on the island by placing decapitated beetles in small metal cages in their natural environment. They found that worms exit dauer 1-week post-beetle death, and first-generation dauers have gone through just one generation in the direct life cycle. The beetle carcass microbiome was dominated by a single family, and was less diverse, but more stable, than the surrounding soil microbiome (Meyer *et al*., 2017). While these studies provided the first insight into the succession of *P. pacificus* in the beetle carcass ecosystem, it was also limited in both spatial (restricted to just the beetle carcass) and temporal (<2 weeks) resolutions. Moreover, in this experimental design, it was impossible to determine if the presence or absence of certain bacteria influenced nematode succession.

Here, we both expanded the space observed and extended the duration of observation. Our aim was to inform on the elusive ecology of insect–nematode–bacteria interactions by exploring *P. pacificus* population density and dispersal via experiments both in the wild and in the laboratory, specifically by answering four questions: what is the nematode succession on the carcass over several months following beetle death; is there a bacterial succession on the carcass and does it correspond to the nematode dispersal pattern; what is the mouth form preference of nematodes after exiting from the dauer stage; and at what rate do nematodes disperse? We answered these questions by following the succession of *P. pacificus* and bacteria on decaying *O. borbonicus* carcasses in the months immediately following beetle death. Every 2 weeks we observed the bacteria and nematode composition, and determined that the worms employ two survival strategies: dauer and predatory morphs. *Pristionchus pacificus* displays a biphasic 'boom and bust' dispersal pattern that reflects bacterial load on the carcass, but, surprisingly, not bacterial type. Strikingly, all post-dauer adults adopted the predatory mouth form. These findings demonstrate novel intricate interactions on decaying insect hosts and reveal that nematode survival strategies incorporate two critical plastic traits. These aspects of their natural lives provide ecological significance for several developmental characteristics often utilized in research and illuminate ubiquitous ecological dynamics.

Methods

Beetle collecting and cage set-ups in both the wild and in lab

Adult scarab beetle *O. borbonicus* were caught at our standard collection site Trois Bassins (TB) on La Réunion Island using an established light trap method (Herrmann *et al*., 2006) (Fig. 2A). Beetles used in island cages were decapitated before placement of both parts (head and abdomen) (Fig. 2B) in cages, while beetles bound for laboratory cages were kept alive individually in aerated falcon tubes with moistened tissues until the cages were assembled in the lab. For island cages, soil from TB was autoclaved; for lab cages, standard gardening soil (Einheitserde, Classic Profisubstrat) was autoclaved. Cages, columns, and compartment dividers were made from sterilised stainless steel mesh (cages and columns were made with 1.25 mm mesh and 0.4 mm wire, compartment dividers with 0.5 mm mesh and 0.25 mm wire). Cages were 15 × 15 cm, with nine columns in each cage, 3 × 3, fitting snuggly with little space between them (Fig. 2C). Each column was separated into three equal parts by dividers (Fig. 2D). Worms could move freely through the dividers and columns. Two beetles (both heads and abdomen) were placed in the middle compartment of the middle column, along with soil. While previous collection years showed that the *P. pacificus* infestation rate of *O. borbonicus* at TB was 90% (Morgan *et al*., 2012), we added two beetles per cage to ensure that each cage would have worms. Thus, any outliers in dispersal time could reflect the lack of both beetles being infested. The rest of the compartments were filled with soil, the columns placed in the cages, and soil added to fill any gaps. On the island, the cages were buried 15 cm deep at various sites to account for any orientation effects in TB (Fig. 2E, Fig. S2). Cages in lab were placed in boxes; the cages sat upon pedestals submerged in water with paper towels covering the cages and dipping into the water in order to imitate the wet environment of

Fig. 2. Beetle catching and cage set-up on La Réunion. A. Light-catches effectively attract adult *O. borbonicus* at our traditional collecting site, Trois Bassins (TB) (panel in the upper right-hand corner is a Google Earth image of La Réunion Island, east of Madagascar in the Indian Ocean, and its flag). B. Before placed in cages, beetles were decapitated to induce death and initiate nematode emergence. C. Nine columns were placed in each cage, 3 × 3. The two beetles were placed in the centre compartment of the centre column. D. Metal-mesh 15 cm-long columns were divided into three compartments by metal-mesh that allows for free movement of nematodes. E. Cages were buried 15 cm deep at various sites around TB (more detailed schematic of burial sites can be seen in Fig. S2).

TB. These boxes were kept in incubators set to 17°C, the average soil temperature of TB as determined in previous studies (Meyer *et al*., 2017). We began our studies in 2017, with 6 and 12 months as the time points on the island and 3 months as the shortest time point in the lab. Unexpectedly, after 3 months there were worms throughout the compartments in lab conditions. Thus, the following 2 years, 2018 and 2019, we had our first time points at 2 weeks (which is roughly equivalent to the longest time point in Meyer *et al*., 2017), and every 2 weeks for 3 months. We omitted time points earlier than 2 weeks, including $t = 0$, as Meyer *et al*. (2017) detailed those, and *O. borbonicus* remains an endangered species. For greater sample size, Figs 3A,B and 4A–C represent combined data from laboratory and field cages. For more insight into the long-term dynamics on the carcasses, we kept two cages originally buried in 2017 on the island that remained for 2 years.

At each given time point (2, 4, 6, 8, 10, and 12 weeks), cages on the island were dug up and the contents of each compartment were transferred to labelled falcon tubes. Whatever was left of the beetle carcasses was separated from the soil of that compartment and placed in its own falcon tube. The tubes were packaged and expedited to our lab in Tübingen, Germany. Baermann funnels were used to isolate the nematodes from the soil (Viglierchio and Schmitt, 1983), which were transferred to nematode growth medium (NGM) plates to be identified by morphology and counted under a dissecting stereomicroscope. Beetle carcasses were placed in 50 ml falcon tubes with 20 ml M9 and spun on a wheel for 30 min at 20°C. The carcasses were then moved to NGM plates,

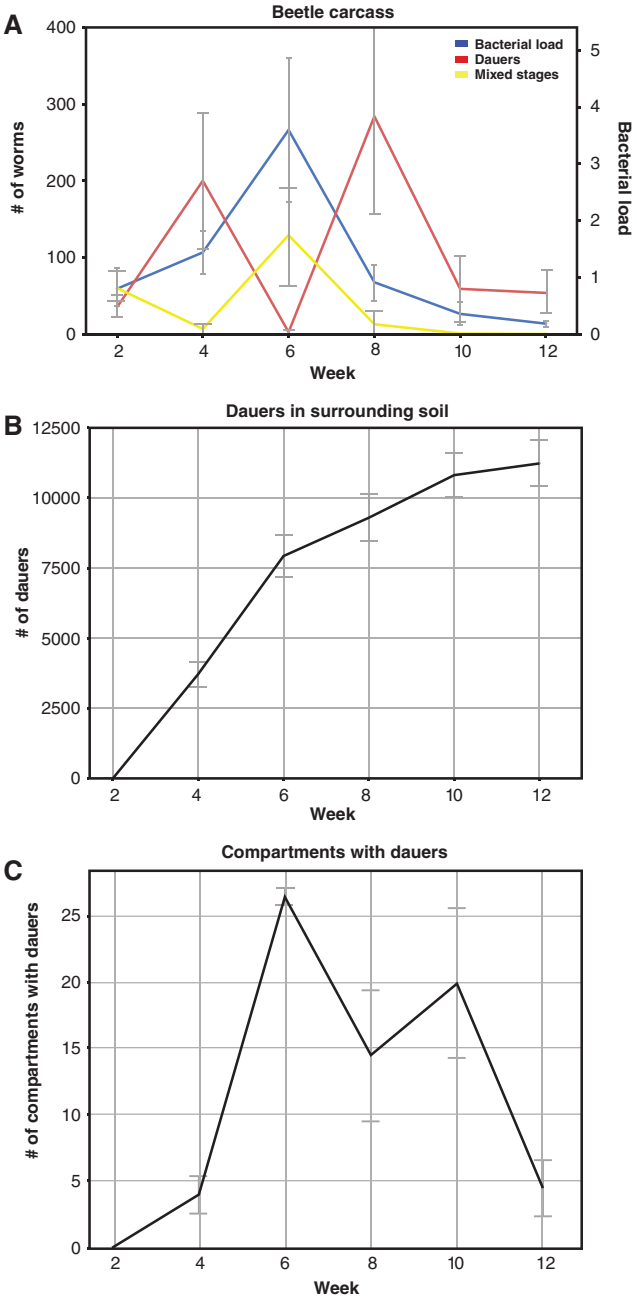

Fig. 3. Biphasic 'boom and bust' dispersal dynamics occur within 3 months post-beetle death. A. Nematode succession and bacterial load were tracked on decaying beetle carcasses every 2 weeks for 3 months (*x*-axis). The bacterial load (blue, right *y*-axis) is 2^{Ct} and normalized to OP50 *E. coli*. The number of dauers (red) and various mixed-staged, non-dauer worms (yellow) are shown using the left *y*-axis. At 4 and 8 weeks, when bacterial load is low, there are two dauer dispersal events. At 6 weeks, when bacteria are at peak abundance, there are many feeding mixed-stage worms and few dauers. At 10 weeks, the bacterial load and number of worms taper off. N = two La Réunion cages, eight lab cages. Error bars are standard deviation. B. The number of dauers in the surrounding compartments of the carcass increases over time. N = two La Réunion cages, eight lab cages. Error bars are standard error of the mean. C. The number of compartments (27 total) containing dauers (N = two La Réunion cages). Only field cages are included here, as dauers in the laboratory cages are more constrained in space and compartmental analysis may misrepresent proximity to origin.

and the beetle wash screened immediately for nematodes. The wash was then used for further investigation, as detailed in 'Beetle bacterial quantification.' Nematodes remaining on the carcasses (that had not washed off) were later counted as they emerged on the NGM plates.

DNA extraction for molecular identification

To verify the identity of nematode species, DNA was extracted from worms using a lysis solution. A polymerase chain reaction (PCR) was used to amplify a 1-kb region of the small subunit rRNA gene (SSU), utilising the following primers: SSU18A (5′-AAAGATTAAGCCATGCATG-3′) and SSU26R (5′-CATTCTTGGCAAATGCTTTCG-3′) (Blaxter *et al*., 1998), and conditions: denaturation at 95°C for 5 min, amplification by 35 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 90 s, concluding with 72°C for 10 min. Using the Applied Biosystems BigDye sequencing master mix, primer SSU9R (5′-AGCTGGAATTACCGCGGCTG-3′) (Blaxter *et al*., 1998), and 0.3 μl of the PCR product, sequences were produced on an Applied Biosystems sequencer (Darmstadt, Germany). Sequence chromatograms were trimmed using SeqMan Pro and 'blasted' for taxonomic identification against the NCBI reference database.

Beetle bacterial quantification

Immediately following screening of nematodes ('Nematode isolation and screening'), the falcon tubes containing the beetle washes were centrifuged (20 min, 4°C, 4000 x g). The supernatants were discarded, while the pellets were used for DNA extraction using the DNeasy PowerSoil Kit and following the manufacturer's instructions. Using 16S rRNA universal primers 515f (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGYCAGCMGCCGCGGTAA-3′) and 806r (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACNVGGGTWTCTAAT-3′) and 1× LightCycler® 480 SYBR Green I Master Mix (Roche), quantitative real-time PCR was utilised to determine bacterial load on a LightCycler 480. Bacterial load was quantified by averaging the Cp values of four technical replicates to obtain 2^{Ct} (that passed Dixon's *Q* test) and normalized to relative quantities of *Escherichia coli* OP50.

16S Metagenomics sequencing sample preparation and sequencing

The same DNA samples extracted from the beetle washes were used for bacterial MiSeq preparation and sequencing. The samples were prepared using Illumina's

16S Metagenomics Sequencing Library Preparation and following the manufacturer's protocol. In addition to the time points from 2 weeks to 12 weeks, we included beetle washes from two cages that remained on La Réunion for 2 years.

Analysis of MiSeq results and data construction

Sequencing results were processed following the MiSeq SOP (Quast *et al*., 2012). Briefly, the paired-end reads were assembled into contigs; the medium length being 465 base pairs. The putative Chimera were detected using Wang classifier (Wang *et al*., 2007). Sequences were then clustered into operational taxonomic units (OTUs) at 97% similarity. Taxonomies were assigned to OTUs using release 123 of the SILVA 16S ribosomal RNA database (Kozich *et al*., 2013) as the reference. The rare OTUs that appeared in fewer than four samples were removed to avoid inclusion of spuriously identified OTUs. Samples with fewer than 4000 reads were not included in the analyses (ranged from 9521 to 427 178 sequences per sample), and the data were subsampled using the size of the smallest sample (size = 9521). The OTUs from each sample were used to calculate the diversity index and principal component analysis (PCA). Tukey–Kramer HSD was used for week-wise multiple comparisons of OTU and alpha diversity. The data can be accessed under NCBI BioProject accession number PRJNA698805.

Dauer induction of laboratory strains and mouth-form phenotyping

Laboratory strains PS312, RS5205, RSC019, and RS5410 were kept on NGM agar plates seeded with OP50 *Escherichia coli*. Adults were transferred to fresh plates every 4 days to prevent starvation and overcrowding; these were used for direct-life cycle mouth-form phenotyping. Egg-filled plates were washed and bleached for synchronization (3.5 ml of M9-washed worms, 1 ml bleach, and 0.5 ml NaOH), filtered carcasses using 120 μm Millipore nylon screens, and washed with 3 ml M9 and centrifuged at 1300 rpm for 2 min. Egg pellets were resuspended in 10 ml S-medium and added to 50 ml Erlenmeyer flasks. For worms undergoing the direct life cycle, 100 ml (per flask) of OP50 *E. coli* grown in LB with an optical density of 0.5 were centrifuged at 4°C at 4000 rpm for 30 min, as described in Werner *et al*. (2017). The pellet was resuspended in the 10 ml S-medium. For dauer induction, 25% more eggs were added per flask and only 25 ml of OP50 *E. coli*. Flasks were kept at 22°C and 180 rpm. At the given time points, worms were removed from flasks, filtered and added to NGM-agar plates seeded with 300 μl OP50 *E. coli*. Dauers were first isolated using 1% sodium dodecyl sulfate and subsequently washed with M9 (Cassada and Russell, 1975). Mouth forms were phenotyped using differential interference contrast on a Zeiss Axioskop.

Results and discussion

Pristionchus *displays biphasic 'boom and bust' dynamics*

To determine the succession of *P. pacificus* on the beetle carcass, we observed carcasses every 2 weeks, starting at 2 weeks post-beetle death and up until 12 weeks. We screened two cages at the sampling site at TB and eight lab cages per time point. Remarkably, we found no differences between nematode succession trends in lab versus wild cages, arguing that the laboratory conditions are effective in simulating natural conditions (Fig. S3). At 2 weeks post-beetle death there is an almost-even amalgam of mixed stage worms, including dauers and feeding worms (developing through the direct life cycle and becoming reproducing adults). However, a drastic change occurs by 4 weeks: there is a surge of dauers and plunge of feeding worms (Fig. 3A). Strikingly, 2 weeks later, this configuration is reversed: there is an increase in the number of feeding worms and virtually no dauers. Yet at 8 weeks, the carcasses are again swarming with dauers and few feeding animals remain. These stark contrasts can be explained by the bacterial load on the carcasses at the corresponding times (Fig. 3A). At 4 weeks on the carcass, dauers dominate while feeding stages are low in number; thus, bacterial populations have an opportunity to bloom. However, as more bacteria become available, dauers are induced to exit and resume development, seen by week 6. Consumption of bacteria progresses until the bacteria become depleted, which then causes worms to enter dauer between weeks 6 and 8. A small population of dauers remain on the carcass after 8 weeks but do not exit; both bacteria and feeding worms dwindle in population. Taken together, these observations reveal two major dispersal events occurring just after 4 and 8 weeks, when dauer numbers are at peaks on the carcass, and then scatter (Fig. 3A and B). Thus, *P. pacificus* displays a biphasic 'boom and bust' dispersal pattern. Note that this general conclusion results from studies under both wild and laboratory conditions and additionally, was confirmed by repeating some of the trials in a second year.

Bacterial composition of beetle carcass is not associated with dauer dispersal. Next, we were interested in whether the 'boom and bust' dispersal pattern corresponds to bacterial succession on the decaying beetle carcass. For that, we performed 16S metagenomics

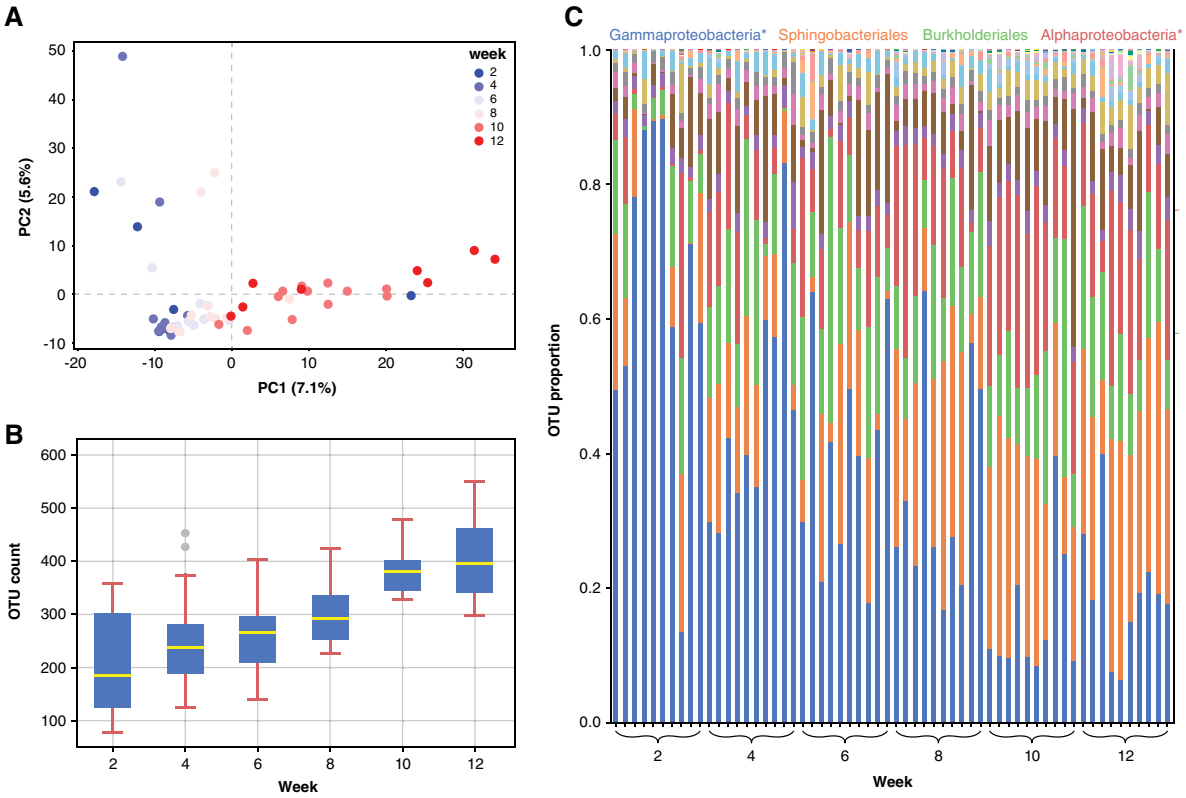

Fig. 4. Bacterial succession on the beetle carcass does not indicate a major influencer in timing of dauer dispersal. A. Principal component analysis (PCA) of the relative abundances of beetle bacterial order for the first 3 months post-beetle death. B. Box plot of the operational taxonomic unit (OUT) of bacterial genera for the first 3 months post-beetle death. C. Relative abundance of bacterial order; the four in highest abundance are Gammaproteobacteria (unclassified), Sphingobacteriales, Burkholderiales, and Alphaproteobacteria (unclassified). A more detailed legend can be found in Fig. S4A and described OTUs can be found in Table S1. *Unclassified. Nematode isolation and screening

sequencing and performed a PCA using the relative abundances of bacterial genera from both lab- and field-cage carcasses (Fig. 4A). The first four time points (2, 4, 6, and 8 weeks) cluster together, while the later time points (10 and 12 weeks) show a slight increase in variability, explained by an increase in diversity. Intriguingly, our findings reveal that Proteobacteria and Bacteroidetes (ubiquitous Gram-negative bacteria involved in a variety of processes, frequently found as the main bacterial populators of various insects, and include the nematode laboratory food source, *Escherichia* [Jones *et al*., 2013]) dominate in abundance over the period from 2 to 8 weeks, and that there is no significant difference in the bacterial composition during this time period (Fig. 4C). Only OTUs at 2 weeks differed significantly from OTUs at 10 and 12 weeks, and OTUs at 6 weeks from at 12 weeks (Tukey's test, $p < 0.05$). These findings are consistent with the previous study that followed bacterial succession for 11 days (Meyer *et al*., 2017), arguing that the bacterial composition of an insect carcass in the soil remains remarkably constant for almost 2 months after death. It is important to add that even at the genus level, there were no bacterial groupings that distinguish periods of nematode dispersal from nematode feasts on the carcass (Fig. S4B). Thus, the bacterial composition on the beetle remains consistent over the first 2 months despite large swings in nematode life cycle frequencies.

While beetle bacterial succession did not correspond to the two nematode dispersal events, bacterial genera abundance does reflect nematode load. A boxplot depicting number of OTUs for each time point reveals that at 10 weeks, when most worms have left the carcass, bacterial diversity increases (Fig. 4B; 2 weeks significantly differs from 10 and 12 weeks, Tukey's test, $p < 0.05$). This is true for diversity at 12 weeks as well, and at 2 years, when very few dauers remain (two cages, 19 and 6 dauers on each pair of carcasses). Note that the samples from the 2-year La Réunion cages display the greatest variability, as shown in a Shannon–Wiener index confirming that diversity increases on the carcass over time (Fig. S4C; 2 weeks differs significantly from 10, 12, and 108 weeks, 4 weeks from 12 and 108 weeks,

6 from 10, 12, and 108, Tukey's test, $p < 0.05$). Thus, the bacterial community decreases in rate of variance in the absence of nematodes on a decaying insect carcass.

Beetle-derived dauers develop predatory mouth forms. We were curious as to the mouth-form frequencies of post-dauer animals on beetle carcasses. Intriguingly, all 180 carcass dauers observed per time point developed Eu (predatory) mouth forms, resulting in a total of 1440 Eu animals (Fig. 5A). Yet, when singled-out and subsequent generations were screened, not all lines maintained highly Eu ratios. Even as early as the F_1 generation (developing directly into adults via J3), several lines exhibited intermediate mouth-form ratios (Fig. 5B). To determine if the high incidence of the predatory morph is an effect of development via dauer or the beetle environment, we induced dauer in four lab strains that under normal conditions have differing mouth-form predilections: PS312 (highly Eu), RS5205 (roughly 50:50), RSC019 (roughly 50:50), and RS5410 (highly St). Surprisingly, these laboratory-induced dauers did not all develop predatory morphs, regardless of the length of the dauer diapause (Fig. 5C and D, S4c-d). Both the highly Eu and highly St strains remained at their respective extremes, and the two 50:50 strains ultimately became slightly more St the longer they were dauer. This is in stark contrast to the wild dauers from the carcasses, which all developed predatory morphs. In addition, worms from the carcass that did not develop via dauer were not all Eu (Fig. S5B), and neither were dauers found in the surrounding soil (Fig. S5A). Together, these findings argue for a substantial link between plastic trait formation in an ecologically relevant context. While it remains currently unknown which effect of the decaying beetle environment causes post-dauer adults to exclusively express the Eu mouth form, it is known that the mouth-form decision and killing efficiency are influenced by various factors, including environmental perturbations (Werner *et al*., 2017) and bacterial diets (Akduman *et al*., 2020).

Nematodes depart host carcass after one generation and disperse rapidly. Finally, we wanted to determine the dispersal rate of *P. pacificus* throughout our cage setup. We followed worm movement from the carcass starting at 2 weeks, for every 2 weeks up until 12 weeks by analysing the content of all 27 compartments individually. While at 2 weeks there were dauers found on the beetle carcass, there were not any seen in the 26 compartments surrounding the beetle compartment (Fig. 3B). At 4 weeks, dauers were seen in the surrounding soil; presumably, the dauers seen on the carcass at 2 weeks began dispersing in search of a new host. Surprisingly, at 6 weeks, there were dauers in all 27 compartments indicating an unexpectedly high dispersal rate. The number of dauers in the surrounding soil increases with time and eventually tapers off after 3 months (Fig. 3B). In addition, the number of compartments positive with worms is high between 6 and 10 weeks, after which not many compartments contain worms (Fig. 3C). Note that worms were dispersed evenly across different compartment types; depth nor proximity to the ground surface appear to play a role in dispersion direction. Also, none of the soil compartments was inhabited with any reproducing nematode stage, and only contained dauers. In summary, these findings reveal multitiered dispersal events following beetle death, possibly representing different behavioural and ecological strategies. For instance, some dauers depart

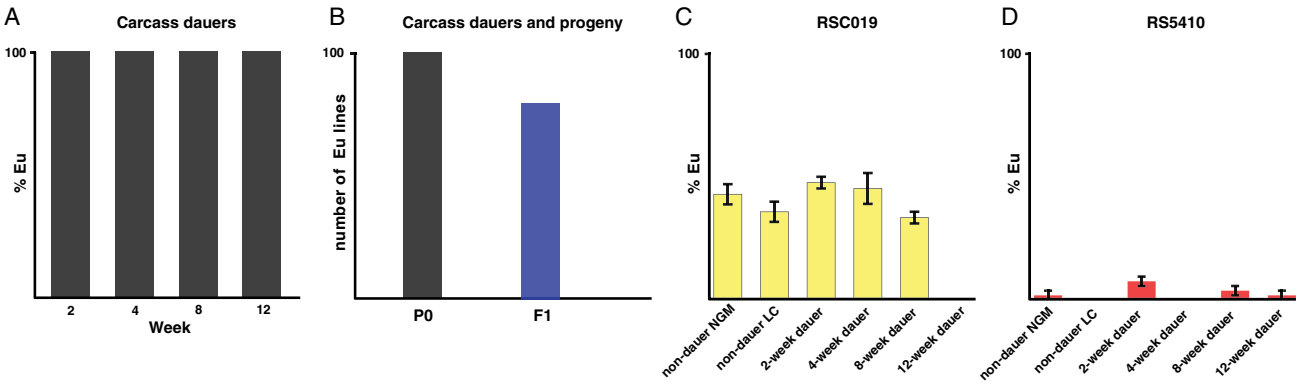

Fig. 5. Wild dauers from the beetles are exclusively predators, unlike domesticated lab dauers. A. All 1440 carcass-derived dauers observed from all time points developed the predatory mouth form, Eu. B. Carcass-derived singled dauers ($n = 100$) and the number of F_1 progeny lines that were strictly Eu (20 F_1s screened per line). Eighty of the 100 P_0 lines had only Eu progeny. (C–D) Laboratory strains raised on nematode growth media (NGM) display the strains' standard MF ratio. Liquid culture (LC) was used to induce dauer; as this culture method has been shown to alter MF ratios, the MF of worms developing via the direct life cycle in LC are also shown. C. RSC019 is roughly a 50:50 strain on NGM and slightly more St in LC. Post-dauer, the strain remains roughly 50:50. The low numbers at 3 months are due to the lack of surviving dauer worms for that duration. D. Highly St RS5410 remains highly St in all conditions. (C–D) Error bars are standard errors of the mean. Three biological replicates ($n = 45$) per condition/time point per strain; one-way ANOVA $p > 0.05$ for both.

the host carcass after as few as 2 weeks, even though there is still ample bacterial load.

Ecological significance of plastic trait dynamics. The analysis of the nematode and bacterial succession on beetle carcasses provides novel insight on both spatial and temporal dynamics and supports a key role for plastic trait development as a nematode survival strategy. Additionally, we observed previously unknown links between the two major polyphenisms of *P*. *pacificus*, dauer and mouth-form development. Dauer formation, as seen in our cage systems and associated dispersal events, could be induced by any of the manifold activities ensuing on the carcass; the beetle host can be co-infested with other nematodes (Meyer *et al*., 2017) and other organisms in the surrounding soil, which could partake in consuming the arising bacterial bloom (Duncan *et al*., 2003; Campos-Herrera *et al*., 2015). Thus, the two dispersal events may reflect the competitive conditions on the carcass; when resources become too scarce, most *P*. *pacificus* enter dauer and disperse, with few remaining in case conditions improve, the latter of which would be indicative of a bet-hedging strategy. Remarkably, dauers remained on the carcass even 2 years after host death, though we do not know how many generations of dauers occurred during this time. This surprising finding suggests an alternative, passive survival strategy, though we cannot rule out the possibility that these dauers have returned to the carcass after originally departing. Hence, we hypothesize that there are two contrasting dauer strategies, stationary and migratory dauer larvae: while these worms employ the same physiological strategy, there are different ecological consequences. Future work will be necessary to understand associated molecular mechanisms that might disentangle stationary (non-dispersal) and migratory (dispersal) dauers, a potential novel form of behavioural plasticity.

Dauer induction is influenced by various factors, both environmental and inter- and intraspecific signals. *Pristionchus pacificus* strains interact utilising ascarosides, a group of small molecule nematode-derived modular metabolites that act as pheromones and can induce dauer entry and exit, with strain-dependent interactions (Bose *et al*., 2012; Bose *et al*., 2014; Falcke *et al*., 2018). The capacity for certain strains to induce dauer entry in other strains and not their own (Mayer and Sommer, 2011; Mayer *et al*., 2015) is an imperative competitive manipulation that may play a role in the boom and bust cycle observed on *O*. *borbonicus*. Furthermore, the complex NDMM dasc#1 specific to *P*. *pacificus* strongly influences mouth form (Bento *et al*., 2010; Bose *et al*., 2012). Interestingly, this molecule is produced almost exclusively by adults and induces juveniles to develop the predatory morph in a density-dependent manner (Werner *et al*., 2018). Future long-term studies with even more sophisticated setups are necessary to reveal the chemistry associated with these observations.

Surprisingly, we did not find any evidence that the bacterial composition of the beetle carcass is associated with nematode succession (Fig. 4); no specific bacterial class or genus corresponds to the dispersal events. Many other nematodes are dependent on bacteria to carry out their life cycles. Entomopathogenic nematodes (EPNs) *Steinernema* and *Heterorhabditis* release mutualistic bacteria to infect and kill their hosts; the juvenile EPNs then develop into reproducing adults as they feed off the host carcass (Dillman *et al*., 2012a, Dillman *et al*., 2012b; Lee and Stock, 2010). *Pristionchus pacificus*' relationship with *O*. *borbonicus* is more flexible; the worm neither induces host death nor relies on certain bacteria to cause host death. The microbiomes of Los Angeles scarab beetles associated with *Pristionchus* are not affected by the nematode (Koneru *et al*., 2016). Though *P*. *pacificus* survival can be impacted by natural bacterial strains (Akduman *et al*., 2018), so a specific cocktail on the beetle carcass beyond the resolution of 16S classification could influence dispersal strategy.

In *C*. *elegans*, recent studies detail derailment and enhanced recovery of dauers depending on the bacterial diet (Bubrig *et al*., 2020). The frequency of *C*. *elegans* dauers is associated with Bacteroidetes-rich communities (Samuel *et al*., 2016); in our study, we found a slight increase in the presence of Bacteroidetes after 2 months. This is consistent with the occurrence of dauers and lack of feeding stages on the carcass after 8 weeks. In *C*. *elegans*, the interactions among the bacterial taxa in the worm gut were different from the interactions of the same taxa in the surrounding environment (Berg *et al*., 2016); while we did not see an effect of bacterial composition on dispersal, there may be subtle factors that went undetected.

Finally, it should be noted that the dispersal tactic may relate to the beetle life cycle (Fig. S1); the short-lived (potentially a few months) adult female beetle dies after egg-laying in the soil, so remaining *P*. *pacificus* may be waiting to colonize beetle grubs, which are known to be relatively stationary, only moving up and down the soil column. There were no grubs in our cages; therefore, future studies must examine the *P*. *pacificus* interaction with and infection of *O*. *borbonicus* grubs; grub presence near the deceased adults may influence the local bacterial composition and affect nematode dispersal. Due to the proximity of grubs to the decaying adults, we hypothesize that nematodes may infect these juveniles, though this remains to be determined. Larval development takes roughly 3 years (Rochat, pers. obs.), providing ample opportunity for infestation, even for the observed stationary dauers that may much later be migratory. However,

whether nematodes are attracted to *Oryctes* grubs is currently unknown and awaits future investigations. Another *P. pacificus* host, the oriental beetle *Exomala orinetalis*, produces a sex pheromone that attracts yet paralyses and arrests various developmental stages of *P. pacificus* (Cinkornpumin *et al*., 2014; Renahan and Hong, 2017), though nematodes also rely on olfactory cues to identify hosts (Hong *et al*., 2008; Dillman *et al*., 2012a, Dillman *et al*., 2012b). This antagonism complicates the ability of different worm stages to infect specific beetle stages. Thus, a study exploring the dynamics of *P. pacificus* with beetle grubs is necessary to further illuminate this intricate relationship.

Conclusions

Our study results in a better understanding of the complex bacteria–nematode–insect dynamics. We detail, for the first time, the dispersal dynamics of *P. pacificus* up to 2 years after the death of one of its primary scarab beetle hosts. In a previous study in our lab, Meyer and coworkers described the early succession of nematodes and bacteria on the beetle carcass but were limited both spatially and temporally, which prevented them from establishing associations between bacteria and *P. pacificus* dispersal. Here, we extended the observed duration time and expanded the space observed to elucidate four aspects: the nematode succession that occurs for months following beetle death, the bacterial succession on the carcass and its influence on nematode dispersal, the mouth forms the nematodes develop, and the rate at which nematodes disperse. Altogether, nematode communities live in highly variable environments that affect species' population sizes, developmental rates, reproductive strategies, and feeding habits. The particular combinations of nematodes and their interactions along with fluctuating microbial blooms contribute to the success of the worms. Unexpectedly, the dispersal dynamic of *P. pacificus* is biphasic and corresponds to bacterial abundance on the carcasses but is not influenced by any particular bacterial type. The multifarious elements on the decaying carcass in conjunction with nematodes dynamically interacting affect two critical plastic traits that aid in survival, dauer and mouth form. The biphasic 'boom and bust' dispersal cycle with the evolution of the predatory morph serves as competition strategies to both broaden dietary range and consume nematode competitors on decaying beetle carcasses. The capacity to develop a predatory morph enables *P. pacificus* to predate and consume other nematodes (Wilecki *et al*., 2015), while avoiding cannibalism by self-recognition (Lightfoot *et al*., 2019). Together, this epitaph describes the details of worms' lives in beetle graves, which provide ecologically relevant information for future studies involving environmental-induced plasticity in *P. pacificus*.

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Author Contributions

T.R. and R.J.S. conceived and designed the experiments. T.R., W.-S.L., M.H., J.R. and R.J.S. were involved in cage set-up on the island. T.R. performed and analysed cage and MF experiments, and conducted 16S library prep for MiSeq. W.-S.L. analysed MiSeq data. T.R. and M.S.W. performed bacterial load experiments. T.R. wrote the manuscript with assistance from R.J.S. and edits from M.S.W.

References

Akduman, N., Lightfoot, J.W., Röseler, W., Witte, H., Lo, W. S., Rödelsperger, C., and Sommer, R.J. (2020) Bacterial vitamin B 12 production enhances nematode predatory behavior. *ISME J* **14**: 1494–1507.

Akduman, N., Rödelsperger, C., and Sommer, R.J. (2018) Culture-based analysis of *Pristionchus*-associated microbiota from beetles and figs for studying nematode-bacterial interactions. *PLoS One* **13**: e0198018.

Archer, H., Deiparine, S., and Andersen, E.C. (2020) The nematode *Caenorhabditis elegans* and the terrestrial isopod *Porcellio scaber* likely interact opportunistically. *PLoS One* **15**: e0235000.

Bento, G., Ogawa, A., and Sommer, R.J. (2010). Co-option of the hormone-signalling module dafachronic acid–DAF-12 in nematode evolution. *Nature* **466**: 494–497. http://dx.doi.org/10.1038/nature09164.

Berg, M., Stenuit, B., Ho, J., Wang, A., Parke, C., Knight, M., *et al*. (2016) Assembly of the *Caenorhabditis elegans* gut microbiota from diverse soil microbial environments. *ISME J* **10**: 1998–2009.

Berta, G., Trotta, A., Fusconi, A., Hooker, J.E., Munro, M., Atkinson, D., *et al*. (1995) Arbuscular mycorrhizal induced changes to plant growth and root system morphology in *Prunus cerasifera*. *Tree Physiol* **15**: 281–293.

Blaxter, M.L., De Ley, P., Garey, J.R., Liu, L.X., Scheldeman, P., Vierstraete, A., *et al*. (1998) A molecular evolutionary framework for the phylum Nematoda. *Nature* **392**: 71–75.

Bordenstein, S.R., and Theis, K.R. (2015) Host biology in light of the microbiome: ten principles of holobionts and hologenomes. *PLoS Biol* **13**: e1002226.

Bose, N., Meyer, J.M., Yim, J.J., Mayer, M.G., Markov, G.V., Ogawa, A., *et al*. (2014) Natural variation in dauer pheromone production and sensing supports intraspecific competition in nematodes. *Curr Biol* **24**: 1536–1541.

Bose, N., Ogawa, A., von Reuss, S.H., Yim, J.J., Ragsdale, E.J., Sommer, R.J., and Schroeder, F.C. (2012) Complex small-molecule architectures regulate phenotypic plasticity in a nematode. *Angew Chem* **124**: 12606–12611.

Bubrig, L. T., Sutton, J. M., & Fierst, J. L. (2020) *C. elegans* dauer recovery varies with worm-bacteria interactions. *BioRxiv*.

Buffington, S.A., Di Prisco, G.V., Auchtung, T.A., Ajami, N.J., Petrosino, J.F., and Costa-Mattioli, M. (2016) Microbial reconstitution reverses maternal diet-induced social and synaptic deficits in offspring. *Cell* **165**: 1762–1775.

Campos-Herrera, R., Jaffuel, G., Chiriboga, X., Blanco-Pérez, R., Fesselet, M., Půža, V., *et al*. (2015) Traditional and molecular detection methods reveal intense interguild competition and other multitrophic interactions associated with native entomopathogenic nematodes in Swiss tillage soils. *Plant Soil* **389**: 237–255.

Cassada, R.C., and Russell, R.L. (1975) The dauerlarva, a post-embryonic developmental variant of the nematode *Caenorhabditis elegans*. *Develop Biol* **46**: 326–342.

Cinkornpumin, J.K., Wisidagama, D.R., Rapoport, V., Go, J. L., Dieterich, C., Wang, X., *et al*. (2014) A host beetle pheromone regulates development and behavior in the nematode *Pristionchus pacificus*. *Elife* **3**: e03229.

Clark, R.I., Salazar, A., Yamada, R., Fitz-Gibbon, S., Morselli, M., Alcaraz, J., *et al*. (2015) Distinct shifts in microbiota composition during *Drosophila* aging impair intestinal function and drive mortality. *Cell Rep* **12**: 1656–1667.

Cosme, M., and Wurst, S. (2013) Interactions between arbuscular mycorrhizal fungi, rhizobacteria, soil phosphorus and plant cytokinin deficiency change the root morphology, yield and quality of tobacco. *Soil Biol Biochem* **57**: 436–443.

Dieterich, C., and Sommer, R.J. (2009) How to become a parasite–lessons from the genomes of nematodes. *Trends Genet* **25**: 203–209.

Dillman, A.R., Chaston, J.M., Adams, B.J., Ciche, T.A., Goodrich-Blair, H., Stock, S.P., and Sternberg, P.W. (2012a) An entomopathogenic nematode by any other name. *PLoS Pathog* **8**: e1002527.

Dillman, A.R., Guillermin, M.L., Lee, J.H., Kim, B., Sternberg, P.W., and Hallem, E.A. (2012b) Olfaction shapes host–parasite interactions in parasitic nematodes. *Proc Natl Acad Sci U S A* **109**: E2324–E2333.

Dirksen, P., Marsh, S.A., Braker, I., Heitland, N., Wagner, S., Nakad, R., *et al*. (2016) The native microbiome of the nematode *Caenorhabditis elegans*: gateway to a new host-microbiome model. *BMC Biol* **14**: 1–16.

Duncan, L.W., Dunn, D.C., Bague, G., and Nguyen, K. (2003) Competition between entomopathogenic and free-living bactivorous nematodes in larvae of the weevil *Diaprepes abbreviatus*. *J Nematol* **35**: 187.

Falcke, J.M., Bose, N., Artyukhin, A.B., Rödelsperger, C., Markov, G.V., Yim, J.J., *et al*. (2018) Linking genomic and metabolomic natural variation uncovers nematode pheromone biosynthesis. *Cell Chem Biol* **25**: 787–796.

Frézal, L., and Félix, M.A. (2015) The natural history of model organisms: *C. elegans* outside the Petri dish. *Elife* **4**: e05849.

Giblin-Davis, R.M. (1993) Interactions of nematodes with insects. In *Nematode Interactions*. Dordrecht: Springer, pp. 302–344.

Gilbert, S.F., Bosch, T.C., and Ledón-Rettig, C. (2015) Eco-Evo-Devo: developmental symbiosis and developmental plasticity as evolutionary agents. *Nat Rev Genet* **16**: 611–622.

Golden, J.W., and Riddle, D.L. (1984) The *Caenorhabditis elegans* dauer larva: developmental effects of pheromone, food, and temperature. *Develop Biol* **102**: 368–378.

Gould, A.L., Zhang, V., Lamberti, L., Jones, E.W., Obadia, B., Korasidis, N., *et al*. (2018) Microbiome interactions shape host fitness. *Proc Natl Acad Sci U S A* **115**: E11951–E11960.

Grant, W., and Viney, M. (2011) The dauer phenomenon. In *Molecular and Physiological Basis of Nematode Survival*. Wallingford: CABI Publishing, pp. 99–125.

Herrmann, M., Kienle, S., Rochat, J., Mayer, W.E., and Sommer, R.J. (2010) Haplotype diversity of the nematode *Pristionchus pacificus* on Réunion in the Indian Ocean suggests multiple independent invasions. *Biol J Linnean Soc* **100**: 170–179.

Herrmann, M., Mayer, W.E., Hong, R.L., Kienle, S., Minasaki, R., and Sommer, R.J. (2007) The nematode *Pristionchus pacificus* (Nematoda: Diplogastridae) is associated with the oriental beetle *Exomala orientalis* (Coleoptera: Scarabaeidae) in Japan. *Zool Sci* **24**: 883–889.

Herrmann, M., Mayer, W.E., and Sommer, R.J. (2006) Nematodes of the genus *Pristionchus* are closely associated with scarab beetles and the Colorado potato beetle in Western Europe. *Zoology* **109**: 96–108.

Hong, R.L., Svatoš, A., Herrmann, M., and Sommer, R.J. (2008) Species-specific recognition of beetle cues by the nematode *Pristionchus maupasi*. *Evol Dev* **10**: 273–279.

Jones, R.T., Sanchez, L.G., and Fierer, N. (2013) A cross-taxon analysis of insect-associated bacterial diversity. *PLoS One* **8**: e61218.

Kaplan, F., Alborn, H.T., von Reuss, S.H., Ajredini, R., Ali, J. G., Akyazi, F., *et al*. (2012) Interspecific nematode signals regulate dispersal behavior. *PLoS One* **7**: e38735.

Koneru, S.L., Salinas, H., Flores, G.E., and Hong, R.L. (2016) The bacterial community of entomophilic nematodes and host beetles. *Mol Ecol* **25**: 2312–2324.

Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K., and Schloss, P.D. (2013) Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl Environ Microbiol* **79**: 5112–5120.

Lee, D.L. (ed). (2002) *The Biology of Nematodes*. Boca Raton, FL: CRC Press.

Lee, M.M., and Stock, S.P. (2010) A multilocus approach to assessing co-evolutionary relationships between *Steinernema* spp. (Nematoda: Steinernematidae) and their bacterial symbionts *Xenorhabdus* spp.(γ-Proteobacteria: Enterobacteriaceae). *Syst Parasitol* **77**: 1–12.

Lightfoot, J.W., Wilecki, M., Rödelsperger, C., Moreno, E., Susoy, V., Witte, H., and Sommer, R.J. (2019) Small

peptide–mediated self-recognition prevents cannibalism in predatory nematodes. *Science* **364**: 86–89.

Mayer, M.G., Rödelsperger, C., Witte, H., Riebesell, M., and Sommer, R.J. (2015) The orphan gene dauerless regulates dauer development and intraspecific competition in nematodes by copy number variation. *PLoS Genet* **11**: e1005146.

Mayer, M.G., and Sommer, R.J. (2011) Natural variation in *Pristionchus pacificus* dauer formation reveals cross-preference rather than self-preference of nematode dauer pheromones. *Proc R Soc B: Biol Sci* **278**: 2784–2790.

McGaughran, A., Morgan, K., and Sommer, R.J. (2013) Natural variation in chemosensation: lessons from an Island nematode. *Ecol Evol* **3**: 5209–5224.

McGaughran, A., Rödelsperger, C., Grimm, D.G., Meyer, J. M., Moreno, E., Morgan, K., *et al*. (2016) Genomic profiles of diversification and genotype–phenotype association in Island nematode lineages. *Mol Biol Evol* **33**: 2257–2272.

Meyer, J.M., Baskaran, P., Quast, C., Susoy, V., Rödelsperger, C., Glöckner, F.O., and Sommer, R.J. (2017) Succession and dynamics of *Pristionchus* nematodes and their microbiome during decomposition of Oryctes borbonicus on La Réunion Island. *Environ Microbiol* **19**: 1476–1489.

Meyer, J.M., Markov, G.V., Baskaran, P., Herrmann, M., Sommer, R.J., and Rödelsperger, C. (2016) Draft genome of the scarab beetle *Oryctes borbonicus* on La Réunion Island. *Genome Biol Evol* **8**: 2093–2105.

Montalvo-Katz, S., Huang, H., Appel, M.D., Berg, M., and Shapira, M. (2013) Association with soil bacteria enhances p38-dependent infection resistance in *Caenorhabditis elegans*. *Infect Immun* **81**: 514–520.

Moreno, E., McGaughran, A., Rödelsperger, C., Zimmer, M., and Sommer, R.J. (2016) Oxygen-induced social behaviours in *Pristionchus pacificus* have a distinct evolutionary history and genetic regulation from *Caenorhabditis elegans*. *Proc R Soc B: Biol Sci* **283**: 20152263.

Morgan, K., McGaughran, A., Ganeshan, S., Herrmann, M., and Sommer, R.J. (2014) Landscape and oceanic barriers shape dispersal and population structure in the Island nematode *Pristionchus pacificus*. *Biol J Linnean Soc* **112**: 1–15.

Morgan, K., McGaughran, A., Villate, L., Herrmann, M., Witte, H., Bartelmes, G., *et al*. (2012) Multi locus analysis of Pristionchus pacificus on La Réunion Island reveals an evolutionary history shaped by multiple introductions, constrained dispersal events and rare out-crossing. *Mol Ecol* **21**: 250–266.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., *et al*. (2012) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* **41**: D590–D596.

Ragsdale, E. J., Kanzaki, N., & Herrmann, M. (2015) Taxonomy and natural history: the genus *Pristionchus*. In *Pristionchus pacificus* **11**, (pp. 77–120). Brill.

Ragsdale, E.J., Müller, M.R., Rödelsperger, C., and Sommer, R. J. (2013) A developmental switch coupled to the evolution of plasticity acts through a sulfatase. *Cell* **155**: 922–933.

Renahan, T., and Hong, R.L. (2017) A species-specific nematocide that results in terminal embryogenesis. *J Exp Biol* **220**: 3238–3247.

Samuel, B.S., Rowedder, H., Braendle, C., Félix, M.A., and Ruvkun, G. (2016) *Caenorhabditis elegans* responses to bacteria from its natural habitats. *Proc Natl Acad Sci U S A* **113**: E3941–E3949.

Sanghvi, G.V., Baskaran, P., Röseler, W., Sieriebriennikov, B., Rödelsperger, C., and Sommer, R.J. (2016) Life history responses and gene expression profiles of the nematode *Pristionchus pacificus* cultured on Cryptococcus yeasts. *PLoS One* **11**: e0164881.

Sommer, R.J., and McGaughran, A. (2013) The nematode *Pristionchus pacificus* as a model system for integrative studies in evolutionary biology. *Mol Ecol* **22**: 2380–2393.

Sudhaus, W. (2010) Preadaptive plateau in Rhabditida (Nematoda) allowed the repeated evolution of zooparasites, with an outlook on evolution of life cycles within Spiroascarida. *Palaeodiversity* **3**: 117–130.

Susoy, V., and Sommer, R.J. (2016) Stochastic and conditional regulation of nematode mouth-form dimorphisms. *Front Ecol Evol* **4**: 23.

Viglierchio, D.R., and Schmitt, R.V. (1983) On the methodology of nematode extraction from field samples: Baermann funnel modifications. *J Nematol* **15**: 438.

Viney, M.E., Gardner, M.P., and Jackson, J.A. (2003) Variation in *Caenorhabditis elegans* dauer larva formation. *Dev, Growth Differ* **45**: 389–396.

Wang, Q., Garrity, G.M., Tiedje, J.M., and Cole, J.R. (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* **73**: 5261–5267.

Werner, M.S., Claaßen, M.H., Renahan, T., Dardiry, M., and Sommer, R.J. (2018) Adult influence on juvenile phenotypes by stage-specific pheromone production. *Iscience* **10**: 123–134.

Werner, M.S., Sieriebriennikov, B., Loschko, T., Namdeo, S., Lenuzzi, M., Dardiry, M., *et al*. (2017) Environmental influence on *Pristionchus pacificus* mouth form through different culture methods. *Sci Rep* **7**: 1–12.

Wilecki, M., Lightfoot, J.W., Susoy, V., and Sommer, R.J. (2015) Predatory feeding behaviour in *Pristionchus* nematodes is dependent on phenotypic plasticity and induced by serotonin. *J Exp Biol* **218**: 1306–1313.

Zhang, F., Berg, M., Dierking, K., Félix, M.A., Shapira, M., Samuel, B.S., and Schulenburg, H. (2017) *Caenorhabditis elegans* as a model for microbiome research. *Front Microbiol* **8**: 485.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1: Supporting information

Table S1. List of OTU orders of beetle bacterial succession corresponding to Figs. 4C and S1a.

Supplementary material for

Nematode biphasic "boom and bust" dynamics are dependent on host bacterial load while linking dauer and mouth-form polyphenisms

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Supplementary Figure Legends

Fig. S1: (A) Fig. S1: (a) *Oryctes borbonicus* life cycle, based off of personal observations of Jacques Rochat, both in the wild on La Réunion and home culture. Adults are observed in the wild from December to April, though in rearing some have lived for a year. A female in rearing is able to lay one egg every two weeks, and eggs incubate from two to four weeks in the summer and up to six in the winter (our experiments were conducted during the island's summer). Larval development takes 2.5 years, and pupation is roughly a month. Photos by Jacques Rochat.

Fig. S2: (A) Schematic of the beetle collection meadow and locations of buried field cages (indicated by a square; pairs were buried in the same hole together). Brown hexagon is the kiosk that can be seen in Fig. 2a, and the accompanying light for the beetle catch. Cages are overrepresented in size. Scale bar is 1 meter. Environments did not differ among pairs.

Fig. S3: (A-B) Figure 3a separated by sample origin. **(A)** Field cages on La Réunion (n=2) and **(B)** laboratory cages (n=8). Nematode succession and bacterial load were tracked on decaying beetle carcasses every two weeks for three months (x-axis). The bacterial load (blue, right y-axis) is 2^{Ct} and normalized to OP50 *E. coli*. The number of dauers (red) and various mixed-staged, non-dauer worms (yellow) are shown using the left y-axis. Error bars are standard deviation.

Fig. S4: (A) OTU legend for Fig. 4c, relative abundance of bacterial order. Described OTUs can be found in Table S1. **(B)** Relative abundance of bacterial genera for the first three months post-beetle death and two years later. **(C)** Shannon-Wiener diversity index of beetle bacterial order for the first three months post-beetle death and two years later.

Fig. S5: (A) MF of dauers from the carcass and dauers from the soil. n=180 for carcass dauers, n=10 for soil dauers per time point. One-tailed Student's t-

test $p < 0.05$ (*). **(B)** MF of carcass-derived dauers and carcass-derived non-dauers (worms developing directly via J3). n=180 per time point for dauers, n=10 per time point for non-dauers. One-tailed Student's t-test $p < 0.05$ (*). **(C)** highly Eu laboratory strain PS312 remains highly Eu post-dauer. n=45 per time point; one-way ANOVA $p > 0.05$. **(D)** 50:50 laboratory strain RS5205 maintains MF frequency post-dauer. n=45 per time point; one-way ANOVA $p > 0.05$.

Table S1: List of OTU orders of beetle bacterial succession corresponding to Figs. 4C and S1a.

Fig. S1

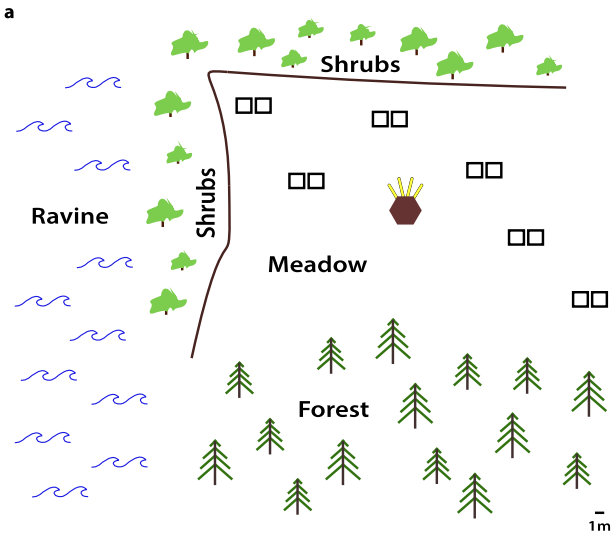

Fig. S2

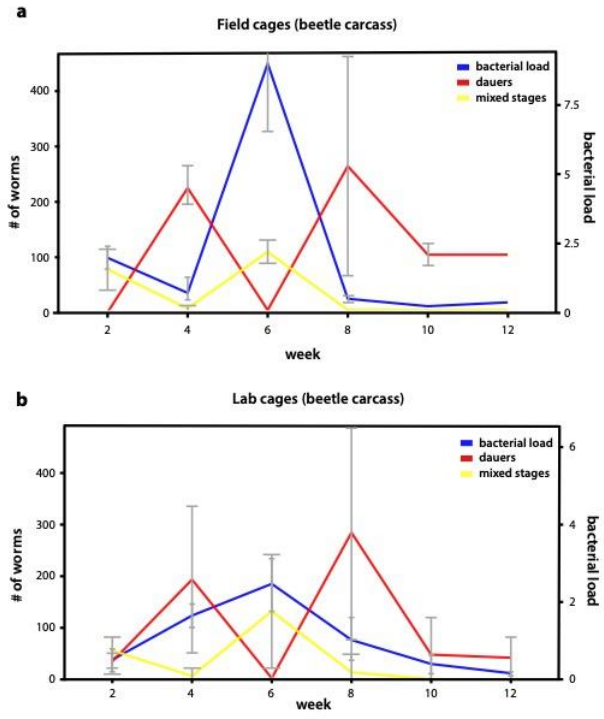
a
Field cages (beetle carcass)
bacterial load
dauers
mixed stages
# of worms
bacterial load
week
b
Lab cages (beetle carcass)
bacterial load
dauers
mixed stages
# of worms
bacterial load
week

Fig. S3

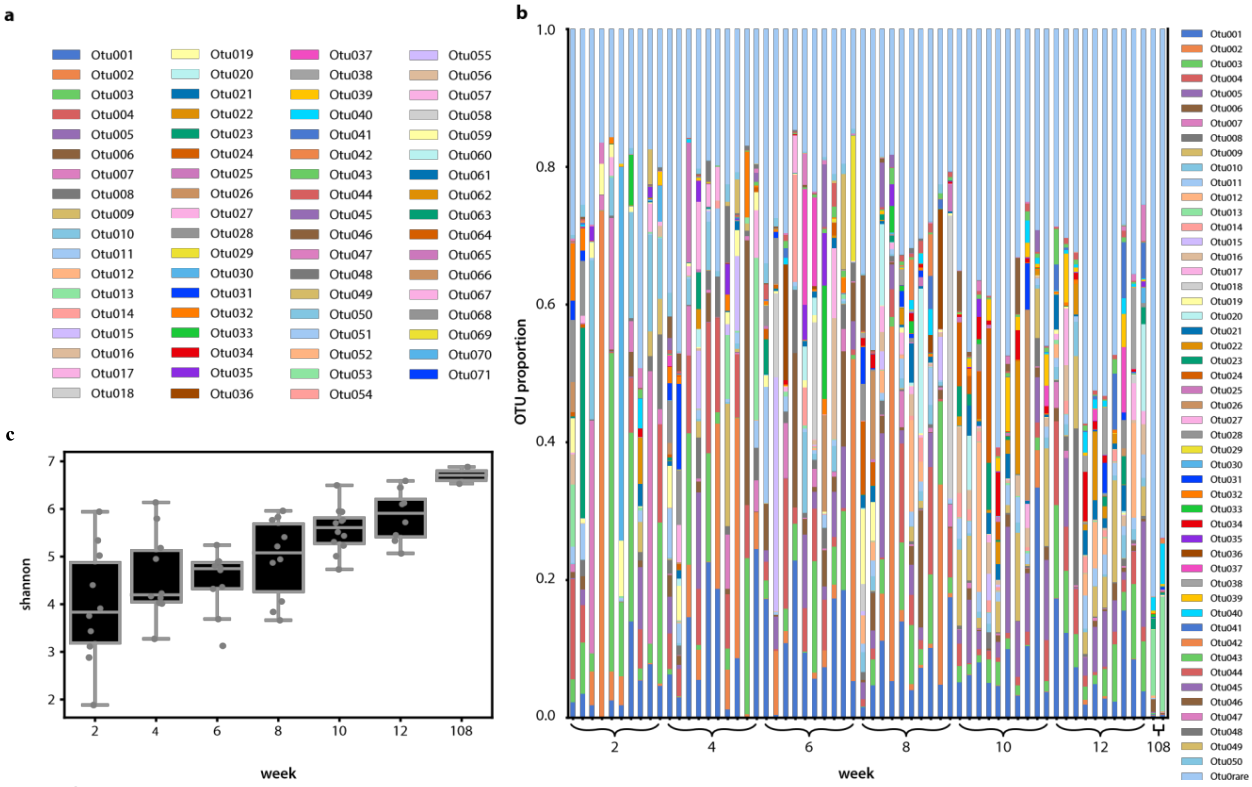
a
Otu001
Otu002
Otu003
Otu004
Otu005
Otu006
Otu007
Otu008
Otu009
Otu010
Otu011
Otu012
Otu013
Otu014
Otu015
Otu016
Otu017
Otu018
Otu019
Otu020
Otu021
Otu022
Otu023
Otu024
Otu025
Otu026
Otu027
Otu028
Otu029
Otu030
Otu031
Otu032
Otu033
Otu034
Otu035
Otu036
Otu037
Otu038
Otu039
Otu040
Otu041
Otu042
Otu043
Otu044
Otu045
Otu046
Otu047
Otu048
Otu049
Otu050
Otu051
Otu052
Otu053
Otu054
Otu055
Otu056
Otu057
Otu058
Otu059
Otu060
Otu061
Otu062
Otu063
Otu064
Otu065
Otu066
Otu067
Otu068
Otu069
Otu070
Otu071
b
OTU proportion
1.0
0.8
0.6
0.4
0.2
0.0
2
4
6
8
10
12
108
week
Otu0rare
c
shannon
7
6
5
4
3
2
2
4
6
8
10
12
108
week

Fig. S4

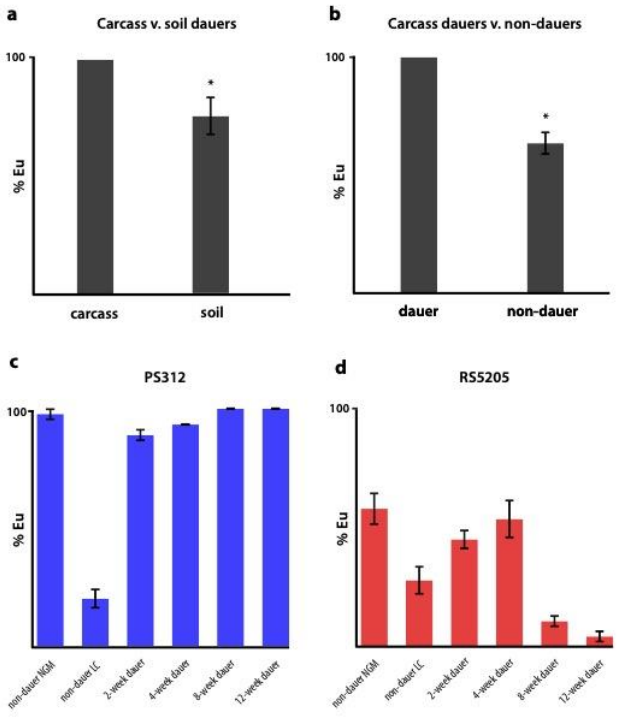
a
Carcass v. soil dauers
100
% Eu
*
carcass
soil
b
Carcass dauers v. non-dauers
100
% Eu
*
dauer
non-dauer
c
PS312
100
% Eu
non-dauer NGM
non-dauer LC
2-week dauer
4-week dauer
8-week dauer
12-week dauer
d
RS5205
100
% Eu
non-dauer NGM
non-dauer LC
2-week dauer
4-week dauer
8-week dauer
12-week dauer

Fig. S5

OTU	Size	Taxonomy
Otu001	2955296	Bacteria(100);Proteobacteria(100);Gammaproteobacteria(100);Gammaproteobacteria_unclassified(100);Gammaproteobacteria_unclassified(100);Gammaproteobacteria_unclassified(100);
Otu002	1436626	Bacteria(100);Bacteroidetes(100);Sphingobacteriia(100);Sphingobacteriales(100);Sphingobacteriaceae(63);Sphingobacteriaceae_unclassified(63);
Otu003	1098964	Bacteria(100);Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(98);Alcaligenaceae(66);Achromobacter(55);
Otu004	927970	Bacteria(100);Proteobacteria(100);Alphaproteobacteria(100);Alphaproteobacteria_unclassified(100);Alphaproteobacteria_unclassified(100);Alphaproteobacteria_unclassified(100);
Otu005	186236	Bacteria(100);Bacteria_unclassified(100);Bacteria_unclassified(100);Bacteria_unclassified(100);Bacteria_unclassified(100);Bacteria_unclassified(100);
Otu006	576475	Bacteria(100);Bacteroidetes(100);Flavobacteriia(100);Flavobacteriales(100);Flavobacteriaceae(95);Chryseobacterium(62);
Otu007	130586	Bacteria(100);Bacteroidetes(100);Bacteroidetes_unclassified(100);Bacteroidetes_unclassified(100);Bacteroidetes_unclassified(100);Bacteroidetes_unclassified(100);
Otu008	120264	Bacteria(100);Proteobacteria(100);Proteobacteria_unclassified(100);Proteobacteria_unclassified(100);Proteobacteria_unclassified(100);Proteobacteria_unclassified(100);
Otu009	143962	Bacteria(100);Actinobacteria(100);Actinobacteria(100);Actinomycetales(97);Actinomycetales_unclassified(97);Actinomycetales_unclassified(97);
Otu010	112002	Bacteria(100);Firmicutes(100);Bacilli(100);Bacillales(94);Bacillales_unclassified(94);Bacillales_unclassified(94);
Otu011	30850	Bacteria(100);Proteobacteria(100);Deltaproteobacteria(100);Myxococcales(93);Myxococcales_unclassified(93);Myxococcales_unclassified(93);
Otu012	30805	Bacteria(100);Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100);Porphyromonadaceae(97);Dysgonomonas(81);
Otu013	14756	Bacteria(100);Planctomycetes(100);Planctomycetia(100);Planctomycetales(100);Planctomycetaceae(100);Planctomycetaceae_unclassified(100);
Otu014	16475	Bacteria(100);Firmicutes(100);Clostridia(100);Clostridiales(100);Clostridiales_unclassified(100);Clostridiales_unclassified(100);
Otu015	15967	Bacteria(100);Bacteroidetes(100);Cytophagia(100);Cytophagales(100);Cytophagaceae(92);Dyadobacter(58);
Otu016	6595	Bacteria(100);Firmicutes(100);Firmicutes_unclassified(100);Firmicutes_unclassified(100);Firmicutes_unclassified(100);Firmicutes_unclassified(100);
Otu017	11598	Bacteria(100);Candidatus_Saccharibacteria(100);Candidatus_Saccharibacteria_unclassified(100);Candidatus_Saccharibacteria_unclassified(100);Candidatus_Saccharibacteria_unclassified(100);Candidatus_Saccharibacteria_unclassified(100)
Otu018	8437	Bacteria(100);Acidobacteria(100);Acidobacteria_Gp1(100);Acidobacteria_Gp1_unclassified(66);Acidobacteria_Gp1_unclassified(66);Acidobacteria_Gp1_unclassified(66);
Otu019	4556	Bacteria(100);Gemmatimonadetes(100);Gemmatimonadetes(100);Gemmatimonadales(100);Gemmatimonadaceae(100);Gemmatimonas(100);
Otu020	3491	Bacteria(100);Verrucomicrobia(100);Opitutae(100);Opitutales(99);Opitutaceae(99);Opitutus(96);
Otu021	3293	Bacteria(100);Verrucomicrobia(100);Verrucomicrobiae(100);Verrucomicrobiales(100);Verrucomicrobiaceae(100);Luteolibacter(91);
Otu022	2626	Bacteria(100);Acidobacteria(100);Acidobacteria_Gp3(100);Gp3(64);Gp3_unclassified(64);Gp3_unclassified(64);
Otu023	2931	Bacteria(100);Verrucomicrobia(100);Spartobacteria(100);Spartobacteria_unclassified(84);Spartobacteria_unclassified(84);Spartobacteria_unclassified(84);
Otu024	2874	Bacteria(100);Acidobacteria(100);Holophagae(100);Holophagales(100);Holophagaceae(100);Holophagaceae_unclassified(88);
Otu025	1730	Bacteria(100);Acidobacteria(100);Acidobacteria_Gp6(100);Gp6(100);Gp6_unclassified(100);Gp6_unclassified(100);
Otu026	1622	Bacteria(100);Acidobacteria(100);Acidobacteria_Gp4(100);Acidobacteria_Gp4_unclassified(100);Acidobacteria_Gp4_unclassified(100);Acidobacteria_Gp4_unclassified(100);
Otu027	1222	Bacteria(100);Verrucomicrobia(100);Subdivision3(100);Subdivision3_unclassified(100);Subdivision3_unclassified(100);Subdivision3_unclassified(100);
Otu028	802	Bacteria(100);Verrucomicrobia(100);Verrucomicrobia_unclassified(100);Verrucomicrobia_unclassified(100);Verrucomicrobia_unclassified(100);Verrucomicrobia_unclassified(100);
Otu029	1283	Bacteria(100);Chloroflexi(100);Thermomicrobia(100);Sphaerobacterales(78);Sphaerobacteraceae(78);Sphaerobacteraceae_unclassified(77);
Otu030	814	Bacteria(100);Chlamydiae(100);Chlamydiia(100);Chlamydiales(100);Chlamydiales_unclassified(53);Chlamydiales_unclassified(53);
Otu031	1010	Bacteria(100);Firmicutes(100);Erysipelotrichia(100);Erysipelotrichales(100);Erysipelotrichaceae(100);Erysipelothrix(84);
Otu032	685	Bacteria(100);candidate_division_WPS-1(100);candidate_division_WPS-1_unclassified(100);candidate_division_WPS-1_unclassified(100);candidate_division_WPS-1_unclassified(100);candidate_division_WPS-1_unclassified(100);
Otu033	497	Bacteria(100);Chloroflexi(100);Chloroflexi_unclassified(100);Chloroflexi_unclassified(100);Chloroflexi_unclassified(100);Chloroflexi_unclassified(100);
Otu034	461	Bacteria(100);Actinobacteria(100);Actinobacteria_unclassified(100);Actinobacteria_unclassified(100);Actinobacteria_unclassified(100);Actinobacteria_unclassified(100);
Otu035	259	Bacteria(100);Firmicutes(100);Negativicutes(100);Selenomonadales(100);Veillonellaceae(91);Veillonellaceae_unclassified(70);
Otu036	204	Bacteria(100);Acidobacteria(100);Acidobacteria_Gp16(100);Gp16(100);Gp16_unclassified(100);Gp16_unclassified(100);
Otu037	218	Bacteria(100);Armatimonadetes(100);Armatimonadetes_gp5(100);Armatimonadetes_gp5_unclassified(100);Armatimonadetes_gp5_unclassified(100);Armatimonadetes_gp5_unclassified(100);
Otu038	157	Bacteria(100);Parcubacteria(100);Parcubacteria_unclassified(100);Parcubacteria_unclassified(100);Parcubacteria_unclassified(100);Parcubacteria_unclassified(100);
Otu039	156	Bacteria(100);Acidobacteria(100);Acidobacteria_Gp2(100);Gp2(100);Gp2_unclassified(100);Gp2_unclassified(100);
Otu040	144	Bacteria(100);Armatimonadetes(100);Armatimonadetes_unclassified(100);Armatimonadetes_unclassified(100);Armatimonadetes_unclassified(100);Armatimonadetes_unclassified(100);
Otu041	78	Bacteria(100);Acidobacteria(100);Acidobacteria_unclassified(100);Acidobacteria_unclassified(100);Acidobacteria_unclassified(100);Acidobacteria_unclassified(100);
Otu042	112	Bacteria(100);Acidobacteria(100);Acidobacteria_Gp17(100);Gp17(100);Gp17_unclassified(100);Gp17_unclassified(100);
Otu043	129	Bacteria(100);Chloroflexi(100);Anaerolineae(100);Anaerolineales(100);Anaerolineaceae(100);Anaerolineaceae_unclassified(99);

Otu044	140	Bacteria(100);Latescibacteria(100);Latescibacteria_unclassified(100);Latescibacteria_unclassified(100);Latescibacteria_unclassified(100);Latescibacteria_unclassified(100);
Otu045	106	Bacteria(100);Nitrospirae(100);Nitrospira(100);Nitrospirales(100);Nitrospiraceae(100);Nitrospira(100);
Otu046	39	Bacteria(100);Planctomycetes(100);Phycisphaerae(100);Tepidisphaerales(54);Tepidisphaeraceae(54);Tepidisphaera(54);
Otu047	57	Bacteria(100);Synergistetes(100);Synergistia(100);Synergistales(100);Synergistaceae(100);Synergistaceae_unclassified(93);
Otu048	30	Bacteria(100);Acidobacteria(100);Acidobacteria_Gp5(100);Gp5(100);Gp5_unclassified(100);Gp5_unclassified(100);
Otu049	25	Bacteria(100);Armatimonadetes(100);Fimbriimonadia(100);Fimbriimonadales(100);Fimbriimonadaceae(100);Fimbriimonas(100);
Otu050	30	Bacteria(100);Chloroflexi(100);Ktedonobacteria(100);Ktedonobacterales(100);Ktedonobacteraceae(90);Ktedonobacter(90);
Otu051	49	Bacteria(100);Acidobacteria(100);Acidobacteria_Gp10(100);Gp10(100);Gp10_unclassified(100);Gp10_unclassified(100);
Otu052	20	Bacteria(100);Spirochaetes(100);Spirochaetia(100);Spirochaetales(100);Spirochaetaceae(80);Spirochaetaceae_unclassified(80);
Otu053	23	Bacteria(100);Acidobacteria(100);Acidobacteria_Gp7(100);Gp7(100);Gp7_unclassified(100);Gp7_unclassified(100);
Otu054	19	Bacteria(100);Planctomycetes(100);Planctomycetes_unclassified(100);Planctomycetes_unclassified(100);Planctomycetes_unclassified(100);Planctomycetes_unclassified(100);
Otu055	29	Bacteria(100);Chloroflexi(100);Caldilineae(100);Caldilineales(100);Caldilineaceae(100);Litorilinea(90);
Otu056	16	Bacteria(100);Acidobacteria(100);Acidobacteria_Gp13(100);Gp13(100);Gp13_unclassified(100);Gp13_unclassified(100);
Otu057	19	Bacteria(100);Fusobacteria(100);Fusobacteriia(100);Fusobacteriales(100);Leptotrichiaceae(100);Sebaldella(95);
Otu058	14	Bacteria(100);Tenericutes(100);Mollicutes(100);Mycoplasmatales(100);Mycoplasmataceae(100);Mycoplasma(100);
Otu059	5	Bacteria(100);Actinobacteria(100);Thermoleophilia(100);Thermoleophilales(100);Thermoleophilaceae(100);Thermoleophilum(100);
Otu060	11	Bacteria(100);Hydrogenedentes(100);Candidatus_Hydrogenedens(100);Candidatus_Hydrogenedens_unclassified(100);Candidatus_Hydrogenedens_unclassified(100);Candidatus_Hydrogenedens_unclassified(100);
Otu061	5	Bacteria(100);BRC1(100);BRC1_unclassified(100);BRC1_unclassified(100);BRC1_unclassified(100);BRC1_unclassified(100);
Otu062	5	Bacteria(100);Armatimonadetes(100);Armatimonadia(100);Armatimonadales(100);Armatimonadaceae(100);Armatimonas/Armatimonadetes_gp1(100);
Otu063	4	Bacteria(100);Proteobacteria(100);Oligoflexia(100);Oligoflexales(100);Oligoflexaceae(100);Oligoflexus(100);
Otu064	4	Bacteria(100);Armatimonadetes(100);Chthonomonadetes(100);Chthonomonadales(100);Chthonomonadaceae(100);Chthonomonas/Armatimonadetes_gp3(100);
Otu065	4	Bacteria(100);Acidobacteria(100);Acidobacteria_Gp11(100);Gp11(100);Gp11_unclassified(100);Gp11_unclassified(100);
Otu066	2	Bacteria(100);candidate_division_WPS-2(100);candidate_division_WPS-2_unclassified(100);candidate_division_WPS-2_unclassified(100);candidate_division_WPS-2_unclassified(100);candidate_division_WPS-2_unclassified(100);
Otu067	2	Bacteria(100);Acidobacteria(100);Acidobacteria_Gp18(100);Gp18(100);Gp18_unclassified(100);Gp18_unclassified(100);
Otu068	3	Bacteria(100);Microgenomates(100);Microgenomates_unclassified(100);Microgenomates_unclassified(100);Microgenomates_unclassified(100);Microgenomates_unclassified(100);
Otu069	1	Bacteria(100);Chloroflexi(100);Chloroflexia(100);Kallotenuales(100);Kallotenuaceae(100);Kallotenue(100);
Otu070	1	Bacteria(100);Acidobacteria(100);Acidobacteria_Gp15(100);Gp15(100);Gp15_unclassified(100);Gp15_unclassified(100);
Otu071	1	Bacteria(100);Verrucomicrobia(100);Subdivision5(100);Subdivision5_unclassified(100);Subdivision5_unclassified(100);Subdivision5_unclassified(100);

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
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Nematode Interactions on Beetle Hosts Indicate a Role of Mouth-Form Plasticity in Resource Competition

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Competition is rampant across kingdoms, arising over potential mates, food resources, and space availability. When faced with opponents, phenotypic plasticity proffers organisms indispensable advantageous strategies to outcompete rivals. This tactic is especially crucial on decaying insect hosts as myriad microbes and numerous nematodes struggle to establish thriving populations and ensure resource availability for future generations. Scarab beetles and their associated nematode symbionts on La Réunion Island have provided exceptional systems to study complicated cross-phylum interactions in soil, and recently we have identified a previously unexplored beetle host, *Gymnogaster bupthalma*, to be reliably co-infested with diplogastrids *Pristionchus mayeri* and *Acrostichus* spp. These nematodes maintain the capacity to plastically respond to environmental conditions by developing disparate mouth forms, a strict bacterial-feeding morph or an omnivorous morph that enables predation on other nematodes. In addition, under stressful settings these worms can enter an arrested development stage called dauer, non-feeding dispersal larvae that resume development into reproducing adults when conditions improve. By investigating this beetle-nematode system in a natural context, we uncovered a novel *Pristionchus* strategy, wherein dauer dispersal from the carcass is gradual and a reproducing population is sustained. Remarkably, usually preferential-bacterial morph *P. mayeri* develop as predators in populations dense with competitors.

Keywords: phenotypic plasticity, nematode, *Pristionchus*, scarab beetle, competition, *Acrostichus*

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INTRODUCTION

"The smallest Worme will turne, being trodden on".

William Shakespeare, 1951.

In the infamous historical composition *Henry VI*, Lord Clifford accurately assessed microscopic worms' capacities for turning to alternative forms when trodden on by competitors (Shakespeare, 2001). Indeed, phenotypic plasticity provides an imperative beneficial strategy in the face of competition and is employed by copious creatures, both flora and fauna (Miner et al., 2005; Stomp et al., 2008; Turcotte and Levine, 2016). Tadpoles of the spadefoot toad genus *Spea* can develop a carnivorous morph that serves as an alternative to the typical omnivorous morph under specific environmental conditions, allowing for consumption of its competitors (Pfennig and Murphy, 2002). Plants exhibit a plethora of plastic phenotypes in response to competition, including stem elongation and modified flowering time when overshadowed by opponents restricting shade, as comprehensively exemplified using

model *Arabidopsis thaliana* (Callahan and Pigliucci, 2002; Donohue, 2003). When resource competition limits dung beetle *Onthophagus taurus's* favorite snack (due to increasing density), the insect can plastically respond in morphology, behavior, and life history traits (Macagno et al., 2016; Casasa and Moczek, 2018). However, beetles are not only prone to competition and the associated plastic responses, but also serve as the underground battlefield for competition among other organisms.

Competition among nematodes on insect cadavers is widespread and the conditions motley, depending on assorted factors including nematode species present, bacterial composition, and external environmental influences that may alter the already fluctuating settings (Dillman et al., 2012; Ali et al., 2013; Bertoloni Meli and Bashey, 2018; Blanco-Pérez et al., 2019; Renahan et al., 2021). The fight for food may be restricted to interspecies combat, but can also include conflict within species (Koppenhöfer et al., 1995; O'Callaghan et al., 2014). Advantages are largely species-dependent, and often as conspicuous as archetypal dietary range and developmental time, though more intricate pros may reflect worms' plastic capacities to expand nutritional sources and modify growth trajectories. Entomopathogenic nematodes (EPNs), obligate and occasional facultative parasites of insects, employ various strategies to dominate after inducing host death utilizing symbiotic bacteria (Burnell and Stock, 2000; Campos-Herrera et al., 2012). While some are quick to develop and populate the carcass, other EPNs rely on significantly larger initial populations; these strategies have been observed to reflect reproductive modes of the worms, with hermaphroditic EPNs depending on quick colonization and gonochoristic on original density (Duncan et al., 2003; Campos-Herrera et al., 2015). To evade highly dense and competitive settings, a critical plastic property conserved across nematodes, an arrested developmental stage, is relied on. Named "infective juvenile" (IJ) in pathogenic worms and "dauer" in others, this alternative developmental pathway is often the dispersal stage; intraspecific density can result in continued dauer persistence (Sommer and Ogawa, 2011; Nermut' et al., 2012; Artyukhin et al., 2013) and on crowded insect cadavers, worms enter IJ when food has been depleted in search of a new host (Koppenhöfer et al., 1997; Rolston et al., 2006).

Necromenic model organism *Pristionchus pacificus* readily enters dauer under unfavorable environmental conditions and in response to competition (Mayer and Sommer, 2011; Bose et al., 2014). This valuable capacity is especially vital on insect carcasses, on which *P. pacificus* is often found (Herrmann et al., 2006, 2007). In addition to dauer, *P. pacificus*, along with a hodgepodge of diplogastrids, maintains mouth-form phenotypic plasticity (Von Lieven and Sudhaus, 2000; Kiontke and Fitch, 2010; Ragsdale et al., 2013), in which either a strict bacterial-feeding morph, stenostomatous (St) (**Figure 1D**), or an omnivorous morph that enables predation on other nematodes, eurystomatous (Eu) (**Figure 1C**), is developed (Sudhaus, 2010; Susoy and Sommer, 2016). The combination of these two polyphenisms has been investigated both in laboratory and ecological contexts, utilizing the reliable association of *P. pacificus* with scarab beetle *Oryctes borbonicus* on La Réunion Island (Bento et al., 2010; Meyer et al., 2017; Renahan et al., 2021). La Réunion Island in the Indian Ocean is home to a mélange of beetles associated with numerous nematodes (Herrmann et al., 2010), including the thoroughly studied *O. borbonicus* and *P. pacificus* (Meyer et al., 2017; Renahan et al., 2021). Nematode infestation rates vary among insects, though nearly 100% of *O. borbonicus* hosts *P. pacificus*, allowing for rare ecological experiments and deep dives into the evolutionary history of the worm (Morgan et al., 2012; McGaughran et al., 2016). While *O. borbonicus* and *P. pacificus* have served as an exceptional system to both study host-microbe interactions and dig into the ecology of a well-established model organism, the dearth of co-infestations of the beetle host with more than one nematode species has made competition studies in a natural context lacking.

Fortunately, we have recently discovered endemic cockchafer *Gymnogaster bupthalma* in abundance (**Figure 1A**), even though this beetle was previously assumed to be rare (see section "Materials and Methods" for details). Specifically, there were fewer than 15 specimens acquired between 1851 and 2018 (Lacroix, 1979; Gomy et al., 2017; Max Planck field work 2008–2018). Excitingly, as we ventured into formerly unexplored island territory, all three of our collection trips since 2019 resulted in copious *G. bupthalma* sightings, allowing for capture of enough specimen to study without disruption of the local population. Adult *G. bupthalma* is host to diplogastrids *Pristionchus mayeri* (**Figures 1C,D**) and species of the genus *Acrostichus* (**Figure 1B**), both capable of the mouth-form dimorphism (the latter depending on the species) (Giblin and Kaya, 1984; Kanzaki et al., 2013). Some beetle individuals only harbor one species, though there is no paucity of co-infestation with both nematodes; we utilized this naturally existing competition arena to investigate how these two nematodes fight for resources.

Globally found *P. mayeri* is strongly preferentially St (**Figure 1D**), even maintaining the bacterial-feeding morph under conventional Eu-inducing conditions, as developed and determined using several *Pristionchus* species (Wilecki et al., 2015; Werner et al., 2017). Since domesticated strains in a laboratory environment may not employ use of this discrete polyphenism, we sought to investigate how *P. mayeri* behaves when faced with competition in a natural setting, and if it utilizes its capacity for phenotypic plasticity. We collected and subsequently decapitated adult *G. bupthalma* (initiating nematode emergence), and tracked the succession and dynamics of the two nematode genera over 3 months post-beetle death. We uncovered a new *Pristionchus* dauer strategy, in which the larvae disperse gradually while maintaining a population on the carcass. In addition, we pinpoint the vital role of mouth-form plasticity in resource competition, wherein a predatory morph proliferates in environments dense with competitors (**Figure 1C**).

MATERIALS AND METHODS

Beetle Collection and Cage Set-Up

Male *G. bupthalma* were collected on La Réunion Island in January 2021 (Parc Nationale permit DIR-I-2020-280 and Nagoya number ABSCH-IRCC-FR-254969-1), when male adults are above ground for mate finding at night. Specimen were captured

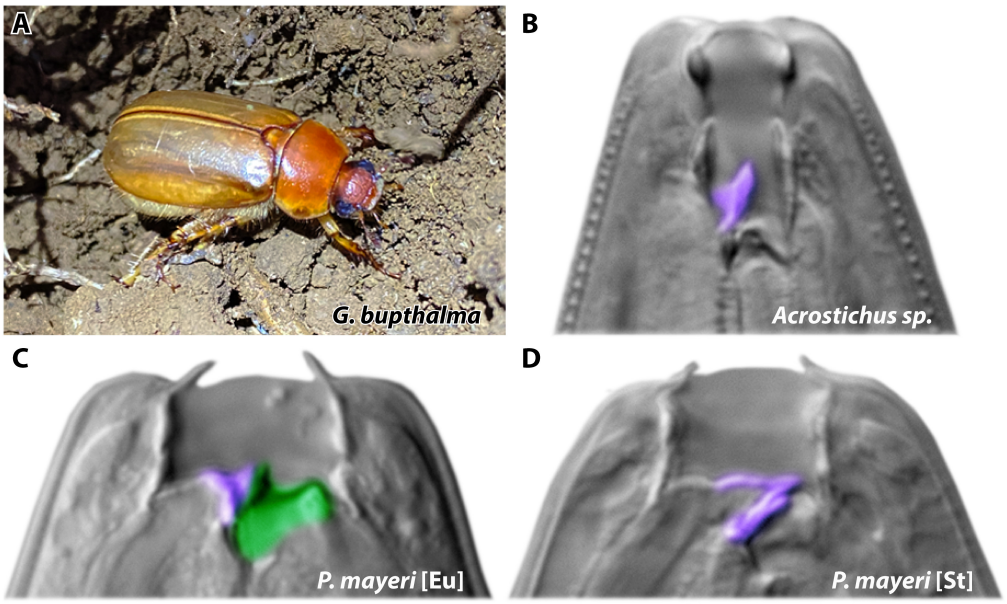

FIGURE 1 | Host beetle and mouth forms of symbiont nematodes. **(A)** Adult male beetle *Gymnogaster bupthalma* on La Réunion Island. Photo by Christian Weiler. **(B–D)** Dorsal tooth marked in purple, subventral in green. **(B)** Single-toothed *Acrostichus* is strictly bacterial-feeding. *P. mayeri* develops either **(C)** the wide predatory Eu morph with both the dorsal and subventral teeth or **(D)** the strict bacterial-feeding St morph with only the dorsal tooth.

by searching among trees post-dusk with headlights and then placed in 50 mL falcon tubes (5 per tube), with holes punctured in the falcon lids for aeration and wet towels added for moisture. The beetles were then flown to our lab in Tübingen, Germany for processing. Beetles were decapitated before being placed in autoclaved standard gardening soil (Einheitserde, Classic Profisubstrat) in 15 × 5 cm cages made from 0.5 mm mesh. Cages were kept at 17°C and were watered to imitate the environment on the island. These methods are consistent with previous experiments establishing this set-up (Renahan et al., 2021). Eight cages were processed at each time point (t = 0, 1, and 4 days, 1, 2, 4, 6, 8, 10, and 12 weeks).

Nematode Isolation, Screening, and Identification

At the appropriate time points, the cages were disassembled, the beetle carcasses moved to nematode growth medium (NGM) plates, and the soil to Baermann funnels to isolate the nematodes (Viglierchio and Schmitt, 1983). Worms were screened for mouth form and screened based on morphology to identify species, then singled and later sequenced for further species verification. DNA was extracted using a lysis solution, followed by a polymerase chain reaction (PCR) to amplify a 1 kb region of the small subunit (SSU) rRNA gene using the primers SSU18A (5′-AAAGATTAAGCCATGCATG-3′) and SSU26R (5′CATTCTTGGCAAATGCTTTCG-3′) (Blaxter et al., 1998). Sanger sequencing was carried out by GENEWIZ in Leipzig using sequencing primer SSU9R (5′-AGCTGGAATTACCGCGGCTG-3′). SeqMan Pro was used to trim sequencing chromatograms, and NCBI reference database to blast for taxonomic identification. Unfortunately, due to limited data availability, we were not able to confidently determine the *Acrostichus* species present, as BLAST hits were consistently tied between *A. nudicapitatus* and *A. halicti*. Thus, potentially two, or even more unidentified, *Acrostichus* species are present on the carcass, but we treat these two as one species throughout the project.

Competition Assays and Mouth-Form Phenotyping

Singled worms from carcasses were left on NGM agar plates seeded with OP50 *Escherichia coli* to repopulate. The subsequent generations quickly entered dauer as the population density increased (more readily than domesticated lab strains tend to), and as worms are typically found in the dauer stage on live hosts (Herrmann et al., 2010; Ragsdale et al., 2015), these dauer worms were then used for competition assays. Worms were washed from plates and dauers isolated using 1% sodium dodecyl sulfate, then subsequently washed with M9 (Cassada and Russell, 1975). Dauers of the same species used as competitors against each other were first stained either CellTracker Green BODIPY (Thermo Fisher) or neutral red (Thomas and Lana, 2008) to differentiate the two populations during phenotyping, as established in Werner et al. (2018). The competition assays were composed of the following combinations: *P. mayeri* vs. *Acrostichus*, *P. mayeri* vs. *P. mayer*i from different carcasses, and *P. mayeri* vs. *P. mayeri* of the same strain. The same number of dauers for each competitor were added to NGM agar plates seeded with OP50 *E. coli* (to induce dauer exit, as on a carcass when microbes bloom), ranging from 10 dauers of each

competitor to 250 dauers of each competitor. As control, dauers of each strain were isolated and grown on seeded plates at the same density as the total number of worms on an assay plate (e.g., in an assay of 250 *P. mayeri* strain 1 vs. 250 *P. mayeri* strain 2, control plates of 500 *P. mayeri* strain 1 and 500 *P. mayeri* strain 2 were tracked). Once the worms had developed into adults, they were mouth-form phenotyped using differential interference contrast (DIC) on a Zeiss Axioskop.

RESULTS

Gymnogaster bupthalma Is Frequently Co-infested With Two Different Nematode Genera

To determine nematode occurrence on *G. bupthalma*, we screened eight beetles per time point and found nearly each carcass was infested with at least one nematode, *P. mayeri* or *Acrostichus*. Almost a third of the carcasses (23 out of 80 beetles, 28.75%) were infested with both nematodes (**Figure 2A**). Time of worm emergence from the carcasses varied among the time points: at t = 0, decapitated carcasses were immediately plated on NGM agar, and nematodes emerged from 1 to 2 weeks after. This was consistently seen among the first four time points (t = 0, t = 1 day, t = 4 days, and t = 1 week), but by t = 2 weeks, nematodes left the carcasses within 5 days. Nematodes in the surrounding soil were consistently observed at t = 2 weeks and later (t = 4 weeks, t = 6 weeks, t = 8 weeks, t = 10 weeks, and t = 12 weeks), with three replicates at t = 1 week having few worms in the surrounding soil, and none in the earlier time points. Succession in the earlier time points were not distinguished (t = 0, 1, and 4 days), likely due to the duration required for worms to emerge; thus a couple days between these time points do not confer any conspicuous differences. In specimen infested with either a single species or with two, time of departure was independent of nematode type. Though, at earlier time points (t = 0 through t = 2 weeks), in co-infested carcasses, *Acrostichus* was more prevalent in numbers of worms than *P. mayeri*, but the two equalized after 1 month on the carcasses. On the carcass, worms were found in both feeding mixed stages and the arrested development dauer stage (**Figure 2B**), independent of whether they were in co-inhabited space or singly lived. From t = 2 to t = 6 weeks there was a steep increase in the number of both dauers and feeding stages (from 30 dauers and 10 feeding stages at t = 2 weeks to 160 dauers and 130 feeding stages at t = 6 weeks). From then, both populations drop, and at t = 12 weeks only 12 dauers and 1 feeding stage worm remain. In the surrounding soil, worms were exclusively in dauer, the dispersal stage.

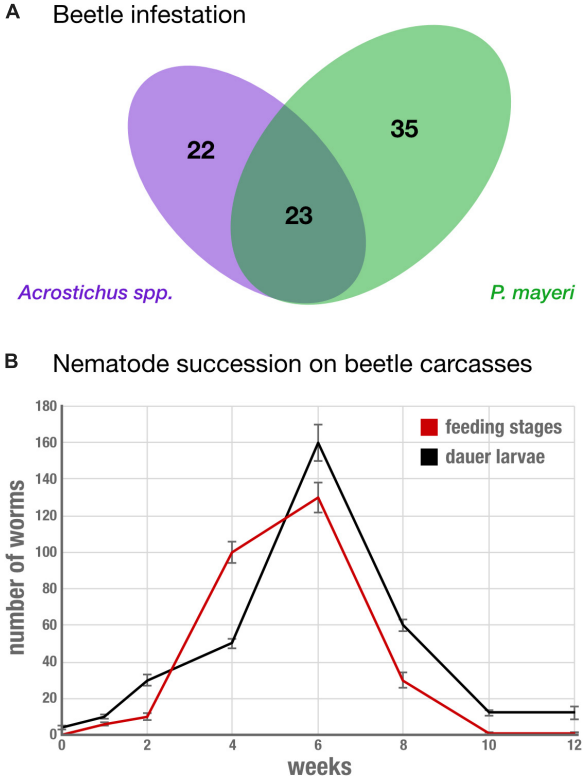

FIGURE 2 | Reliably co-infested *G. bupthalma* remains home to various stages of both *P. mayeri* and *Acrostichus*. **(A)** 80 adult *G. bupthalma* were investigated across 3 months. A total of 22 individuals harbored only *Acrostichus spp.* and 35 only *P. mayeri*. 23 individuals were infested with both *Acrostichus* and *P. mayeri*. **(B)** Nematode succession on the carcass over 3 months. The populations of both dauers (black) and feeding mixed stages (red) increase during the first 6 weeks, reaching a peak at 6 weeks (160 dauers and 130 feeding stages), and then decrease until the population is almost depleted at 12 weeks (12 dauers and 1 feeding stage). N = 8 beetles per time point, average number shown. Error bars are standard error of the mean.

Succession of *Pristionchus mayeri* Mouth Form Over 3 Months

We tracked the mouth forms of *P. mayeri* over the course of 3 months post-beetle death when co-infested with *Acrostichus* (**Figure 3A**). *P. mayeri* developed only the strict bacterial-feeding St morph at early time points (t = 0–1 week), when population densities on the carcasses are relatively low. But, at t = 2 weeks as population increases, *P. mayeri* starts also developing into the predatory morph, with 36.5% being Eu. The number of Eu animals increases with some fluctuation (38% at t = 2 weeks, 84% at t = 4 weeks, 62% at t = 6 weeks), and reaches 100% Eu at t = 8 weeks. While the number of worms reaches its peak at t = 6 weeks (160 dauers, 130 feeding stages), at the dip during t = 8 weeks (60 dauers and 30 feeding stages), *P. mayeri* still employs the predatory morph at 100% frequency. Though, at t = 10 and 12 weeks there are few worms on the carcasses, and the predatory prevalence drops to 20% and 17%, respectively. Thus, as population density increases alongside the presence of competitors, more *P. mayeri* become predatory.

Competition Experiments Reveal Cross-Genera Induction of the Predatory Morph

To determine if the increase in predatory mouth forms in *P. mayeri* indeed reflects the presence of competition, we

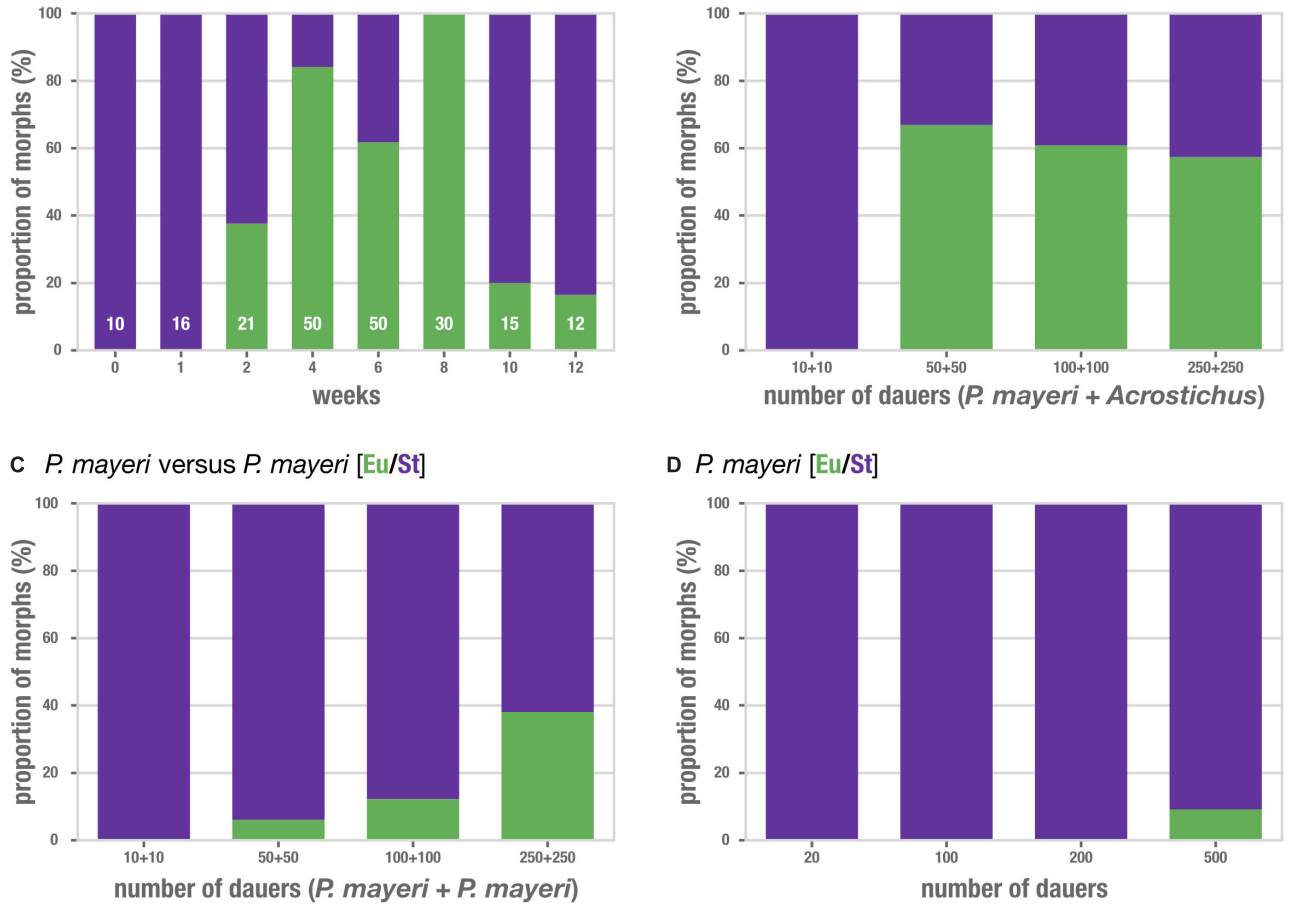

FIGURE 3 | *Pristionchus mayeri* develops the predatory morph in populations dense with competitors. **(A)** Succession of mouth-form ratios of *P. mayeri* on beetle carcasses co-infested with *Acrostichus.* N is shown in white for each time point; the range reflects the variability of number of individuals present as displayed in **Figure 2B**. The number of predators increases as the density on the carcass increases (t = 2–8 weeks). Though, when the population is low at t = 0, 1, 10, and 12 weeks, the prevalence of predators decreases. Competition assays **(B–D)** using dauer lines from the worms observed in panel **(A)**. **(B)** *P. mayeri* vs. *Acrostichus* in increasing density of dauers shows an increase in the predatory morph. Fisher's exact test on summed Eu counts against the respective dauer density as shown in panel **(D):** $p < 0.05$ for densities 50:50, 100:100, and 250:250. N = 100 per bar, 10 strains of each competitor in various combinations. **(C)** *P. mayeri* vs. *P. mayeri* from different carcasses in increasing density of dauers. Fisher's exact test on summed Eu counts against the respective dauer density as shown in panel **(D):** $p < 0.05$ for densities 100:100 and 250:250. N = 100 per bar, 10 strains of each competitor in various combinations. **(D)** Control *P. mayeri* assays in which singled strains were grown at the same densities as in the competition assays of **(B,D)**. N = 100 per bar, 10 strains each.

maintained lines of both genera from multiple carcasses and time points, and induced dauer simply by allowing the NGM agar plates to become overcrowded. While we isolated and grew lines of *P. mayeri* that were Eu on the carcass, once on plates these strains became St. Dauers were isolated, stained, and mixed on fresh plates with food in the following combinations: *P. mayeri* vs. *Acrostichus*, *P. mayeri* vs. *P. mayer*i from different carcasses, and *P. mayeri* against itself. As few as 10 dauers of each strain were added to reflect the low number of worms at early time points, and as many as 250 dauers of each strain to reflect the high population densities at t = 4 and 6 weeks seen in **Figure 2B**. *P. mayeri* maintained the St morph in all assays with 10 + 10 dauers, regardless of whether the competition was *Acrostichus*, another *P. mayeri* strain, or itself (**Figures 3B–D**). This is consistent with the lack of predatory *P. mayeri* observed in early carcass time points when nematode density is rather low. Though, once the population in the competition assays increased to 50 vs. 50, two-thirds (66%) of *P. mayeri* developed the predatory morph when the competition was *Acrostichus* (**Figure 3B**). The same *P. mayeri* strain in competition with itself exclusively exhibited the St morph (**Figure 3D**). Though, when 50 *P. mayeri* were paired with 50 *P. mayeri* of a different strain, 6% became Eu (**Figure 3C**), while those strains against themselves remained St. This trend continues as the number of dauers increases: *P. mayeri* vs. *Acrostichus* results in over 50% Eu (**Figure 3B**) and even *P. mayeri* vs. *P. mayeri* become 40% Eu when the density is 250 vs. 250 (**Figure 3C**). Isolated *P. mayeri*, however, even in high density (500), fail to produce many Eu, with the highest being just shy of 10% (**Figure 3D**). Thus, *P. mayeri* is prone to develop the predatory morph in competitive

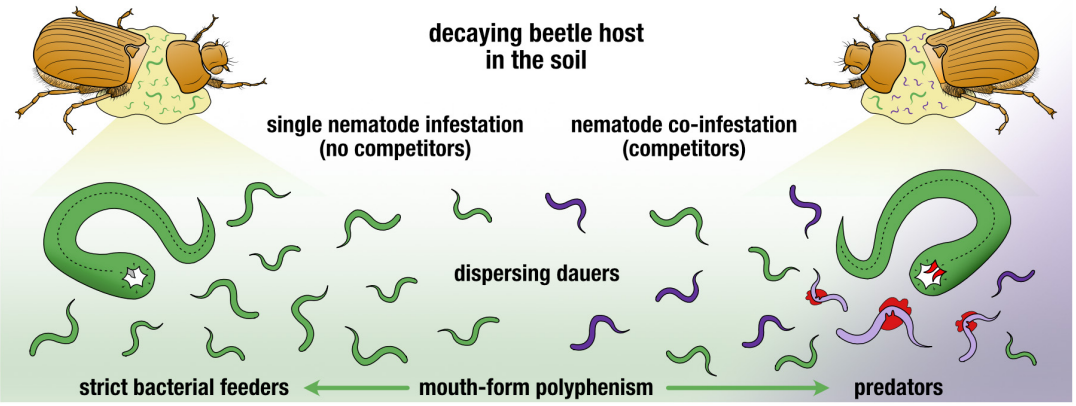

FIGURE 4 | Graphical summary of two nematode plastic responses in face of resource competition post-beetle death. Dauers continuously disperse and *P. mayeri* develops as predators when co-infesting the host with *Acrostichus*.

and dense environments, especially when the competitor is of another genus (*Acrostichus*) and even when the competitor is of the same species but another line. The mouth-form pattern observed in the competition assays mirror that of the observed mouth-form pattern in the natural carcass setting: as competitor density increases, so does the occurrence of the predatory morph.

DISCUSSION

For the first time, we explored the dynamics of co-occurring nematodes on the previously uninvestigated beetle *G. bupthalma*. We found the adult beetle to be reliably infected with at least one nematode genus, *Pristionchus* or *Acrostichus*, and often co-infested with both, allowing for unique studies of nematode competition in a natural setting. By decapitating beetles and thus inducing nematode emergence, we were able to track the succession and plastic responses of worms over 3 months. Strikingly, we found that *P. mayeri* utilizes its capacity to develop the predatory Eu morph when faced with dense competition. This trend toward predation was verified using freshly isolated *P. mayeri* lines in competition assays with also newly cultured strains of *Acrostichus*, revealing the vital role of mouth-form plasticity in resource competition. Our combined field and laboratory study system resulted in three major conclusions.

First, we found a novel dispersal strategy of *Pristionchus* dauer larvae. As we tracked nematode succession on the carcasses, we expected a pattern of feeding stages and the arrested developmental and dispersal stage, dauer. Surprisingly, we did not observe an inverse presence/absence of feeding stages versus dauer; worms consistently developed via both pathways (**Figure 2B**). Though, this is in contrast to previous studies on *O. borbonicus* and *P. pacificus*, in which feeding stages were in low abundance while dauers were numerous, and vice versa; this was found to reflect bacterial load, and thus food availability (Renahan et al., 2021). Perhaps since *P. pacificus* is often exclusively the solitary diplogastrid on its host, its dauer plasticity response is weighted toward resource abundance, while on *G. bupthalma*, the two nematode genera must also factor in the occurrence of the other worm. While *P. pacificus* displays a biphasic boom and bust strategy with two major dispersal events from the carcass, *P. mayeri* and *Acrostichus* dauers steadily and gradually disperse from the carcass, while also sustaining a feeding and reproducing population in order to maintain a competitive advantage, and do so throughout the first 2 months until food is depleted. Thus, on hosts in which two diplogastrids frequently co-occur, the worm dispersal strategy differs from that of worms that infest hosts often solitarily. These findings add to the complexity of behavioral strategies of dauer-associated traits.

Second, nematode interactions indicate a role of mouth-form plasticity in resource competition. In addition to entering the dauer stage to disperse, these nematodes retain the plastic ability to develop either a strict bacterial-feeding (St) morph or the omnivorous (Eu) morph that allows for predation on nematode larvae. Domesticated strains of *P. mayeri* tend toward the St morph, rarely developing Eu, even when Eu induction is attempted by established methods (Wilecki et al., 2015; Werner et al., 2017). Intriguingly, *P. mayeri* is preferentially St when few worms populate the carcass, but as density increases temporally, more and more *P. mayeri* become predators (**Figure 3A**). While certain species of *Acrostichus* have also displayed the polyphenism, dimorphism was not observed during this perhaps limited exploration. Though these observations are consistent with recent studies detailing the association of reproductive mode and mouth form, in which hermaphrodites display predatory tendencies to incite competition, while gonochoristic species are preferentially bacterivorous to deter conflict between parents and offspring (Lightfoot et al., 2021). Accordingly, hermaphroditic *P. mayeri* becomes predatory without risk of compromising its own kin, while *Acrostichus* remains non-predatory and avoids potential kin consumption.

Third, we confirmed that *P. mayeri*'s increase in the predatory morph is a consequence of higher competitor density by conducting assays pairing worms against each other. Indeed, *P. mayeri* becomes more predatory with higher abundances of *Acrostichus* (**Figure 3B**), and to a more muted extent, with higher

abundances of other *P. mayeri* strains (**Figure 3C**). Though, the occurrence of predators in these assays is not as high as observed in the natural setting of the beetle carcass. Myriad of factors can explain this aberration, as copious affairs are ongoing on the carcass, both seemingly detectable and not, and that we did not fully replicate or pinpoint in our assays. Mainly, the bacteria present on the decaying carcass may play roles both in worm developmental stages and thus dispersal, and mouth form tendencies. Bacterial influence on nematode polyphenisms is well-explored, with several studies demonstrating microbial impact on dauer induction, Eu formation, and predatory rates (Samuel et al., 2016; Akduman et al., 2018, 2020; Bubrig et al., 2020).

Furthermore, nematodes chemically communicate via small molecules, nematode-derived modular metabolites (NDMMs), that regulate various behaviors and trigger plastic responses, including dauer entry and exit and mouth form (Bento et al., 2010; Bose et al., 2012). In particular, the complex ascaroside dasc#1 induces the predatory morph in *P. pacificus* in a density and age-specific manner (Werner et al., 2018). *P. mayeri* also produces this pheromone, though not to the same extent that *P. pacificus* does, while it is undetected in *Acrostichus* (Dong et al., 2020). The presence of a known Eu-inducing molecule in *P. mayeri* and its absence in *Acrostichus* may explain the development of predators in the former genus and lack of in the latter. Though, as the abundance of dasc#1 is relatively low in *P. mayeri,* and Eu worms are not observed in dense populations of singled *P. mayeri* strains (**Figure 3D**), it is clear that other factors maintain vital roles in influencing development of the predatory morph.

All together, we have demonstrated a plastic response of a preferentially bacterivorous species to become predatory when faced with competition in a natural setting (**Figure 4**). While population density and pheromone communication explain aspects of this trend, supplementary exploration of the prominent carcass microbiome may reveal further factors influencing competitive strategies. In addition, deeper investigating of the co-occurrence of these two nematode genera in other beetle life stages will shed light on the dynamics of the nematodes with its host, alongside the bacterial succession on the beetle developmental stages. Conclusively, we have established that when *P. mayeri* is trodden on by competition, it turns predatory.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

TR and RS conceived the study. TR conducted all experiments, analyzed all data, and wrote the manuscript with edits from RS. Both authors contributed to the article and approved the submitted version.

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REFERENCES

Akduman, N., Lightfoot, J. W., Röseler, W., Witte, H., Lo, W. S., Rödelsperger, C., et al. (2020). Bacterial vitamin B 12 production enhances nematode predatory behavior. *ISME J.* 14, 1494–1507. doi: 10.1038/s41396-020-0626-2

Akduman, N., Rödelsperger, C., and Sommer, R. J. (2018). Culture-based analysis of *Pristionchus*-associated microbiota from beetles and figs for studying nematode-bacterial interactions. *PLoS One* 13:e0198018. doi: 10.1371/journal.pone.0198018

Ali, J. G., Campos-Herrera, R., Alborn, H. T., Duncan, L. W., and Stelinski, L. L. (2013). Sending mixed messages: a trophic cascade produced by a belowground herbivore-induced cue. *J. Chem. Ecol.* 39, 1140–1147. doi: 10.1007/s10886-013-0332-x

Artyukhin, A. B., Schroeder, F. C., and Avery, L. (2013). Density dependence in *Caenorhabditis larval* starvation. *Sci. Rep.* 3, 1–7. doi: 10.1038/srep02777

Bento, G., Ogawa, A., and Sommer, R. J. (2010). Co-option of the hormone-signalling module dafachronic acid–DAF-12 in nematode evolution. *Nature* 466, 494–497. doi: 10.1038/nature09164

Bertoloni Meli, S., and Bashey, F. (2018). Trade-off between reproductive and anti-competitor abilities in an insect–parasitic nematode–bacteria symbiosis. *Ecol. Evol.* 8, 10847–10856. doi: 10.1002/ece3.4538

Blanco-Pérez, R., Bueno-Pallero, F. Á, Vicente-Díez, I., Marco-Mancebón, V. S., Pérez-Moreno, I., and Campos-Herrera, R. (2019). Scavenging behavior and interspecific competition decrease offspring fitness of the entomopathogenic nematode *Steinernema feltiae*. *J. Invertebr. Pathol.* 164, 5–15. doi: 10.1016/j.jip.2019.04.002

Blaxter, M. L., De Ley, P., Garey, J. R., Liu, L. X., Scheldeman, P., Vierstraete, A., et al. (1998). A molecular evolutionary framework for the phylum Nematoda. *Nature* 392, 71–75. doi: 10.1038/32160

Bose, N., Meyer, J. M., Yim, J. J., Mayer, M. G., Markov, G. V., Ogawa, A., et al. (2014). Natural variation in dauer pheromone production and sensing supports intraspecific competition in nematodes. *Curr. Biol.* 24, 1536–1541. doi: 10.1016/j.cub.2014.05.045

Bose, N., Ogawa, A., von Reuss, S. H., Yim, J. J., Ragsdale, E. J., Sommer, R. J., et al. (2012). Complex small-molecule architectures regulate phenotypic plasticity in a nematode. *Angew. Chem* 124, 12606–12611. doi: 10.1002/ange.201206797

Bubrig, L. T., Sutton, J. M., and Fierst, J. L. (2020). Caenorhabditis elegans dauer recovery varies with worm-bacteria interactions. *Ecol. Evol.* 10, 9886–9895. doi: 10.1002/ece3.6646

Burnell, A., and Stock, S. P. (2000). Heterorhabditis, Steinernema and their bacterial symbionts—lethal pathogens of insects. *Nematology* 2, 31–42. doi: 10.1163/156854100508872

Callahan, H. S., and Pigliucci, M. (2002). Shade-induced plasticity and its ecological significance in wild populations of *Arabidopsis thaliana*. *Ecology* 83, 1965–1980.

Campos-Herrera, R., Barbercheck, M., Hoy, C. W., and Stock, S. P. (2012). Entomopathogenic nematodes as a model system for advancing the frontiers of ecology. *J. Nematol.* 44:162.

Campos-Herrera, R., Pùža, V., Jaffuel, G., Blanco-Pérez, R., Èepulytë-Rakauskienë, R., and Turlings, T. C. (2015). Unraveling the intraguild competition between *Oscheius* spp. nematodes and entomopathogenic nematodes: implications for their natural distribution in Swiss agricultural soils. *J. Invertebr. Pathol.* 132, 216–227. doi: 10.1016/j.jip.2015.10.007

Casasa, S., and Moczek, A. P. (2018). The role of ancestral phenotypic plasticity in evolutionary diversification: population density effects in horned beetles. *Anim. Behav.* 137, 53–61. doi: 10.1016/j.anbehav.2018.01.004

Cassada, R. C., and Russell, R. L. (1975). The dauerlarva, a post-embryonic developmental variant of the nematode *Caenorhabditis elegans*. *Dev. Biol.* 46, 326–342. doi: 10.1016/0012-1606(75)90109-8

Dillman, A. R., Chaston, J. M., Adams, B. J., Ciche, T. A., Goodrich-Blair, H., Stock, S. P., et al. (2012). An entomopathogenic nematode by any other name. *PLoS Pathog.* 8:e1002527. doi: 10.1371/journal.ppat.1002527

Dong, C., Weadick, C. J., Truffault, V., and Sommer, R. J. (2020). Convergent evolution of small molecule pheromones in *Pristionchus nematodes*. *eLife* 9:e55687. doi: 10.7554/eLife.55687

Donohue, K. (2003). Setting the stage: phenotypic plasticity as habitat selection. *Int. J. Plant Sci.* 164, S79–S92. doi: 10.1086/368397

Duncan, L. W., Dunn, D. C., Bague, G., and Nguyen, K. (2003). Competition between entomopathogenic and free-living bactivorous nematodes in larvae of the weevil *Diaprepes abbreviatus*. *J. Nematol.* 35:187.

Giblin, R. M., and Kaya, H. K. (1984). *Aduncospiculum halicti* n. gen., n. sp.(Diplogasterida: Diplogasteroididae), an associate of bees in the genus *Halictus* (Hymenoptera: Halictidae). *Rev. Nématol.* 7, 189–197.

Gomy, Y., Lemagnen, R., and Poussereau, J. (2017). *Les Coléoptères de l'Île de La Réunion*. London: Nhbs Ltd.

Herrmann, M., Kienle, S., Rochat, J., Mayer, W. E., and Sommer, R. J. (2010). Haplotype diversity of the nematode *Pristionchus pacificus* on Réunion in the Indian Ocean suggests multiple independent invasions. *Biol. J. Linn. Soc.* 100, 170–179. doi: 10.1111/j.1095-8312.2010.01410.x

Herrmann, M., Mayer, W. E., Hong, R. L., Kienle, S., Minasaki, R., and Sommer, R. J. (2007). The nematode *Pristionchus pacificus* (Nematoda: Diplogastridae) is associated with the oriental beetle *Exomala orientalis* (Coleoptera: Scarabaeidae) in Japan. *Zool. Sci.* 24, 883–889. doi: 10.2108/zsj.24.883

Herrmann, M., Mayer, W. E., and Sommer, R. J. (2006). Nematodes of the genus *Pristionchus* are closely associated with scarab beetles and the Colorado potato beetle in Western Europe. *Zoology* 109, 96–108. doi: 10.1016/j.zool.2006.03.001

Kanzaki, N., Ragsdale, E. J., Herrmann, M., Susoy, V., and Sommer, R. J. (2013). Two androdioecious and one dioecious new species of *Pristionchus* (Nematoda: Diplogastridae): new reference points for the evolution of reproductive mode. *J. Nematol.* 45:172.

Kiontke, K., and Fitch, D. H. (2010). Phenotypic plasticity: different teeth for different feasts. *Curr. Biol.* 20, R710–R712. doi: 10.1016/j.cub.2010.07.009

Koppenhöfer, A. M., Baur, M. E., Stock, S. P., Choo, H. Y., Chinnasri, B., and Kaya, H. K. (1997). Survival of entomopathogenic nematodes within host cadavers in dry soil. *Appl. Soil Ecol.* 6, 231–240. doi: 10.1016/S0929-1393(97)00018-8

Koppenhöfer, A. M., Kaya, H. K., Shanmugam, S., and Wood, G. L. (1995). Interspecific competition between steinernematid nematodes within an insect host. *J. Invertebr. Pathol.* 66, 99–103. doi: 10.1006/jipa.1995.1070

Lacroix, M. (1979). Gymnogaster buphthalma Blanchard, espèce énigmatique de l'île de la Réunion (Coleoptera Melolonthidae). *Rev. Franç. D'entomol.* 1988, 25–29.

Lightfoot, J. W., Dardiry, M., Kalirad, A., Giaimo, S., Eberhardt, G., Witte, H., et al. (2021). Sex or cannibalism: polyphenism and kin recognition control social action strategies in nematodes. *Sci. Adv.* [Epub ahead of print].

Macagno, A. L., Moczek, A. P., and Pizzo, A. (2016). Rapid divergence of nesting depth and digging appendages among tunneling dung beetle populations and species. *Am. Nat.* 187, E143–E151. doi: 10.1086/685776

Mayer, M. G., and Sommer, R. J. (2011). Natural variation in *Pristionchus pacificus* dauer formation reveals cross-preference rather than self-preference of nematode dauer pheromones. *Proc. R. Soc. B Biol. Sci.* 278, 2784–2790. doi: 10.1098/rspb.2010.2760

McGaughran, A., Rödelsperger, C., Grimm, D. G., Meyer, J. M., Moreno, E., Morgan, K., et al. (2016). Genomic profiles of diversification and genotype–phenotype association in island nematode lineages. *Mol. Biol. Evol.* 33, 2257–2272. doi: 10.1093/molbev/msw093

Meyer, J. M., Baskaran, P., Quast, C., Susoy, V., Rödelsperger, C., Glöckner, F. O., et al. (2017). Succession and dynamics of *Pristionchus nematodes* and their microbiome during decomposition of Oryctes borbonicus on La Réunion Island. *Environ. Microbiol.* 19, 1476–1489. doi: 10.1111/1462-2920.13697

Miner, B. G., Sultan, S. E., Morgan, S. G., Padilla, D. K., and Relyea, R. A. (2005). Ecological consequences of phenotypic plasticity. *Trends Ecol. Evol.* 20, 685–692. doi: 10.1016/j.tree.2005.08.002

Morgan, K., McGaughran, A., Villate, L., Herrmann, M., Witte, H., Bartelmes, G., et al. (2012). Multi locus analysis of *Pristionchus pacificus* on La Réunion Island reveals an evolutionary history shaped by multiple introductions, constrained dispersal events and rare out-crossing. *Mol. Ecol.* 21, 250–266. doi: 10.1111/j.1365-294X.2011.05382.x

Nermuť, J., Pùža, V., and Mráèek, Z. (2012). The effect of intraspecific competition on the development and quality of *Phasmarhabditis hermaphrodita* (Rhabditida: Rhabditidae). *Biocontrol Sci. Technol.* 22, 1389–1397. doi: 10.1080/09583157.2012.730604

O'Callaghan, K. M., Zenner, A. N., Hartley, C. J., and Griffin, C. T. (2014). Interference competition in entomopathogenic nematodes: male Steinernema kill members of their own and other species. *Int. J. Parasitol.* 44, 1009–1017. doi: 10.1016/j.ijpara.2014.07.004

Pfennig, D. W., and Murphy, P. J. (2002). How fluctuating competition and phenotypic plasticity mediate species divergence. *Evolution* 56, 1217–1228. doi: 10.1111/j.0014-3820.2002.tb01433.x

Ragsdale, E. J., Kanzaki, N., and Herrmann, M. (2015). Taxonomy and natural history: the genus *Pristionchus*. *Pristionchus Pacificus* 1, 77–120. doi: 10.1163/9789004260306_005

Ragsdale, E. J., Müller, M. R., Rödelsperger, C., and Sommer, R. J. (2013). A developmental switch coupled to the evolution of plasticity acts through a sulfatase. *Cell* 155, 922–933. doi: 10.1016/j.cell.2013.09.054

Renahan, T., Lo, W. S., Werner, M. S., Rochat, J., Herrmann, M., and Sommer, R. J. (2021). Nematode biphasic 'boom and bust' dynamics are dependent on host bacterial load while linking dauer and mouth-form polyphenisms. *Environ. Microbiol.* [Epub ahead of print]. doi: 10.1111/1462-2920.15438

Rolston, A. N., Griffin, C. T., and Downes, M. J. (2006). Emergence and dispersal patterns of two isolates of the entomopathogenic nematode *Steinernema feltiae*. *J. Nematol.* 38:221.

Samuel, B. S., Rowedder, H., Braendle, C., Félix, M. A., and Ruvkun, G. (2016). Caenorhabditis elegans responses to bacteria from its natural habitats. *Proc. Natl. Acad. Sci. U.S.A.* 113, E3941–E3949. doi: 10.1073/pnas.1607183113

Shakespeare, W. (2001). *Henry VI.: Part Three*. New York, NY: Oxford University Press, USA.

Sommer, R. J., and Ogawa, A. (2011). Hormone signaling and phenotypic plasticity in nematode development and evolution. *Curr. Biol.* 21, R758–R766. doi: 10.1016/j.cub.2011.06.034

Stomp, M., van Dijk, M. A., van Overzee, H. M., Wortel, M. T., Sigon, C. A., Egas, M., et al. (2008). The timescale of phenotypic plasticity and its impact on competition in fluctuating environments. *Am. Nat.* 172, E169–E185. doi: 10.1086/591680

Sudhaus, W. A. (2010). Preadaptive plateau in Rhabditida (Nematoda) allowed the repeated evolution of zooparasites, with an outlook on evolution of life cycles within Spiroascarida. *Palaeodiversity.* 3(Suppl.), 117–130.

Susoy, V., and Sommer, R. J. (2016). Stochastic and conditional regulation of nematode mouth-form dimorphisms. *Front. Ecol. Evol.* 4:23. doi: 10.3389/fevo.2016.00023

Thomas, M. C., and Lana, P. D. (2008). Evaluation of vital stains for free-living marine nematodes. *Brazil. J. Oceanogr.* 56, 249–251.

Turcotte, M. M., and Levine, J. M. (2016). Phenotypic plasticity and species coexistence. *Trends Ecol. Evol.* 31, 803–813. doi: 10.1016/j.tree.2016.07.013

Viglierchio, D. R., and Schmitt, R. V. (1983). On the methodology of nematode extraction from field samples: baermann funnel modifications. *J. Nematol.* 15:438.

Von Lieven, A. F., and Sudhaus, W. (2000). Comparative and functional morphology of the buccal cavity of *Diplogastrina* (Nematoda) and a first outline of the phylogeny of this taxon. *J. Zool. Syst. Evol. Res.* 38, 37–63. doi: 10.1046/j.1439-0469.2000.381125.x

Werner, M. S., Claaßen, M. H., Renahan, T., Dardiry, M., and Sommer, R. J. (2018). Adult influence on juvenile phenotypes by stage-specific pheromone production. *Iscience* 10, 123–134. doi: 10.1016/j.isci.2018.11.027

Werner, M. S., Sieriebriennikov, B., Loschko, T., Namdeo, S., Lenuzzi, M., Dardiry, M., et al. (2017). Environmental influence on *Pristionchus pacificus* mouth form through different culture methods. *Sci. Rep.* 7, 1–2. doi: 10.1038/s41598-017-07455-7

Wilecki, M., Lightfoot, J. W., Susoy, V., and Sommer, R. J. (2015). Predatory feeding behaviour in *Pristionchus nematodes* is dependent on phenotypic plasticity and induced by serotonin. *J. Exp. Biol.* 218, 1306–1313. doi: 10.1242/jeb.118620

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